

Novel Fibroblast Activation Protein Inhibitor- Based Radiopharmaceuticals for Improved Theranostic Application in Various Cancer Types

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Abstract

Tumors do not only consist of tumor cells, but are surrounded by a tumor microenvironment that includes various components such as activated fibroblasts, immune cells, vascular cells, extracellular matrix, etc. The so-called cancer-associated fibroblasts account for a large proportion of the total tumor mass in many epithelial tumors. Fibroblast activation protein alpha (FAP) is a particularly interesting oncological target because it is highly expressed by activated fibroblasts and is present in more than 90% of epithelial tumors. FAP as a target is very interesting because this pan-tumor approach can be applied in a very broad range of cancer types.

FAP is a transmembrane serine protease. In addition to being strongly expressed in the tumor microenvironment, it is also involved in processes such as wound healing, (chronic) inflammation, fibrosis (liver, pancreas, lung) or rheumatoid arthritis. In contrast, FAP expression in adult healthy tissue is usually not detectable or very low. This selective expression is essential for its suitability as an oncological target. It allows selective accumulation of a diagnostic or therapeutic drug in the tumor or tumor microenvironment if the drug contains a FAP-selective targeting vector.

In 2018, the high-affinity and selective FAP inhibitor (FAPi) UAMC-1110 was used as a targeting vector in the first small-molecule radiotracers that successfully visualized various epithelial tumors via positron emission tomography (PET). Within a few years, molecular imaging with these mainly ^{68}Ga -labeled radiotracers showed very promising diagnostic results in PET. However, translation into effective therapeutic radiopharmaceuticals for radioligand therapy (RLT) has proven to be and still is challenging. The main problem is the short tumor residence time. Substantial progress regarding the tumor-absorbed radiation dose could be achieved with the FAPi homodimer DOTAGA.(SA.FAPi)₂, which contains two FAPi targeting vectors and a bifunctional chelator for radiolabeling.

Novel chelator-based FAPi radiopharmaceuticals were developed, prepared and investigated within this work for improved theranostic (diagnostic and therapeutic) application in various epithelial tumors. After organic synthesis of the compounds, the radiolabeling properties including the complex stability and lipophilicity of the labeled compounds were investigated. Furthermore, binding affinity studies for FAP and the closely related and competitive proteases PREP, DPP4, DPP8 and DPP9 were performed *in vitro*. In addition, some of the compounds were used in first clinical studies.

Projects 1 and 2 particularly focused on improved therapeutic radiopharmaceuticals. For this purpose, a new molecular design for FAPi homodimers was developed in which the two FAPi targeting vectors and the bifunctional chelator are linked via a central linker unit.

The compounds DOTAGA.Glu.(FAPi)₂ and DO3A.Glu.(FAPi)₂ were synthesized in project 1. Subsequently, radiolabeling with gallium-68, lutetium-177, actinium-225, and yttrium-90 was investigated. In all cases, high to quantitative radiochemical conversion (RCC) was achieved and radiopharmaceuticals of high radiochemical purity (RCP > 98%) were synthesized. All radiopharmaceuticals showed high complex stability over two half-lives (of the respective radiometal) in different media and were hydrophilic. The *in vitro* studies revealed very high affinity FAP with subnanomolar *IC*₅₀ values and high selectivity over the related proteases.

The promising results could be confirmed in a first clinical application. [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ showed similar tumor retention in a thyroid cancer patient compared to the previous treatment with [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂. In addition, improved pharmacological properties with faster excretion kinetics and low uptake in the liver and colon were observed that reduced the radiation dose to these organs.

More than 70 heavily pretreated patients have been and are being treated with [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂, some of whom were previously treated with [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂. These have mainly been thyroid and breast cancer patients, but also included one glioblastoma patient, for example, who experienced a significant decrease in primary tumor volume after RLT with [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂. Preliminary results in 19 breast cancer patients show a positive response with 95% pain response and partial remission of tumor volume in at least four of the nine patients still alive after 14 months. Over 50% of 39 thyroid cancer patients show a decrease in the tumor marker thyroglobulin.

In Project 2, five additional FAPi homodimers were synthesized. The linker unit was varied or additional spacer units were introduced compared to [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂. Quantitative RCC and high RCP over 98% were achieved in the radiolabeling with gallium-68 and lutetium-177 except for the ⁶⁸Ga-labeling of one compound. [⁶⁸Ga]Ga-DOTAGA.PEG2.Glu.(FAPi)₂ showed improved labeling properties as it could be equally labeled in several buffer systems. All compounds showed similarly hydrophilicity than [⁶⁸Ga]Ga- or [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂. All prepared radiolabeled compounds exhibited complex stability over 90% over two half-lives except for one. The *in vitro* studies yielded similar results as for DOTAGA.Glu.(FAPi)₂. Both compounds DOTAGA.NPyr.(FAPi)₂ and DOTAGA.PEG2.Glu.(FAPi)₂ showed slightly improved DPP4 selectivity. DO3A.NPyr.(FAPi)₂ exhibited improved DPP4 and PREP selectivity.

The promising radiolabeling and *in vitro* properties of the new FAPi homodimers encourage following therapeutic applications *in vivo*.

The third project aimed to develop new FAPi radiotracers to improve diagnosis via ^{68}Ga -PET before and after RLT with $[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu.(FAPi)}_2$. FAPi monomers were prepared that differed structurally only in one moiety. One FAPi targeting vector was substituted by an "inert" group (pyrrolidine, Pyr).

After organic synthesis of the compounds DATA^{5m-}, DO3A- and DOTAGA.Glu(Pyr).FAPi, the precursors were radiolabeled with gallium-68. Quantitative RCC and high RCP over 98% was achieved for all compounds in each of three different buffer systems investigated. $[^{68}\text{Ga}]\text{Ga-DO3A.Glu(Pyr).FAPi}$ and $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu(Pyr).FAPi}$ were similarly hydrophilic than $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu.(FAPi)}_2$ and both new radiotracers were ca. 95% stable in different media over two hours. *In vitro* studies of the precursors and natGa complexes showed improved affinity and selectivity (the DO3A and DOTAGA derivatives in particular) compared to $[^{68}\text{Ga}]\text{Ga-DOTA.SA.FAPi}$, the FAPi radiotracer that is used in ^{68}Ga -PET-guided ^{177}Lu -FAPi homodimer RLT to date.

$[^{68}\text{Ga}]\text{Ga-DO3A.Glu(Pyr).FAPi}$ and $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu(Pyr).FAPi}$ showed high tumor accumulation in a first human application. Physiological uptake in the pancreas comparable to $[^{68}\text{Ga}]\text{Ga-DOTA.SA.FAPi}$ was observed. Otherwise, accumulation in healthy organs was low except that $[^{68}\text{Ga}]\text{Ga-DO3A.Glu(Pyr).FAPi}$ showed some liver uptake while $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu(Pyr).FAPi}$ still had some activity circulating in the blood one hour post-injection. Overall, the results are promising. Whether one or several of the new compounds provides advantages over $[^{68}\text{Ga}]\text{Ga-DOTA.SA.FAPi}$ when used in PET before and after ^{177}Lu -FAPi dimer therapy needs to be systematically investigated in further (pre)clinical studies.

$^{99\text{m}}\text{Tc}$ -radiotracers for use in single photon emission computed tomography (SPECT) were developed in the fourth project. The two chelators N4 and MAG3 were chosen for the complexation of $^{99\text{m}}\text{Tc(V)}$ and squaric acid (SA) was utilized as a linker analogous to DOTA.SA.FAPi. The precursors NA.SA.FAPi and S-Bz-MAG3.SA.FAPi were successfully prepared in a seven- and nine-step organic synthesis, respectively. The subsequent *in vitro* studies demonstrated high affinity, similar PREP and improved DPP4 selectivity compared to DOTA.SA.FAPi. In addition, the radiolabeling of $[^{99\text{m}}\text{Tc}]\text{Tc-MAG3.SA.FAPi}$ was investigated at room temperature and at 90 °C and quantitative RCC was achieved in both cases. High stability ($\geq 90\%$) was observed after four hours of incubation in diverse media. Furthermore, a first automated synthesis was performed.

$[^{99\text{m}}\text{Tc}]\text{Tc-MAG3.SA.FAPi}$ seems to be a suitable candidate for *in vivo* SPECT studies.

Zusammenfassung

Tumore bestehen nicht nur aus Krebszellen, sondern sind von einer Tumormikroumgebung umgeben, die sich aus verschiedenen Komponenten wie aktivierten Fibroblasten, Immunzellen, vaskulären Zellen, extrazellulärer Matrix etc. zusammensetzt. Die sogenannten Krebs-assoziierten Fibroblasten machen in vielen epithelialen Tumoren einen großen Anteil an der Gesamttumormasse aus. Das Fibroblasten-Aktivierungs-Protein alpha (FAP) ist eine besonders interessante onkologische Zielstruktur, da es von aktivierten Fibroblasten stark exprimiert wird und in mehr als 90% epithelialer Tumore vorkommt. Diese Adressierung ist sehr interessant, da dieser Pan-Tumor-Ansatz die Anwendung in sehr vielen verschiedenen Krebsarten ermöglicht.

FAP ist eine Transmembran-Serinprotease und ist neben der starken Expression in der Tumormikroumgebung auch in Prozessen wie Wundheilung, (chronischen) Entzündungen, Fibrosen (Leber, Bauchspeicheldrüse, Lunge) oder rheumatoider Arthritis beteiligt. Im Gegensatz dazu ist in gesundem Gewebe nicht oder nur sehr schwach exprimiert. Diese selektive Expression ist eine essentielle Bedingung für die Eignung als onkologisches Target. Es ermöglicht die selektive Anreicherung eines diagnostischen oder therapeutischen Medikaments im Tumor bzw. der Tumormikroumgebung, wenn dieses einen FAP-selektiven Targetvektor beinhaltet.

Ein hochaffiner und selektiver FAP-Inhibitor (FAPi) UAMC-1110 wurde 2018 als Targetvektor für die ersten niedermolekularen Radiopharmaka verwendet, die verschiedenste epitheliale Tumore mittels Positronen-Emissions-Tomographie (PET) erfolgreich visualisieren konnten. Innerhalb weniger Jahre konnte gezeigt werden, dass die molekulare Bildgebung mittels PET dieser hauptsächlich ^{68}Ga -markierten Radiopharmaka sehr vielversprechende diagnostische Ergebnisse erbringt. Die Translation hin zu wirksamen therapeutischen Radiopharmaka für die Endoradiotherapie (ERT) ist jedoch weiterhin herausfordernd. Problematisch ist hier vor allem die zu geringe Verweildauer im Tumor. Fortschritte bzgl. der im Tumor deponierten Strahlendosis konnten mit dem FAPi Homodimer DOTAGA.(SA.FAPi)₂ erreicht werden, welches zwei FAPi Targetvektoren und einen bifunktionellen Chelator für Radiomarkierung besitzt.

Im Rahmen dieser Arbeit wurden Chelator-basierte FAPi-Radiopharmaka entwickelt, hergestellt und untersucht, die eine verbesserte theranostische, d.h. diagnostische und therapeutische, Anwendung in verschiedenen epithelialen Tumoren ermöglichen. Nach der organischen Synthese der Verbindungen wurden die Radiomarkierungseigenschaften inklusive Komplexstabilität und Lipophilie der hergestellten Radiopharmaka untersucht. Weiterhin wurden Bindungs-affinitätsstudien bzgl. FAP und den konkurrierenden Proteasen PREP, DPP4, DPP8 und DPP9 durchgeführt, um die Affinität und Selektivität *in vitro* zu bestimmen. Einige der Verbindungen wurden darüber hinaus in ersten klinischen Studien eingesetzt.

Besonderer Fokus lag auf verbesserten therapeutischen Radiopharmaka, weshalb in Projekt 1 und 2 dieser Arbeit neue FAPi-Homodimere hergestellt wurden. Dafür wurde ein neues molekulares Design konzipiert, bei dem die beiden FAPi-Targetvektoren und der Chelator über eine zentrale Linker-Einheit miteinander verknüpft werden.

Die Verbindungen DOTAGA.Glu.(FAPi)₂ und DO3A.Glu.(FAPi)₂ wurden in Projekt 1 synthetisiert. Anschließend wurde die Radiomarkierung mit Gallium-68, Lutetium-177, Actinium-225 und Yttrium-90 untersucht. In allen Fällen konnte eine (nahezu) quantitative radiochemische Umsetzung (RCU) erzielt und Radiopharmaka in hoher radiochemischer Reinheit (RCP > 98%) hergestellt werden. Alle Radiopharmaka zeigten hohe Komplexstabilitäten über zwei Halbwertszeiten in verschiedenen Medien und waren hydrophil. Die *in vitro*-Studien ergaben eine sehr hohe FAP-Affinität mit subnanomolaren IC₅₀-Werten und hohe Selektivität gegenüber den Konkurrenzproteasen.

Die vielversprechenden Ergebnisse wurden in einer ersten klinischen Anwendung bestätigt. [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ zeigte eine ähnlich lange Tumorverweilzeit in einem Patient mit Schilddrüsenkrebs im Vergleich zur vorherigen Behandlung mit [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂. Darüber hinaus wurden verbesserte pharmakologische Eigenschaften, eine schnellere Ausscheidungskinetik und geringere Aufnahme in Leber und Darm beobachtet, welches die Strahlendosis für diese Organe reduziert.

Über 70 stark vorbehandelte Patienten wurden und werden mit [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ behandelt, wovon einige vorher mit [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂ therapiert wurden. Dies sind hauptsächlich Schilddrüsen- und Brustkrebs-Patienten, aber auch ein Glioblastoma-Patient, der einen deutlichen Rückgang des Primärtumors nach Radioligandentherapie mit [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ verzeichnete. Die vorläufigen Ergebnisse an 19 Brustkrebspatienten zeigten eine positive Ansprache mit verringerten Schmerzen in 95% und eine partielle Remission des Tumolvolumens in mindestens vier der neun nach 14 Monaten noch lebenden Patienten. Bei über 50% der 39 Schilddrüsenkrebspatienten konnte ein Rückgang des Tumormarkers Thyreoglobulin beobachtet werden.

In Projekt 2 wurden fünf weitere FAPi-Homodimere synthetisiert, bei denen im Vergleich zu [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ die Linker-Einheit variiert oder zusätzliche Spacer-Einheiten eingeführt wurden. In den Radiomarkierungen mit Gallium-68 und Lutetium-177 wurden bis auf die ⁶⁸Ga-Markierung eines Derivates quantitative RCU und hohe RCP über 98% erreicht. [⁶⁸Ga]Ga-DOTAGA.PEG₂.Glu.(FAPi)₂ zeigte verbesserte Markierungseigenschaften und ließ sich in mehreren Puffersystemen gleich gut markieren. Alle Verbindungen waren ähnlich hydrophil wie [⁶⁸Ga]Ga- bzw. [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂. Bis auf einen Komplex, wiesen alle hergestellten Radiopharmaka eine Komplexstabilität über 90% über zwei Halbwertszeiten auf. Die *in vitro*-Bindungsstudien ergaben ähnliche Ergebnisse wie für DOTAGA.Glu.(FAPi)₂. Die beiden

Verbindungen DOTAGA.NPyr.(FAPi)₂ und DOTAGA.PEG2.Glu.(FAPi)₂ zeigten leicht verbesserte DPP4-Selektivität, DO3A.NPyr.(FAPi)₂ verbesserte DPP4- und PREP-Selektivität.

Die vielversprechenden Eigenschaften machen die neuen FAPi-Homodimere interessant für folgende *in vivo*-Applikationen.

Das dritte Projekt hatte das Ziel neue FAPi-Radiopharmaka herzustellen, welche die Diagnostik mittels ⁶⁸Ga-PET vor und nach einer Therapie mit [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ verbessern sollen. Es wurden FAPi-Monomere hergestellt, die sich strukturell nur dadurch unterscheiden, dass ein FAPi-Targetvektor durch eine „inerte“ Gruppe (Pyrrolidin, Pyr) substituiert wurde.

Nach der organisch-chemischen Synthese der Verbindungen DATA^{5m}-, DO3A- und DOTAGA.Glu(Pyr).FAPi wurden diese mit Gallium-68 markiert. Quantitative RCU und hohe RCP > 98% wurde für alle Verbindungen in jeweils drei verschiedenen Puffersystemen erreicht. [⁶⁸Ga]Ga-DO3A.Glu(Pyr).FAPi und [⁶⁸Ga]Ga-DOTAGA.Glu(Pyr).FAPi waren ähnlich hydrophil wie [⁶⁸Ga]Ga-DOTAGA.Glu.(FAPi)₂ und beiden neuen Radiotracer waren nach zwei Stunden in verschiedenen Medien zu ca. 95% stabil. *In vitro*-Studien der Präkursoren und ^{nat}Ga-Komplexe zeigten verbesserte Affinität und Selektivität gegenüber dem bisher in der Therapiekontrolle verwendeten [⁶⁸Ga]Ga-DOTA.SA.FAPi, besonders für das DO3A- und DOTAGA-Derivat.

Beide Verbindungen zeigten in einer ersten humanen Anwendung hohe Tumoranreicherung. Es wurde physiologischer Uptake im Pankreas vergleichbar zu [⁶⁸Ga]Ga-DOTA.SA.FAPi beobachtet. Ansonsten war die Akkumulation in gesunden Organen gering, außer dass [⁶⁸Ga]Ga-DO3A.Glu(Pyr).FAPi etwas Leber-Uptake zeigte und [⁶⁸Ga]Ga-DOTAGA.Glu(Pyr).FAPi eine Stunde nach Injektion noch etwas Aktivität im Blut zirkulierte. Insgesamt sind die Ergebnisse vielversprechend. Ob eine oder mehrere der neuen Verbindungen Vorteile gegenüber [⁶⁸Ga]Ga-DOTA.SA.FAPi bei der PET vor und nach ¹⁷⁷Lu-FAPi-Dimer-Therapie bringt, muss in weiteren (prä)klinischen Studien systematisch untersucht werden.

Im Rahmen des vierten Projektes wurden ^{99m}Tc-Radiopharmaka für die Anwendung in der Einzelphotonen-Emissionscomputertomographie (SPECT) entwickelt. Dafür wurden die beiden Chelatoren N4 und MAG3 wurden für die Komplexierung von ^{99m}Tc(V) gewählt, sowie die Quadratsäure (SA) als Linker analog zu DOTA.SA.FAPi. Die beiden Markierungsvorläufer NA.SA.FAPi und S-Bz-MAG3.SA.FAPi wurden in einer sieben- bzw. neunstufigen Synthese erfolgreich hergestellt und *in vitro* untersucht. Es konnten hohe Affinitäten, ähnliche PREP- und verbesserte DPP4- Selektivitäten im Vergleich zu DOTA.SA.FAPi gemessen werden. Außerdem wurde die Radiomarkierung von [^{99m}Tc]Tc-MAG3.SA.FAPi bei Raumtemperatur und 90 °C untersucht und in beiden Fällen konnte quantitative RCU erreicht werden. Es wurde eine hohe Stabilität (≥ 90%) nach vier Stunden Inkubation in diversen Medien beobachtet. Des Weiteren wurde eine erste Synthese-Automation durchgeführt.

[^{99m}Tc]Tc-MAG3.SA.FAPi scheint ein geeigneter Kandidat für *in vivo*-SPECT-Untersuchungen.

Für meine Liebsten

*„Man braucht nichts im Leben zu fürchten,
man muss nur alles verstehen.“*

Marie Curie

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1. Introduction: From Tumor Microenvironment to FAP-targeting Radiopharmaceuticals

Cancer is still the number two cause of death worldwide after cardiovascular diseases (ca. 10 million fatalities per year) and due to aging and growing population the total number of cases are still increasing [1]. The most common cancer type is breast cancer in women and prostate cancer in men and the most death cases result from lung cancer in both sexes [2]. For example, approximately 40% of the US population will be diagnosed with cancer at some point in their lifetime [2].

The wide use of computed tomography (CT) and magnetic resonance imaging (MRI) improved morphological imaging substantially compared to X-ray imaging. Molecular imaging with positron emission tomography (PET) and single-photon emission computed tomography (SPECT) is able to deliver totally different valuable diagnostic information. Biochemical, metabolic processes *in vivo* are visualized by using radiotracers as established by GEORGE DE HEVESY (Nobel Prize in Chemistry, 1943) [3]. Small amounts of a suitable radiotracer do not cause any pharmacological interactions but do participate in the metabolism. Positron emitters are required for PET whereas gamma-ray emitting radionuclides are needed for SPECT. Modern scanners like PET or SPECT combined with CT or MRI unite the respective advantages and enable a much more precise visualization and diagnosis [4].

New forms of therapy improve anti-tumor efficacy and minimize adverse side effects since systemic chemotherapy is unspecific and surgery or external beam therapy are not suited to treat metastasized cancer [5]. In nuclear medicine, β^- - or α -emitting radionuclides that are characterized by short range are used for endoradiotherapy (ERT), also called radionuclide therapy (RNT). The ionizing radiation causes DNA and cell damage and leads to apoptosis [6,7]. Over 80 years ago, SAUL HERTZ pioneered RNT with radioiodine [^{131}I]I-NaI to treat thyroid diseases [8].

The radiopharmaceutical must accumulate in the tumor lesion selectively. Off-target accumulation will damage healthy tissue and might lead to side effects. This can be achieved by coupling the radionuclide to a targeting vector (small molecule inhibitor, peptide, antibody etc.) with high affinity and selectivity to the biological target (enzyme, receptor, antigen etc.) [9,10].

The optimal synergy of diagnosis and treatment is one of the keys to optimize patient care which is referred to as “theranostics”. In the context of nuclear medicine, this means to find a diagnostic and a therapeutic radiopharmaceutical that can form a more or less matched pair for precise PET/SPECT diagnosis (pre- and post-treatment) to support treatment planning and assessment.

Practically all radiometal-based theranostic strategies rely on a tumor-selective targeting vector (TV) addressing a specific oncologic molecular target. For example, octreotide derivatives like TOC or TATE target somatostatin receptors (SSTRs) strongly expressed in neuroendocrine tumors. Inhibitors address the prostate specific membrane antigen (PSMA) in prostate cancer or bisphosphonates accumulate in bone metastases (hydroxyapatite) etc. [7,11,12].

1.1. Tumor Microenvironment

Tumors are not just accumulations of more or less rapidly proliferating cancer cells. It rather represents an extremely complex composition of multiple cell types that interact with each other as well as with the surrounding tissue in various forms. The non-malignant cells of tumor lesions are grouped under the term tumor microenvironment (TME) or tumor stroma.

In an attempt to logically organize the complexity and diversity of neoplastic diseases HANAHAN et al. [13] identified 6 key characteristics of cancer [13–15]:

1. keeping a sustained proliferative signaling
2. resistance to apoptosis
3. escaping growth suppressing mechanisms
4. unlimited replication (“immortal cells”)
5. stimulating angiogenesis
6. triggering tumor invasion and metastasis

These hallmarks were later updated and extended by the following [14,15]:

7. customizing the cellular energy metabolism
8. escaping immune response and thus avoiding immunological destruction
9. genome instability leading to facilitative mutation
10. continuous tumor-promoted and -promoting inflammation

All these points already consider the active role of the tumor microenvironment (TME) in tumorigenesis. Tumorigenesis can be subdivided into three stages: initiation, progression and metastasis. The TME plays a crucial and active role in all three stages and is not just a passive bystander [13,14,16]. The TME is composed of different non-malignant cell types such cancer-associated fibroblasts (CAFs), leukocytes and lymphocytes (innate and adaptive immune cells, inflammatory cells), vascular cells (blood and lymphatic endothelial cells, pericytes), adipocytes, mesenchymal stem cells (MSCs) as well as extracellular matrix (ECM) [15,17]. The molecular cross-talk between the cancer cells and its surrounding TME is mediated through direct cell-cell interactions or through a multitude of secreted molecules such as cytokines, chemokines, growth factors, proteases and their respective inhibitors (autocrine and paracrine signaling) [18,19].

The tumor stroma fraction of epithelial cancers is dependent on the cancer type, the lesion diameter and the disease stage. For example, a machine-learning supported meta-analysis in 16 cancer types gave mean values of stroma percentage between 19% (kidney cancer) and 73% (periampullary cancer) with strong differences from patient to patient and over 90% in some of the patients [5,20]. In contrast to previous assumptions, no clear correlation between tumor stroma fraction and overall survival was found [20]. However, this does not question the crucial role of TME in tumorigenesis.

Typical features of the TME include hypoxia [21–23], increased aerobic glycolysis rate (WARBURG effect) [24,25], acidosis [23,26], interstitial fluid pressure [27] and glutamine addiction [28–32] among others. Hypoxia triggers many signaling pathways that are connected to the key characteristics of cancers such as the metabolic reprogramming, also referred to as the “*metabolic switch*” (cf. hallmark 7.). It was found that the isoform pyruvate kinase M2 (PKM2) is exclusively found in TCs and that the switch from PKM1 to PKM2 is the reason for the switch from oxidative phosphorylation to aerobic glycolysis [33]. VANDER HEIDEN et al. [34] proposed that the facilitated anabolic metabolism (synthesis of essential biomolecules like nucleotides, amino acids, etc.) for increased cell proliferation is in favor of the less efficient ATP production. Later, the underlying mechanism could be elucidated why PKM2 is formed in TCs that have higher energy demand although PKM2 has lower glycolytic activity [35]. Another ATP-independent pathway produces pyruvate directly instead of normal ATP-mediated pyruvate production via pyruvate kinase [35]. The metabolic reprogramming is not limited to the tumor cells (TCs) but also involves the TME. Because of the dominating aerobic glycolysis that produces ATP less efficiently than oxidative phosphorylation, glucose uptake as well as glucose transporters and the enzymes involved in glycolysis are upregulated [36,37]. This generates a lot of lactate which is secreted by lactate transporter monocarboxylate transporters and directly leads to acidosis (low pH) in the TME.

The different cell types that are present in the TME will be discussed in the following sections. Figure 1 shows the composition of a tumor lesion schematically.

1.1.1. Immune and Inflammatory Cells

Immune cells can be either tumor suppressing or tumor promoting depending on the context. In parallel to the three steps of tumorigenesis, it is suppressive in the first stage (“elimination”) but gradually transforms from “equilibrium” to “escape” of the immune surveillance by phenotype changes like epithelial-mesenchymal transition (EMT). This can alternatively be described as the immune system helping the tumor to mutate to a favoring, dominant cell type that avoids immune response [15,38]. The immune cell distribution can be classified in immune infiltrated (“hot”, high homogenous leukocyte infiltration), immune excluded (only in the periphery) and immune silent (“cold”, no infiltration) [16,39].

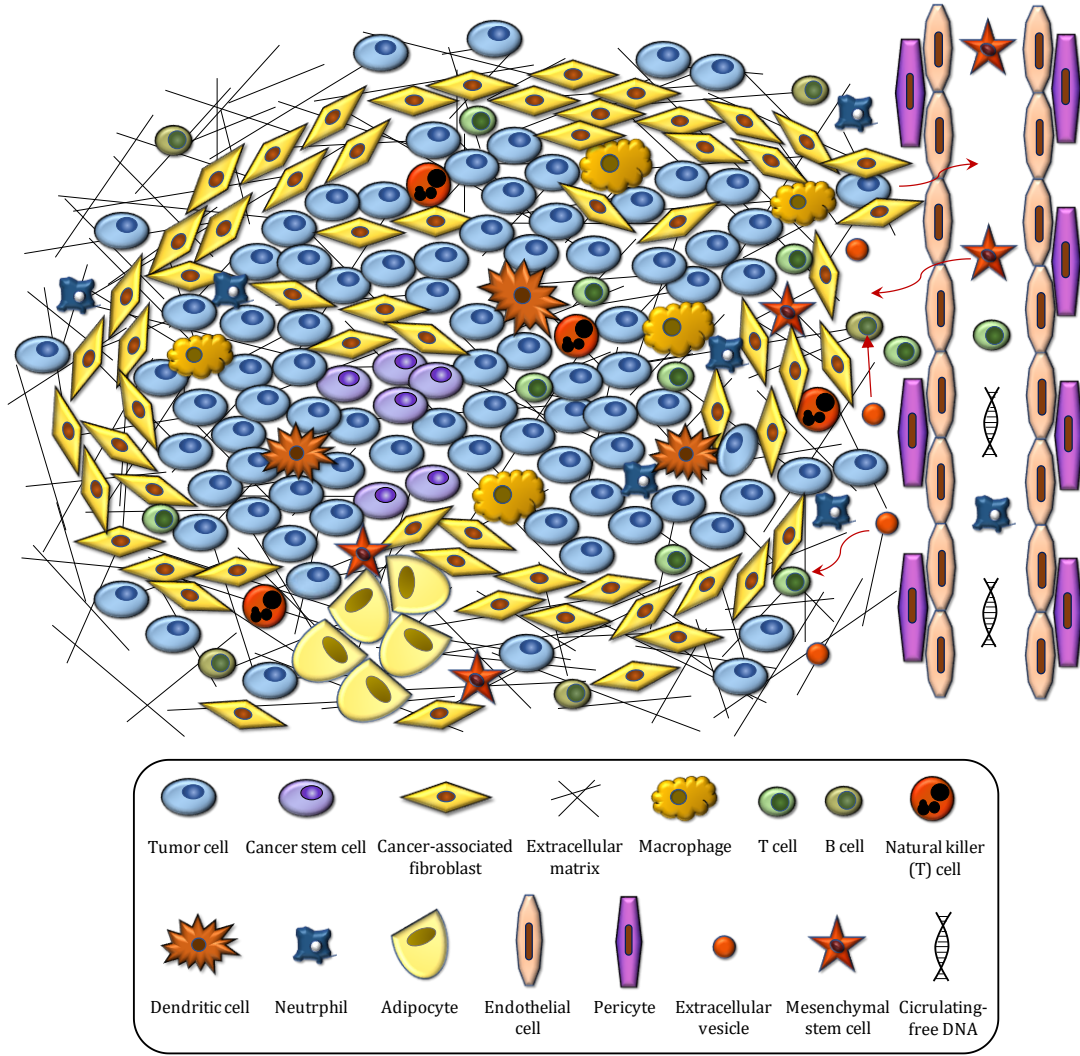


Figure 1: Schematic composition of the tumor and tumor microenvironment including all of the important cellular components (inspired by BAGHBAN et al. [40]).

Adaptive immune cells including T and B cells are activated by specific antigens [15,16,38,41]. T lymphocytes like CD4⁺ T cells are responsible for the cellular immune response and important in regulating the immune system and differentiate into different T-helper cell (T_H) subtypes. For example, differentiation into proinflammatory T_H1 by interleukin-12 (IL-12) [39] leads to recruiting cytotoxic CD8⁺ T cells and natural killer cells (NK) that are especially associated with positive cancer prognosis [15,16,38,42,43]. In contrast, T_H2 are immunosuppressive by secreting many different interleukins such as IL-4 [41,43,44]. Regulatory T cells (T_{Reg}) control the cytotoxic T cell population and extinguish their activity. There are different mechanisms of T cell inhibition and thus have an immunosuppressive effect: 1. secreting cytokines like IL-10 and transforming growth factor β (TGF- β), 2. consuming tryptophan that is essential for the cytotoxic T cell, 3. producing toxic metabolites (reactive oxygen species, ROS) to interfere with T cell activity [39,42,44]. B cells are also lymphocytes but trigger a humoral (non-cellular) immune response by antibody production and antigen recognition. They are mostly found in lymph nodes and are not as vastly found in the TME as T cells. Regulatory B cells (B_{Reg}) behave similar to T_{Reg} [15,16].

Macrophages, neutrophils, dendritic cells and natural killer cells belong to the innate immune cells. There are two main types of macrophages. Macrophages M1 kill cells through phagocytosis (a form of endocytosis) and antigen presentation. M1 are polarized by cytokines that are secreted by T_H1 species [41,43,45]. In contrast, tumor-associated macrophages (TAMs) are mainly polarized as M2 by T_H2 because of hypoxia and the presence of cytokines such as IL-4 or CAF-produced FAP, IL-4/6/8 which resembles a constantly inflamed state [16,43,45–49]. The M2 phenotype is and one of the main immunosuppressive cells among T_{Reg} and myeloid-derived suppressor cells (MDSCs) that are all associated with poor prognosis [15,41,50–52]. Increased or sustained M2 recruitment, e.g. via Hedgehog ligands, contributes to TAMs making up to 50% of the tumor mass and thus being one of the central players in the TME [53–56]. TAMs are also one of the key promoters of angiogenesis. They induce formation of new blood vessels by secreting vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), endothelial growth factor (EGF) and TGF- α/β . On top, they also produce matrix metalloproteinases (MMPs) known for ECM remodeling which indirectly influences the availability of the aforementioned pro-angiogenic growth factors [16,37,43,57,58].

MDSCs share overlapping markers with neutrophils and are sometimes seen as a polarized neutrophil subtype that primarily target T cells [43,59]. Several cytokines (IL-6/10, TGF- β) recruit myeloid cells from the bone marrow that cannot differentiate properly and then become immature MDSCs [60]. Neutrophils are short-lived and quick first responders to pathogens and modulate inflammation mainly by ROS production. In contrast, they are pro-tumorigenic in later stages due to increased release of cytokines like MMPs leading to ECM remodeling and angiogenesis similar to the pro-angiogenic mechanisms of TAMs [16,43].

Dendritic cells are in between the innate and adaptive immune system as they are recognizing, capturing and presenting antigens to T cells at secondary lymphoid organs [16,43]. The role of dendritic cells is very complex as it is dependent on the tumor stage as well as the tumor type and even subtype [43,61–63]. But like with immune cells in general, they tend to become more tumor-promoting with advancing cancer.

Natural killer cells (NK) are extremely efficient tumor cell (TC) eliminators. Unfortunately, they are less efficient in the TME than in the blood stream as solid tumors develop mechanisms to protect themselves, for example by protective collagen or platelet layers or expressing NK-inhibitory receptors [64,65].

1.1.2. The Vascular System

After a tumor lesion reaches a certain size ($\geq 1 - 2$ mm) it starts to build a blood and lymphatic vascular network to improve tumor supply with all necessary nutrients, to reduce hypoxia and thus improve oxygen supply, and remove metabolic waste [15,16,37]. These networks are

characterized by their leaking and twisted nature, increased interstitial fluid pressure, reduced blood pressure and thus are less efficient [15,40,66]. The crosstalk not only involves TCs but immune cells as well. One of the main functions is to support the TCs in evading immune response directly where MDSCs and dendritic cells are able to inhibit the T cell activity [15,67,68].

Endothelial cells (ECs) build a thin monolayer to separate blood vessels and tissue [16]. It is important for supplying nutrients and permeating or carrying other cell types like immune cells [16]. They can undergo TGF- β mediated endothelial-mesenchymal transition (EndMT) and differentiate into CAFs which leads to increasing loss of direct cell-cell contact. This facilitates cell migration and intravasation, a process where TCs adhere to ECs and can enter the blood stream, which is an important factor in tumor invasion and metastasis [16].

Pericytes on the other hand are localized among the outer (abluminal) side of the endothelial cell layer [40]. Their role includes escorting leukocytes and lymphocytes to exit the blood vessel and regulating their activity as well as basement membrane remodeling during angiogenesis [40,69]. As mentioned above vascular endothelial growth factor (VEGF) is probably the most important pro-angiogenic factor [15,37]. VEGF also recruits and mobilizes new progenitor ECs from the bone marrow and drives their differentiation [37,70]. One especially interesting mechanism is the “*angiogenic switch*”. Endothelial cells express the Notch receptor [71,72]. The ligand DLL4 inhibits the Notch receptor whereas the Jagged1 ligand activates it. Therefore, Notch acts like a switch that is more or less permanently turned on in the TME and allows for VEGF-induced signaling that initiates cell division and drives angiogenesis [71,73].

1.1.3. Other Cell Types

Adipocytes regulate energy homeostasis and store excess fat. Overweight generally correlates with poor cancer prognosis. The crosstalk with TCs can trigger lysis of adipocytes to make the stored fatty acids available for TC uptake [16]. Adipocytes secrete over 50 different cytokines. Exemplary, the increased release of the important hormone-like cytokine leptin directly influences tumor metabolism and correlates with the amount of adipose tissue [74,75]. In addition, leptin closely interacts with CAFs and also ECM components like fibronectin and thus seems to play a role in EMT (by TGF- β upregulation), ECM remodeling (by MMP upregulation) and macrophage activation [75–78].

Mesenchymal stem cells (MSCs) originate from bone marrow and are also found in adipose tissue and in the blood. MSCs are able to transdifferentiate into a multitude of different cell subtypes including activated fibroblasts, adipocytes and pericytes etc. [79]. One of the important tumor-promoting mechanisms seems to be the production of exosomes that then induce EMT [80,81].

1.1.4. Extracellular Matrix Components

The extracellular matrix is a heterogeneous network of proteins and other macromolecules such as collagens (type I), glycoproteins (fibronectin and laminins), proteoglycans, hyaluronan, elastin, different integrins and polysaccharides. All cytokines, growth factors and signaling (macro)molecules produced by the surrounding TME cells are secreted into and deposited in the ECM [15,80,82,83]. In early stages of tumorigenesis, it acts as a physical and biological barrier that hampers TC proliferation and tumor invasion into new tissue [80]. But the ECM is progressively remodeled for example by excessive TGF- β -induced collagen production and cross-linking which results in stiffening and fibrosis [83–85]. This can result in overpressure and TCs respond via mechanosensors (e.g. integrin-ECM complexes) that facilitates migration to compensate for the solid stress by compressing the cells [80,86–88]. Solid stress can also lead to poor perfusion exacerbating hypoxia which is compensated by increased expression of VEGF and other pro-angiogenic factors [80,89]. Expression of hypoxia inducible factors (HIFs) is also increased. Hypoxia inhibits prolyl 4-hydroxylase that is responsible for HIF-degradation [83,90,91]. Furthermore, HIF-1 regulates the increasing aerobic glycolysis (WARBURG effect) and thus the lactate production (“*metabolic switch*”) [92,93]. HIF-induced cancer stem cell modulation favors cancer cells to survive better in acidic conditions compared to normal cells [94].

Extracellular vesicles (EVs) such as exosomes are nanoparticles for intercellular communication and constantly secreted into the ECM [95,96]. Exosomes seem to play a role in organ-specific metastasis by preparing cells to be used as pre-metastatic niches that migrating TCs can then interact with and start reprogramming their metabolism [40].

Circulating free DNA (cfDNA) are remnants from former apoptotic or necrotic (tumor) cells. They are short double-stranded DNA fragments that are involved in horizontal gene transfer and thus transforming healthy cells even in distant tissues (“*genometastasis*”) [97–99]. They can cause increased radioresistance because it resembles DNA damage by radiation-induced ROS production and already activates DNA-repairing pathways [100].

The role of TME in tumor invasion and metastasis can be described as a sequence of events [101]. Firstly, ECM remodeling promoted by CAFs is an important mechanism, e.g. by disrupting cell-cell junctions (E-cadherin) [37]. Secondly, formation of invadopodia (small TC agglomerates) and EMT increases cell mobility (further E-cadherin disruption, loss of cell adhesion) leading to facilitated intravasation [102,103]. Thirdly, the survival in the bloodstream is supported by adhesion of TCs to ECs. Similarly, platelets can form a physical barrier to protect the TCs from immune cells [37]. Finally, the primary tumor also secretes cytokines to weaken the cell-cell junctions of the ECs at the distant site. This facilitates the subsequent extravasation [37,104]. Pre-metastatic niches are preferred sites that favor metastasis and the concept was derived from the “seed and soil” concept from STEVEN PAGET over 100 years ago [105,106].

1.2. Cancer-Associated Fibroblasts

Fibroblasts are one of the key cell types in the TME and shall be discussed in more detail. Fibroblasts are of mesenchymal origin and a heterogenous class of spindle- or stellate-shaped cells that build the basic tissue framework (connective tissue) [107,108]. They maintain tissue homeostasis and regulate epithelial differentiation and inflammation processes [49].

During tumor progression, fibroblasts in the TME are activated to a myofibroblast-like phenotype that goes along with morphologic changes and altered expression patterns. There are several mechanisms on how fibroblasts can become cancer-associated fibroblasts (CAFs). The dominant pathway seems to be the activation of “normal” fibroblasts that are in a quiescent “hibernating” state and embedded within the ECM around the tumor. These normal fibroblasts are primarily activated by TGF- β 1 and the process is called mesenchymal-mesenchymal transition (MMT) [19,109,110]. Other cytokines that seem to be involved in fibroblast activation include platelet-derived growth factor (PDGF), FGF2, hematopoietic growth factor (HGF) and different integrins [111–115]. Other pathways are epithelial-mesenchymal transition (EMT) of epithelial cells, endothelial-mesenchymal transition (EndMT) of endothelial cells (cf. Figure 2) [116–119]. Furthermore, CAFs can be recruited from bone marrow-derived mesenchymal stem cells and possibly also from hematopoietic stem cells and adipocytes [80,116,119].

Fibroblast activation is a process that also occurs during wound healing and fibrosis. In wound healing, however, the activation is temporary and the myofibroblasts are removed by apoptosis [120]. In contrast, tumors were described by DVORAK et al. [121] as “wounds that do not heal”, referring to the permanent inflammatory state. Due to the permanent activation, CAFs do not undergo apoptosis [19,116].

CAFs differ from quiescent fibroblasts by enhanced proliferation and cell motility as well as increased secretion of ECM components (collagen, fibronectin, elastin etc.), cytokines etc. [49,122,123]. Morphologically, they are mainly characterized by an enlarged nucleus, endoplasmic reticulum and Golgi apparatus [49,124]. There are some markers that CAFs can be usually identified by, at least by the combination of several of these markers since they are not exclusively expressed by CAFs. The most important are α -smooth muscle actin (α -SMA), additionally expressed by pericytes, smooth muscle cells and some normal fibroblasts, and fibroblast activation protein alpha (FAP: see chapter 1.3 for detailed description) [15,116,125]. α -SMA and FAP expression allow for the classification of different activated subtypes [122,126,127]. Other potential markers include fibroblasts-specific protein 1, vimentin and platelet-derived growth factor (PDGF) receptor [116,128–130].

Two attempts were made to further classify CAFs despite its heterogenous nature. Firstly, CAFs in breast cancer were classified into four subtypes (S1 – S4) according to the expression of 6

markers (FAP, α -SMA, FSP1, CD29, Caveolin 1 and PDGF receptor β) [131]. CAF-S1 were characterized through high expression of all markers except Caveolin 1 and interacted with T lymphocytes in a complex mechanism that increases T_{Reg} level in a positive feedback loop and thus inhibits effector T cell ($CD4^+/CD8^+$ ratio) activity [131,132]. Furthermore, activated TGF- β and CXCL12 pathways lead to increased EMT whereas CAF-S4 induce invasion via Notch pathway [131,133]. Secondly, two different subtypes, an inflammatory cell type (iCAFs) and myofibroblasts (myCAFs), were found in several cancer types [122,131,134]. MyCAFs are localized nearby tumor cells, predominantly interact via TGF- β pathway and are characterized by high levels of α -SMA. On the other hand, iCAFs are more distant, and characterized by IL-1 β -activated NF- κ B pathway and thus secreting inflammatory cytokines like IL-6, or the chemokines CXCL1 and CXCL12 [122,131,134]. Furthermore, exclusive hyaluronan synthases expression was found on iCAFs, that release synthesized hyaluronan into the ECM [122,135]. In addition, antigen-presenting CAFs (apCAFs) were identified in pancreatic cancer where they seem to promote immunosuppressive T_{Reg} production [122,135].

1.2.1. CAF-Crosstalk With Other Cell Types

It is worth to take a closer look at the interaction and crosstalk between TCs and CAFs which is schematically summarized in Figure 2.

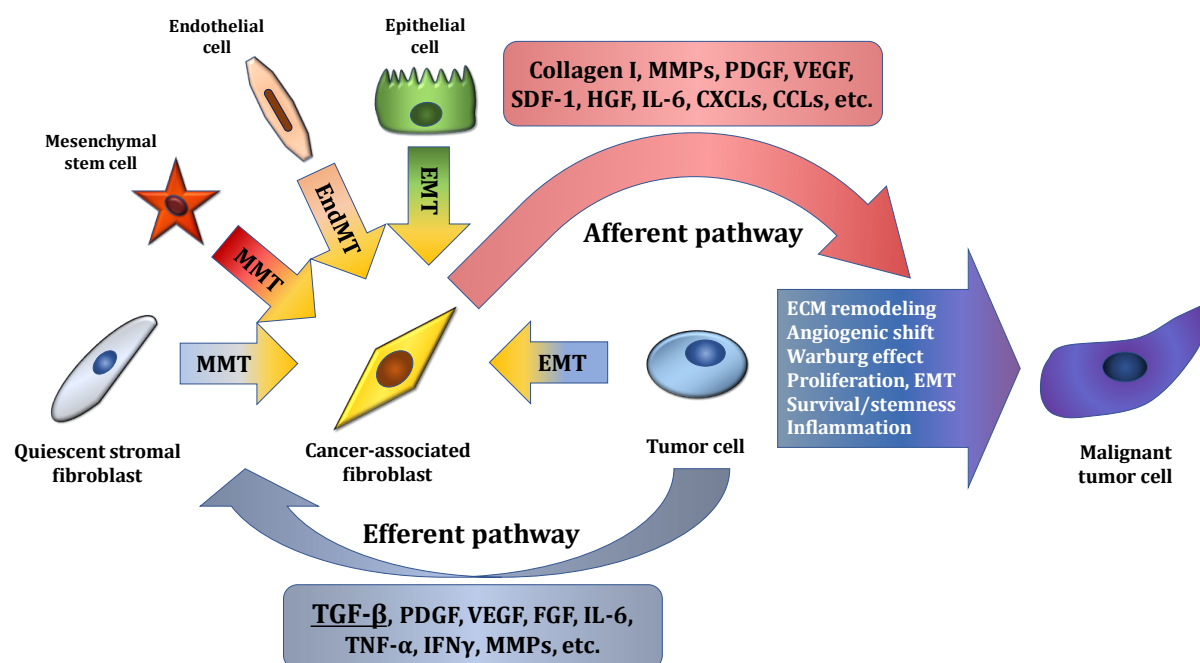


Figure 2: Crosstalk of stromal fibroblasts and tumor cells creating a feedback loop with afferent and efferent pathway (inspired by CIRRI et al. [119]).

It was suggested that epithelial TCs are capable of inducing aerobic glycolysis in neighboring stromal fibroblasts upon their activation. The excessively produced lactate and pyruvate are

released via MCT4 into the ECM and can be taken up by the MCT1 transporters of the TCs which enhances their oxidative phosphorylation capacity to fuel their energy production. This phenomenon was termed “Reverse WARBURG Effect” because of the **afferent pathway** of CAFs to the TCs [119,136]. Interestingly, RATTIGAN et al. [137] reported that this also seems to work in the opposite direction. CAFs are able to take up the lactate, released from TCs. Lactate is then transformed to pyruvate via lactate dehydrogenase (LDH-B) that can then supply the CAFs with energy through ATP production via oxidative phosphorylation (TCA cycle) [137].

The “**efferent pathway**” in Figure 2 summarizes the tumoral reprogramming of the TME by TCs that leads to fibroblast activation. This is mainly driven by transforming growth factor β (TGF- β) via mesenchymal-mesenchymal transition (MMT) of stromal fibroblasts [119].

TGF- β was already mentioned several times before and shall be discussed in more detail due to its central role in fibroblast activation. TGF- β can be subdivided into three subtypes (β 1, β 2, β 3) that are secreted into the ECM [49]. TGF- β 1 binds to a heterodimeric TGF- β receptor type I and II complex on fibroblast cell membranes [49]. Translocation of the heterodimer complex to the nucleus after phosphorylation occurs where different gene expressions are regulated, including transcription of PDGF, VEGF, MMPs, EndMT processes, etc. [18,49,138]. TGF- β 1 plays a critical role in the wound healing process and recruits inflammatory cells as well as being predominantly responsible for stimulating type I and III collagen production, also indirectly via MMP-inhibition [110,139]. Interestingly, its role is highly contextual. It has an immune- and proliferation-regulating role in healthy tissue (and early stages of tumorigenesis). In contrast, it is one of the most important factors in tumor growth as the key player in fibroblast activation into an invasive, tumor-promoting phenotype [19,49,123]. In cancer, TGF- β is mainly expressed by TCs directly and thus strongly upregulated in the TME [140,141].

TGF- β 1 also seems to upregulate β 1-integrins through PI3K/Akt signaling which activated NF- κ B pathway in human lung cancer [19,142]. Simplified, PI3K/Akt signaling is involved in the proliferation of cells [143] and NF- κ B signaling results in the production of pro-inflammatory and anti-apoptotic genes and cytokines which will improve cell survival [143,144]. Additionally, TGF- β was found to be responsible for increased ROS levels that seem to downregulate paracrine signaling via gap junctions in CAFs as a consequence [145,146]. Tumor invasion was indirectly promoted by TGF- β 1 through HGF-dependent invasion pathways [147,148].

Other complex mechanisms in the crosstalk of CAFs and TCs also take place with other cytokines but only a couple should be mentioned briefly. As discussed above, VEGF is the central cytokine promoting angiogenesis [70,72,149]. High IL-6 levels lead to increased VEGF production in several fibroblasts upon stimulation with TNF- α and seem to transform into CAFs [116,150]. IL-6 supports the TCs repair mechanisms by activating anti-apoptotic pathways [151,152]. Platelet-derived growth factor (PDGF) and FGF are mainly regulating the fibroblast proliferation, survival

and differentiation in wound healing. Their role is highly contextual and can be exploited by TCs to work in a tumor-promoting way [18,19,153–155]. PDGF can indirectly recruit CAFs by stimulating TGF- β in macrophages [18,19,153]. Besides that, PDGF helps to stabilize blood vessels and can indirectly enhance angiogenesis by promoting VEGF production [116,156]. Other CAF-secreted cytokines like CXCL12 and FGFs (mainly FGF-2) are also involved in angiogenesis by VEGF-independent pathways [116,157,158].

Beyond local tumor invasion, CAFs can also support the metastasis to distant tissues. They form adhesive complexes with the metastatic TCs that improve their viability and help to prevent direct contact to immune cells in the bloodstream after intravasation to evade immune response [18,159].

1.2.2. ECM Remodeling

One of the central attributes of CAFs is the remodeling of the ECM as CAFs can synthesize many ECM components such as type I and IV collagens, laminin, fibronectin as well as connective tissue growth factors or other cytokines like MMPs [19,80,160]. The ECM remodeling leads to increased tissue stiffness that is characteristic of many epithelial tumors [80,123]. In turn, this mechanical stress stimulates fibroblast activation [49,161]. On top, it has an effect on integrin signaling and the actin microfilaments in the cytoskeleton, thus influencing the cell cycle [162].

MMPs are considered central ECM-degrading proteases [19]. MMP-2 and -9 are known to degrade type IV collagen and laminin which are key components of the basement membrane, a thin layer of ECM that separates epithelial from connective tissue [116]. MMP-9 is either activated by other MMPs (e.g. MMP-3 and -13) or by TNF- α or TGF- β -triggered production in CAFs [116,163]. Actually, MMPs are involved in many more tumor-promoting mechanisms that indirectly result out of degrading ECM components. It includes regulation of growth factors, adipocytes, inflammation and angiogenesis as well as preparation of pre-metastatic niches [164].

Another factor that crucially impacts local tumor invasion is the weakening of cell-cell junctions that facilitates migration of TCs in between other TME cells. In addition, the degradation of cell adhesion via E-cadherin interrupts paracrine signaling [165]. Secretion of type I collagen down-regulates E-cadherin gene expression and stabilizes EMT by loosening the direct cell-cell contacts [166]. Loss of cell-cell contacts is one of key changes during EMT. CAFs can also induce EMT in TCs, creating more motile mesenchymal phenotypes and thus supporting the transformation of local TCs into motile metastasizing TCs, e.g. via MMP-3 [167–170].

CAFs also interact with immune cells to create a physical immune barrier in the ECM. They promote the polarization of macrophages into the immunosuppressive M2 subtype (cf. chapter 1.1.1.) whereas TAMs secrete IL-6 and CXCL12 which in turn activates fibroblasts, creating another positive feedback loop [49,160,171].

1.2.3. The Role of CAFs in Therapy Resistance

CAFs and other components of the TME are genetically more stable than TCs which reduces the emergence of genetically induced resistance to therapy although this was observed in a few studies [172,173]. In short, CAF-mediated drug resistance includes ECM remodeling, TC communication via cytokine secretion and exosomes, metabolic reprogramming in cooperation with TCs and inducing genetic alterations in TCs [79].

The role of CAFs (or TME in general) in therapy resistance became noticeable for the first time when differences between drug resistance *in vivo* vs. *in vitro* was noted [174,175]. High interstitial pressure hampers the extravasation of the drug, which can be a result of TME stiffness or high PDGF levels [119,174,176]. Besides that, the heterogenous blood supply because of the poorly developed vascular system can lead to long interstitial transport distances for the agent to reach the tumor target [174]. CAF-driven increase of hyaluronic acid and type I collagen in the stroma improves the tumoral physical barrier and reduces uptake of a chemotherapeutic drug. Combined treatment with chemotherapy and hyaluronan- or collagen-degrading enzymes seem to show improved overall survival in pancreatic ductal adenocarcinoma xenograft models [177,178].

An extensive analysis with multiple stromal and TC lines as well as anticancer agents demonstrated resistance in many cases. CAFs induce the cancer stem cell (CSC) phenotype via EMT which was found to be induced by extracellular acidification, *inter alia*, due to increased carbonic anhydrase IX production. This triggered MMP-2 and -9 in prostate cancer cells and was found to be correlated with high levels of autophagy [179–181]. CSC population can grow after chemotherapy in some patients and is correlated with (metastatic) recurrence and also therapy resistance of cancer [116,180,182].

Co-culturing of CAFs with TCs leads to facilitated tumor invasion and accelerated tumor growth and angiogenesis in xenograft models in comparison to TCs alone via CXCL12 secretion [157,183]. In another example, high levels of IL-8 have been associated with poor chemotherapy response (e.g. cisplatin) which is believed to be achieved by NF- κ B activation [160,184]. CAFs also contribute to radioresistance by various mechanism such as insulin-like growth factor IGF-1 signaling as well as increased ROS levels arising from CAF-secreted factors [185,186]. The co-culturing experiments clearly indicate the contribution of CAFs in tumor growth.

CAFs also plays a role in resistance to immunotherapy because ECM remodeling and cytokine secretion contributes to an immunosuppressive TME [187]. The influence of CAFs on immunosuppressive M2 polarization of TAMs by different interleukins and FAP was mentioned in chapter 1.1.1. [16,43,49]. CAFs show increased production of certain immune checkpoint inhibitors such as programmed cell death ligand 1 (PD-L1). Binding of PD-L1 to PD-1 on activated T cells can inhibit their anti-tumor activity [187,188]. Indirectly, CAFs can induce similar upregulation in other cell types with cytokines like IL-6 triggering PD-L1 expression in neutrophils [187,189].

1.3. Fibroblast Activation Protein Alpha (FAP)

Fibroblast activation protein alpha was identified independently by two research groups. In 1986, RETTIG et al. [190] described a surface glycoprotein on activated fibroblasts of human astrocytoma cells which they later named fibroblast activation protein alpha (FAP) [191] whereas AOYAMA et al. [192] reported a large gelatinase in 1990, later called Seprase, on malignant melanoma cells [192,193]. Protein sequencing later revealed that FAP and Seprase were identical [194].

1.3.1. Expression Pattern of FAP

The expression of FAP in most adult, healthy tissue is very low or undetectable [5,195,196]. In contrast, it is highly expressed by activated fibroblasts in tissues of wound healing, chronic inflammation and fibrosis (e.g. fibrosis or cirrhosis in liver or pancreas, arthritis), as well as in neoplastic tissues where they are usually referred to as cancer-associated fibroblasts [197–203]. High FAP expression is found in over 90% of epithelial tumors such as lung, breast, colon, gastric, ovarian, prostate cancer and others [5,126,204,205]. In addition to expression in the TME by CAFs, it can also be expressed by other cell types in the tumor (microenvironment). For example, different types of mesenchymal cells such as bone marrow-derived MSCs, endothelial cells, cancer-associated adipocytes upon de-differentiation and TAMs can express FAP [5,201,206–208]. Very importantly, FAP is found on several types of sarcoma (bone, soft tissue, etc.) as well as melanoma cells [195,209].

Although isolated studies have reported FAP expression directly on (neuro)epithelial cancer cells such as astroglia/glioma cells in glioblastoma, this should be interpreted cautiously as most of the expression patterns were not confirmed with several independent methods, for example only immunohistochemical with antibodies that lack selectivity [201,210–212]. Similarly, reports of FAP expression in healthy tissue can be misleading since tissue samples are often taken at a distant site of a malignant organ [201]. Nonetheless, non-malignant FAP expression in pancreas, skin, muscle, bone (marrow) as well as blood plasma was found in several studies with different methods and thus seems to be valid [201,210,213–217].

There are some controversial results in isolated cancer types such as hepatocellular carcinoma, where no correlation with the amount of FAP expression and clinical prognosis (stage or grade, overall survival) was found [218]. Nevertheless, a higher expression of FAP correlates with tumor progression and overall survival in most patients, as supported by a meta-analysis of 15 studies in various cancer types [201,219].

1.3.2. Biology and Enzymatic Activity of FAP

FAP is a type II transmembrane glycoprotein that belongs to the serine integral membrane peptidases family. Together with the closely related dipeptidyl peptidases (DPPs) it belongs to the S9b subfamily [197,220]. FAP is most similar to DPP4. The amino acid sequence and the catalytic domain are identical to around 50% and 70%, respectively, but the DPPs are expressed ubiquitously [194,204,221–223]. DPP8 and DPP9 are intracellular, cytoplasmic proteins [224,225]. FAP consists of 760 amino acids and has a predominantly extracellular C-terminal domain (734 amino acids) while the cytoplasmic tail only consists of 6 and the transmembrane domain of 20 amino acids [204]. In its enzymatically active form, FAP is a 170 kDa homodimer but it was also found to form heterodimers with DPP4 [197,201,226]. The catalytic triad is typical of serine proteases and consists of three amino acids Ser⁶²⁴, Asp⁷⁰² and His⁷³⁴ with the same Gly-Trp-Ser⁶²⁴-Tyr-Gly sequence around Ser⁶²⁴ as in DPP4 [194,220,227,228]. The dimeric crystal structure was first described by AERTGEERTS et al. [196] where they also investigated different potential substrates. A soluble form of FAP (160 kDa), enzymatically active as a homodimer, was identified that lacked intracellular and transmembrane domain [220,229]. Furthermore, the distribution pattern on the cell surface revealed that FAP is concentrated at invadopodia along with MMPs such as MMP-2. Invadopodia are actin-based protrusions that are rich in lipids and proteins and have ECM adhesive functions. This exposed site facilitates the ECM degrading properties of FAP and MMPs [193,226,230].

FAP has a unique dual enzymatic activity and is able to cleave peptides after proline units in two ways as schematically shown in Figure 3a and b. As proline is the only proteogenic amino acid with a cyclic backbone and a secondary amino group, specific properties are required for the active site of the prolyl peptidases to hydrolyze post-proline bonds [197].

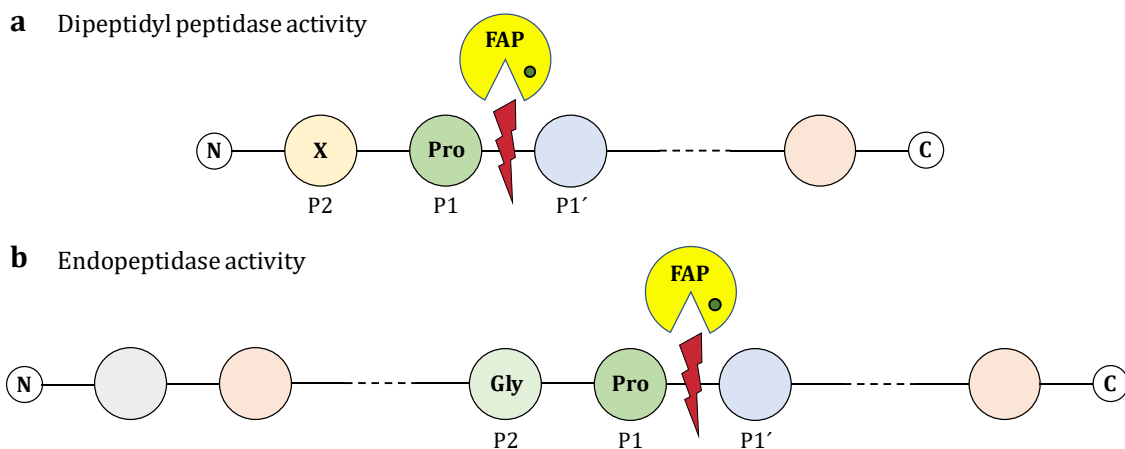


Figure 3: a) Dipeptidyl peptidase activity of FAP: FAP can cleave dipeptides from the N-terminus after a proline (Pro) unit (P1 position) whereby the P2 position allows for a variety of amino acids (X = Gly, Lys, Tyr, Arg, Ser, etc.); b) Endopeptidase activity of FAP: FAP can cleave peptides after a Gly-Pro unit (inspired by HAMSON et al. [197]).

FAP has dipeptidyl peptidase (DPP) activity to cleave dipeptides with proline at the second to last position as other DPPs with the same exopeptidase activity such as DPP4, 8 and 9 [231]. Preferred terminal amino acids (P2 position) include Gly, Lys, Tyr, Arg and Ser but more distant exosite binding seems also to determine whether a X-Pro dipeptide is efficiently cleaved by FAP [232,233]. In contrast, DPP4 cleaves X-Ala and X-Pro dipeptides and has no restriction at X = P2 position [222,223]. Possible physiological substrates include neuropeptides like neuropeptide Y, peptide YY, substance P and B-type natriuretic peptide although this is only confirmed *in vitro* [232]. Only Neuropeptide Y was found to be cleaved efficiently by FAP. DPP4 showed higher enzymatic activity for all the other abovementioned neuropeptides [232,234]. The terminal dipeptides at both C- and N-terminus of the fibroblast growth factor 21 (FGF21) have also been reported to be cleaved by FAP and FGF21 was identified as a natural FAP substrate [235]. Increased levels of intact FGF21 were observed upon FAP inhibition which might suggest a role of FAP in regulation of glucose and lipid metabolism that FGF21 is known for [228,236,237].

Compared to the dipeptidyl peptidase activity, FAP is a more efficient endopeptidase and known substrates include α_2 -antiplasmin, gelatin and type I collagen [196,197,200,229,231,238–242]. α_2 -antiplasmin is a plasmin inhibitor. Plasmin degrades fibrin clots that are formed during tissue repair and wound healing. Cleavage of α_2 -antiplasmin by the soluble form of FAP leads to higher plasmin inhibition which, in turn, decreases fibrinolysis and increases scar formation [229,238,240]. The soluble form of FAP is also a much more efficient gelatinase than membrane-bound FAP [243]. There is only one competitive enzyme with similar prolyl endopeptidase activity: prolyl endopeptidase (PREP). The expression of PREP is ubiquitous and seems to be membrane-bound as well as intracellularly. PREP preferably cleaves short peptides (≤ 10 kDa) [228,244,245]. FAP has the highest endopeptidase efficiency for cleaving peptides after Gly-Pro (P2-P1) units [196,246]. Some other peptides with Ala, Arg, Asp, Glu, Lys, Ser and Val at P2 position can also be cleaved although less efficiently than Gly-Pro units [247]. Other more distant positions (P3–P7) and P1'/P2' seem to be less restricted [220,233,248]. Ser and Ala seem to be slightly preferred at P3 while bulky aromatic acids are less favorable. Similarly, Phe or Met are favored and His or Glu are unfavored at P1' [220,233,248]. The conserved residues (Glu²⁰³-Glu²⁰⁴) and Ala⁶⁵⁷ are both essential for endopeptidase substrate binding at the active site and stabilization of the transition state. In contrast, DPP4 contains a negatively charged Asp⁶⁶³ instead of Ala⁶⁵⁷ that coordinates the free N-terminal amino group of the dipeptide whereas the affinity of FAP for N-terminal amines is decreased [196,249]. This explains the lower DPP activity of FAP.

FAP gene is localized on chromosome 2 (2q23) close to DPP4 suggesting that the pair might be the result of gene duplication and shares the same origin [228,250]. Transcription of FAP seems to be very stable. Even small mutations at a critical part of the transmembrane domain (the three

amino acids Gly¹⁰, Ser¹⁴, Ala¹⁸ in particular) can inhibit dimerization and thus strongly influence endopeptidase activity [251]. Early growth response 1 was identified to bind to the FAP promoter. Downregulation of FAP mRNA was demonstrated by mutation of early growth response 1 [252]. A mutated version with Ser³⁶³ instead of Leu³⁶³ is exclusively expressed in the endoplasmic reticulum which hinders dimerization and instead enables other enzymatic functions as it seems to be involved in endoplasmic reticulum-associated degradation [253]. In the TME, FAP seems to be strongly involved in fibrillar collagen degradation in the ECM. But before FAP can help to degrade collagen with its many Gly-Pro units, MMPs have to initiate the degrading process by cleaving collagen into smaller chunks. Subsequently, the chunks can be further fragmented by FAP [200,231,242,254,255].

Although several physiological FAP substrates have been identified and FAP is expressed in some healthy organs and present in the plasma in its soluble form, its original role *in vivo* in healthy living organisms is not fully understood. FAP knockout in mice resulted in a healthy phenotype which suggest a non-essential role in healthy mice, and maybe also humans [221,256]. Of course, FAP has its specific role when upregulated in cases of inflammation, wound healing or fibrosis. FAP expression by hepatic or pancreatic stellate cells strongly correlates with liver or pancreatic fibrosis progression [200,215,257–259]. FAP has also been associated with atherosclerosis as thinner fibrous caps that are more prone to rupture were found to upregulate FAP [260,261].

1.3.3. *The Tumorigenic Role of FAP*

In the TME, FAP mainly seems to be involved in the ECM/tissue remodeling especially via degradation of type I collagen and preparing the ECM for TC migration and invasion. Interestingly, ECM remodeling is also one of the main mechanisms of FAP-expressing CAFs which might show that FAP might be a central contributor to the tumor-promoting role of CAFs (see previous chapter) [197,220,262,263]. Unsurprisingly, a strong correlation between upregulated TGF- β and FAP was observed as TGF- β induces fibroblast activation which, in turn, triggers FAP expression [245,264].

The influence of FAP expression on tumor growth (tumorigenesis, cell proliferation, metastasis, angiogenesis etc.) was investigated in numerous studies regarding tumor cells of different cancer types [264–271]. In most studies high FAP expression correlated with accelerated cell proliferation, enhanced tumor growth and angiogenesis as well as higher tendency to invade and metastasize. This correlation has been generally accepted although some studies have reported the contrary [197,201,272,273]. In medullary and papillary thyroid cancer patients for example, a strong correlation was not only found between FAP expression and the histological degree of desmoplasia but also in regard to the incidence of lymph node metastases [274]. It was

hypothesized that the cellular context might influence the enzymatic activity and interaction with other proteases (e.g. formation of heterodimers with DPP4, MMPs or $\alpha_3\beta_1$ integrin) and thus its biological function that can either be tumor-promoting or -suppressing which can also be cancer type-dependent [220,245,275]. In contrast to FAP, DPP4 is often downregulated in malignant diseases and this usually has tumor-promoting effects [222,276]. Therefore, the two proteases in particular can influence each other as they can form heterodimers [226]. Another aspect that is important to consider in xenograft studies is the cancer model. The results of studies where tumor cells are artificially transfected to express FAP and are not co-cultured with CAFs have limited validity as they differ significantly from the actual composition of epithelial tumors with tumor stroma in cancer patients [5].

The effects of FAP expression on tumor growth can be divided into direct and indirect effects on tumor cells and the TME, respectively, that can be enzymatic or non-enzymatic [201]. In the TME, slightly reduced production of ECM components and cell proliferation was observed when FAP expression in fibroblasts was downregulated [264,277]. In breast cancer xenografts, a 3-fold increase in blood vessel sprouting in FAP⁺ compared to FAP⁻ TCs was found and another xenograft study with MDA MB-231 cells clearly showed that FAP promotes tumor growth at least partially by enforcing angiogenesis [278–280]. In TCs that directly express FAP, higher FAP levels correlated with accelerated proliferation or integrin signaling pathways which play a role in TC/ECM interactions [272,281]. Indirect effects on TCs include enhanced survival towards chemotherapy with cisplatin and reduced T cell activity [282,283]. First and foremost, FAP has pro-fibrogenic enzymatic activity. Beyond that, non-enzymatic effects only dependent on FAP expression, but not on FAP inhibition, seem to correspond with cell apoptosis and migration etc. [215,278]. For example, FAP⁺ CAFs secreted more CCL2 than FAP⁻. Upregulation of MDSCs and enhanced tumor growth was observed as a consequence. A mediating role could be associated to CCL2 and FAP inhibition did not decrease CCL2 levels [284].

1.3.4. FAP inhibitors (FAPi)

The selective expression pattern in fibrotic or malignant diseases makes FAP an interesting therapeutic target, in particular a pan-tumor target that offers a very broad application potential in many cancer types. FAP was addressed with several therapeutic approaches over the last years: 1. monoclonal antibodies (mAbs) and chimeric antigen receptor (CAR) T cell immunotherapy; 2. small-molecule FAP inhibitors (FAPi): this chapter; 3. FAP-targeted drug delivery with FAP-cleavable prodrugs (FAP substrates): chapter 1.3.5. and 4. radioligand therapy (RLT) with FAP-targeting radiopharmaceuticals: chapter 1.5.

Firstly, the mAb and CAR T cell approach shall be mentioned briefly. Shortly after the identification of FAP, the humanized monoclonal antibody (mAb) F19 (Sibrotuzumab) was developed and also labeled with iodine-131 (see chapter 1.5) [285]. Several groups genetically altered CAR T cells to express FAP. This showed little to some immunotherapeutic efficacy depending on the murine tumor model but exhibited serious side including cachexia, anemia and bone toxicity [215,286,287]. New mAb were developed and a CAF-internalizing anti-FAP mAb was able to deplete FAP expression which led to tumor suppression [288].

Secondly, the optimization of small molecule FAP inhibitors has been ongoing since the discovery of FAP in the 1990s. The selectivity of FAPi for FAP over DPPs is very important since the ubiquitous expression of DPP4 in healthy tissue might lead to severe side effects when addressed with inhibitors.

The inhibitor concentration that is needed to achieve a 50% decrease of enzymatic activity is defined as the half-maximal inhibitory concentration (IC_{50}). The IC_{50} value is dependent on the measurement conditions such as substrate concentration $[S]$, enzyme concentration as well as the inhibition mechanism. In the case of competitive, reversible inhibition, it is connected to the inhibition equilibrium constant K_i , an enzyme concentration-independent value, via CHENG-PRUSOFF equation [289–291]:

$$IC_{50} = K_i \left(1 + \frac{[S]}{K_M} \right) \quad (1)$$

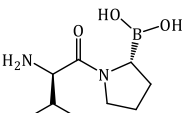
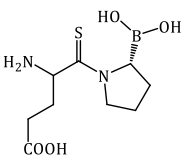
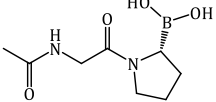
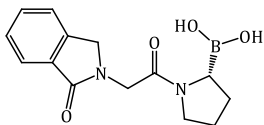
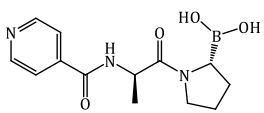
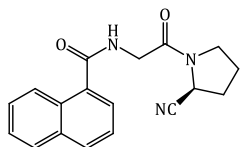
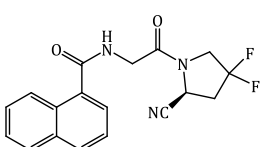
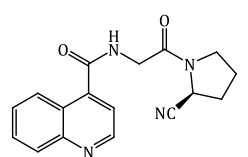
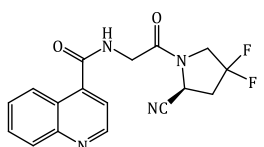
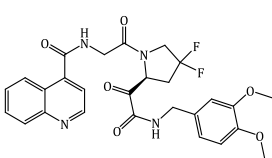
K_M is the MICHAELIS constant of the substrate and the concentration at which the reaction rate v of the enzyme is half-maximal ($v = 0.5 \cdot v_{max}$) [289–291].

Table 1 gives an overview of different FAP inhibitors. Each one is the lead structure of a structure-activity relationship (SAR) study that involved many more similar compounds. The *in vitro* affinity for FAP (IC_{50} or K_i) and their selectivity over DPP4 and PREP is shown. The selectivity is expressed as a selectivity index (SI) which is the ratio of IC_{50} or K_i (DPP4/PREP) to IC_{50} or K_i (FAP).

The first generation of small molecule FAPi was based on a derivatized proline with boronic acid warhead [292]. Warhead is the reactive (electrophilic) functional group that binds to the enzyme covalently. The first lead compound PT-100 (Talabostat, Val-boroPro, cf. Table 1) was investigated in several phase I/II studies [293,294]. PT-100 like other boronic acid derivatives lacked FAP/DPP4 selectivity [292,294]. The anti-tumor efficacy of PT-100 was not totally clear although some studies reported somewhat of anti-tumor efficacy, possibly indirectly through upregulation of certain cytokines [295,296]. Anyhow, the studies were discontinued. Combinatorial treatment with e.g. oxaliplatin are still under investigation and enhanced efficacy was observed [297]. A second-generation derivative PT-630 showed indirect inhibitory effect on

tumor cell proliferation in CT26 colon cancer xenograft model but this might also be due to DPP4 inhibition [264]. Irreversible inhibitors like the diphenyl phosphonates Tyr- and Gly-Pro(OPh)₂ (not shown) had lower affinity for FAP (micromolar) and no DPP4 selectivity [298].

Table 1: Most relevant FAP inhibitor lead compounds including the respective structure, IC₅₀ or K_i value and selectivity index (SI) over DPP4 and PREP.

Compound	Structure	IC ₅₀ (FAP) K _i (FAP)	SI (DPP4/FAP)	SI (PREP/FAP)	Literature
PT-100 (Talabostat, Val-boroPro)		K _i = 6.2 nM	3.5	158	COUTTS et al. [292,294]
PT-630		IC ₅₀ = 23 nM K _i = 5.0 nM	0.6	—	SANTOS et al. [228,264]
Ac-Gly-boroPro		K _i = 23 nM	16.4	9.2	EDOSADA et al. [233]
N-blocked- boroPro		K _i = 7.5 nM	3027	0.37	TRAN et al. [299]
ARI-3099		IC ₅₀ = 36 nM	> 2778	360	POPLAWSKI et al. [300]
N-(1- Naphthoyl)-Gly- cyanoPro		IC ₅₀ = 610 nM	> 164	5.4	TSAI et al. [301,302]
N-(1- Naphthoyl)-Gly- cyanoPro(F,F)		IC ₅₀ = 110 nM	> 909	44	RYABTSOVA et al. [303]
N-(4- Quinolinoyl)-Gly- cyanoPro		IC ₅₀ = 10 nM	> 10 000	86	JANSEN et al. [302]
UAMC-1110		IC ₅₀ = 0.43 nM	23256	4186	JANSEN et al. [304,305]
“compound 6d ”		IC ₅₀ = 89 pM	> 33708	2.9	SIMKOVA et al. [306]

The FAP/DPP4 selectivity can quite easily be directed by derivatizing the *N*-terminus since DPP4 only has exopeptidase activity. This can be realized by *N*-acylation or more generally speaking *N*-blocking. Two examples are Ac-Gly-boroPro with modest FAP/PREP and FAP/DPP4 selectivity and “*N*-blocked-boroPro” with high DPP4 but no PREP selectivity [233,299].

The FAP/PREP selectivity is generally more challenging. POPLAWSKI et al. [300] were able to synthesize two similar inhibitors with high FAP/PREP selectivity, the FAP-specific *N*-(pyridine-4-carbonyl)-D-Ala-boroPro (ARI-3099), and the PREP-specific *N*-(pyridine-3-carbonyl)-Val-boroPro (ARI-3531). In addition, both inhibitors showed low DPP4 affinity.

TSAI et al. [301] were the first to introduce a nitrile warhead instead of boronic acid in compounds with a 2-cyanopyrrolidine group (cyanoPro) like *N*-(1-Naphthoyl)-Gly-cyanoPro. Nitrile warheads can reversibly bind to serine or cysteine proteases by forming an (thio)imide [307]. In the case of FAP the nitrile group reacts with the hydroxy group of Ser⁶²⁴ of the catalytic triad in an electrophile addition where His⁷³⁴ mediates the proton transfer as depicted in Figure 4a.

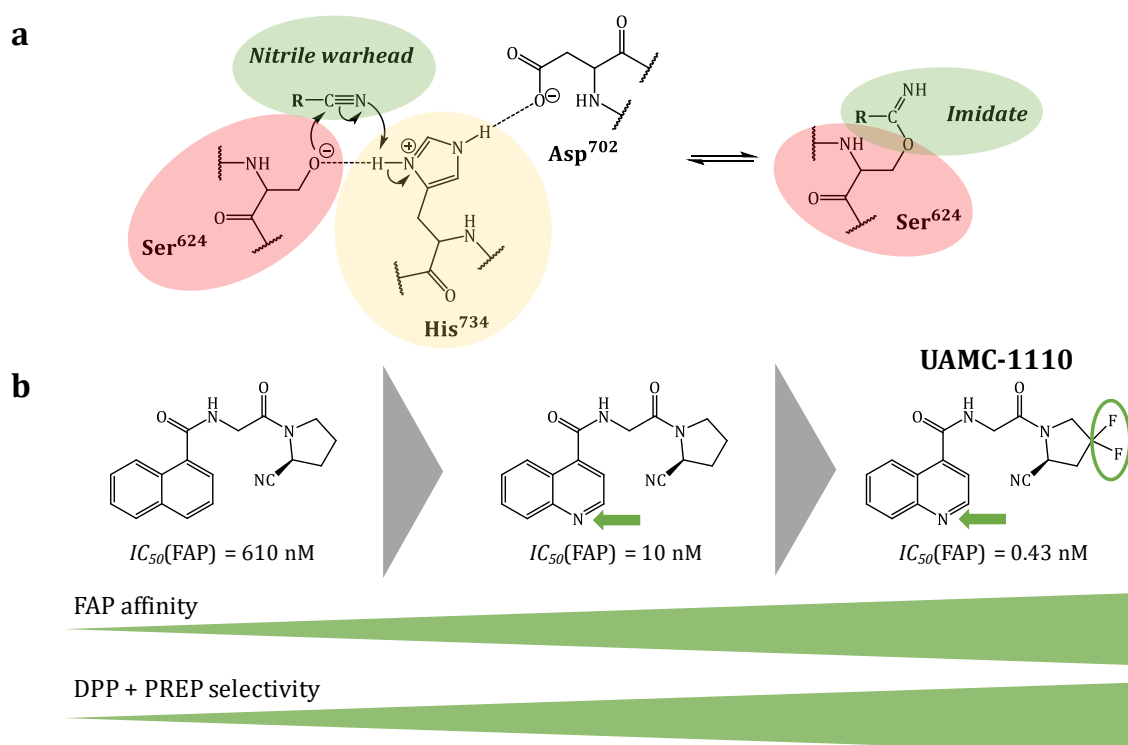


Figure 4: a) Reversible binding of a FAP inhibitor with nitrile warhead (e.g. UAMC-1110) to Ser⁶²⁴, by forming an imide (inspired by POWERS et al. [307]); b) continuous optimization of FAP inhibitors by the group of PIETER VAN DER VEKEN via heteroaromatic quinoline moiety and difluorination to obtain UAMC-1110 (bottom right) with very high FAP affinity and selectivity over DPPs and PREP.

The group of PIETER VAN DER VEKEN from the University of Antwerp carried out extensive structure-activity relationship (SAR) studies and was able to optimize the cyano-based approach in several steps which is graphically highlighted in Figure 4b.

They introduced a difluorination in the pyrrolidine ring for the first time, 2-cyano-4,4-difluoropyrrolidine (cyanoPro(F,F)) to be precise, and found improved affinity and selectivity compared to previous SAR studies by TSAI et al. [301,303]. Furthermore, the introduction of the *N*-(4-quinolinoyl)-Gly-cyanoPro scaffold by JANSEN et al. [302] not only improved FAP affinity with low nanomolar IC_{50} values but significantly improved both DPP4 and PREP selectivity. The combination of *N*-(4-quinolinoyl) residue and difluorination culminated in the compound (*S*)-*N*-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide (**UAMC-1110**) with subnanomolar $IC_{50} = 0.43 \pm 0.02$ nM and very high selectivity over DPP4 and PREP with an $SI(DPP4/FAP) = 23256$ and $SI(PREP/FAP) = 4186$, respectively [304,305]. Recently, Simkova et al. reported a new FAP inhibitor with α -ketoamide warhead and picomolar IC_{50} value. However, “compound 6d” showed low PREP selectivity ($SI = 2.9$) [306].

1.3.5 FAP substrates and FAP-targeted drug delivery

FAP can also be utilized as an oncological target for FAP-targeted drug delivery for example by synthesizing prodrugs that address CAFs in the TME. These FAP substrates contain of a cytostatic drug conjugated to a FAP-targeting moiety that contains a FAP-cleavable amide bond. The conjugated cytostatic has no therapeutic efficacy (inactive form) but cleavage by FAP releases the cytostatic payload into the TME in its active form. The endopeptidase activity is utilized in this approach.

The first published study demonstrated the drug delivery of the promelittin protoxin, a 40 – 50 amino acid peptide derived from the toxic honeybee peptide melittin, but promelittin was toxic when injected intravenously [308]. A similar approach was chosen by conjugating the toxin thapsigargin to a peptide sequence that was supposed to be cleaved by FAP [309]. Thapsigargin is highly toxic by increasing calcium levels in cells due to calcium pump inhibition which leads to apoptosis. The thapsigargin-FAP peptide conjugate only showed increased apoptosis of stromal cells but not of TCs [309,310]. The pharmaceutical formulation of promelittin was altered by a combination of promelittin loaded onto reduced graphene oxide nanosheets that contained the cytostatic doxorubicin (Dox), too. The new formulation allowed intravenous injection and the cytotoxic effect of Dox was slightly higher in combination with the FAP-targeting promelittin loaded nanosheets than administration of free Dox [311]. Dox is an anthracycline that is intercalating in the DNA double helix which inhibits topoisomerase II and therefore leads to irreversible DNA strand breaks.

In Z-Gly-Pro-Dox (cf. Figure 5a), the favored Gly-Pro unit was simply protected with a carboxybenzyl (Z) protecting group conjugated directly to Dox. A similar study with a modified *N*-terminus PhAc-ALGP-Dox (cf. Figure 5b) has also been published [312].

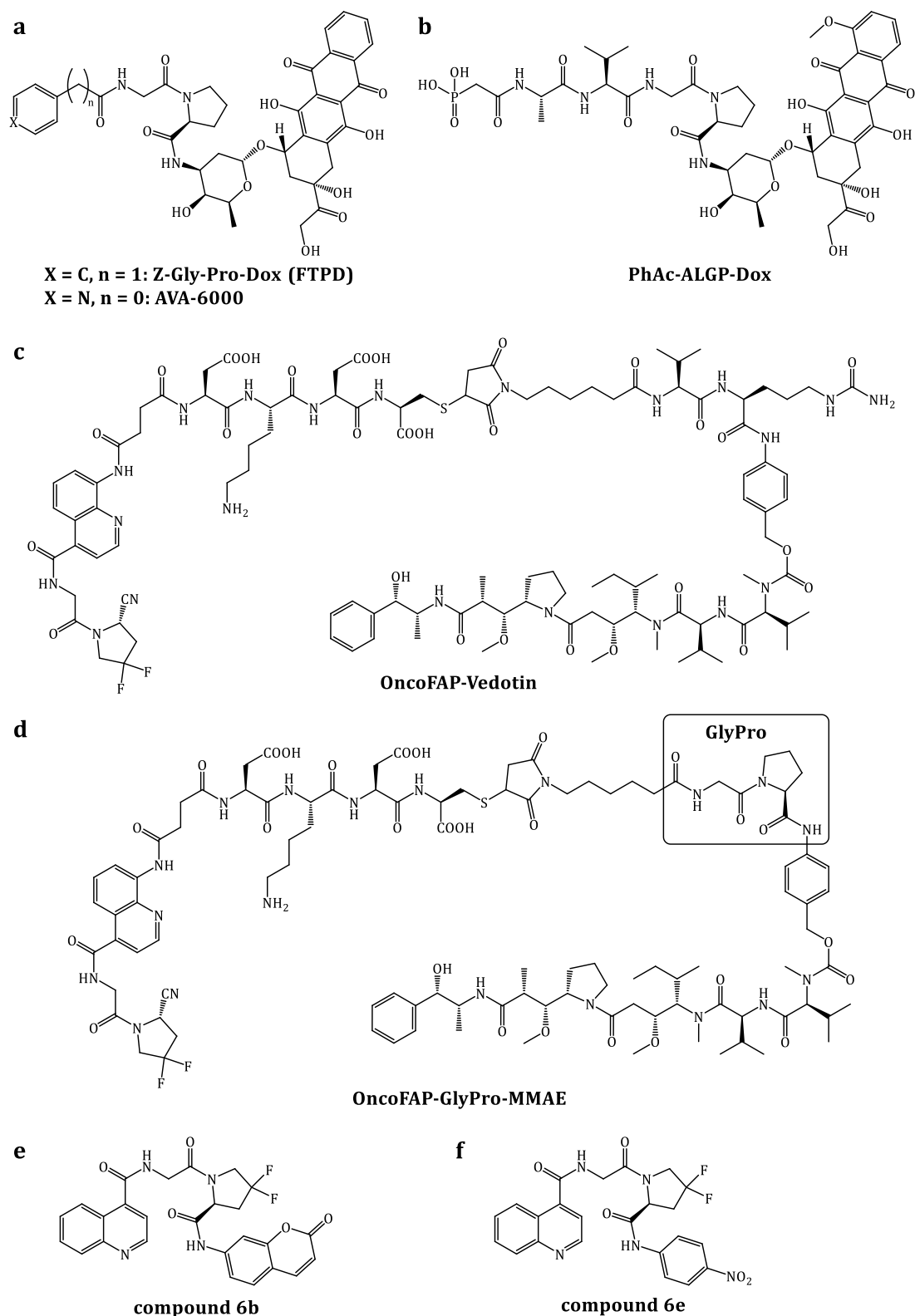


Figure 5: Structure of the FAP-cleavable doxorubicin prodrugs a) Z-Gly-Pro-Dox (FTPD, X = C, n = 1), AVA-6000 (X = N, n = 0) and b) PhAc-ALGP-Dox; c) FAPi-based prodrug OncoFAP-ValCit-MMAE (OncoFAP-Vedotin) cleavable by Cathepsin B and d) the FAPi-based and simultaneously FAP-cleavable prodrug OncoFAP-GlyPro-MMAE; highly PREP-selective FAP substrates from DE DECKER et al. [313]: e) compound 6b and f) compound 6e.

These works demonstrated some anti-tumor efficacy of FAP-targeted drug delivery and showed that the prodrugs are well tolerated in contrast to free Dox. The systemic use of Dox is often problematic due to its cardiotoxicity. Regarding the different Gly-Pro sequences that were used no selectivity over the other related proteases (PREP, DPPs) was investigated. Comparison with the extensive SAR studies on FAP inhibitors (cf. chapter 1.3.4.) indicates that DPP4 selectivity was achieved by blocking the *N*-terminus with the Z- or PhAc-ALGP-group to avoid cleavage by DPPs. But it is much more challenging to achieve PREP selectivity and since no data on FAP/PREP selectivity were reported together with the experience from other groups like DECKER et al. [313] it can be assumed that both PhAc-ALGP-Dox and Z-Gly-Pro-Dox lacked PREP selectivity. Nevertheless, these first prodrugs demonstrated the potential of FAP-targeted drug delivery in first proof-of-principle studies. Recently, a slightly modified version of Z-Gly-Pro-Dox was published, AVA-6000, where a 4-pyridinoyl instead of the benzyl unit was used and showed significant decrease of Dox cytotoxicity. First clinical trials are currently ongoing [314].

MILLUL et al. [315] designed a molecule with a FAP inhibitor Onco-FAP that is similar to UAMC-1110 that was coupled via a peptide sequence to Vedotin. Vedotin consists of the cytostatic monomethyl auristatin E (MMAE) in a prodrug form as it is coupled to a Val-Cit linker via the self-immolative linker PABC (*p*-aminobenzyl carbamate) as shown in Figure 5c. MMAE is a tubulin polymerization inhibitor that inhibits cell division (antimitotic agent) and is a very toxic agent and thus only used in conjugated prodrugs. The Val-Cit linker can be cleaved by Cathepsin B which is strongly expressed in various cancers. The therapeutic effect of OncoFAP-Vedotin was tested in SKRC52-hFAP tumor bearing mice along with the combination with the monoclonal antibody-cytokine fusion protein L19-IL2. The combination was well tolerated and demonstrated high therapeutic effect simultaneously [315].

The same group recently extended the approach where different dipeptide sequences instead of Val-Cit were introduced like Gly-Pro, Ala-Pro or Val-Pro [316]. The idea is that the prodrug is cleaved by FAP itself at post-proline amide bond. Thus, FAP has a dual role in this approach. Firstly, it is the oncological target to ensure high and selective accumulation in the TME by using a suitable FAP inhibitor. Secondly, once the prodrug is accumulated in the tumor/TME, FAP cleaves the prodrug to release the free cytostatic in its active form. The Gly-Pro derivative OncoFAP-GlyPro-MMAE (cf. Figure 5d) showed superior performance in SKRC52-hFAP tumor bearing mice compared to OncoFAP-Vedotin. The treatment protocol was optimized afterwards and the best results with 50% complete response were achieved with weekly administration. The anti-tumor effect was further demonstrated with HT-1080.hFAP xenografts where one dose was sufficient to achieve complete response in 4/4 mice [316].

Based on the SAR studies on FAP inhibitors and UAMC-1110, the group of PIETER VAN DER VEKEN were the first that have proven to synthesize a synthetic small molecules FAP substrates that are

highly selective towards PREP and still have high cleavage rates (k_{cat}) and catalytic efficiency (k_{cat}/K_m), respectively [313]. The nitrile warhead was substituted by a FAP-cleavable amide bond with different fluorogenic residues to quantify cleavage efficiency. Three variable positions were investigated by DE DECKER et al. [313]: D-Ala besides L-Gly at P2 position, L-Pro as well as its difluorinated derivative at 4,4'-position as in UAMC-1110 and three different fluorogenic residues 7-amino-4-methylcoumarin (AMC), *para*-nitroaniline (pNA) and 4-aminonaphthol (4-AN). The 4-quinolinoyl unit was an essential part of all molecules. Compound **6b** with AMC (cf. Figure 5e) and **6e** with pNA (cf. Figure 5f) were identified as the most promising candidates. They both had a Gly and a 4,4'-difluorinated proline unit and had the highest PREP selectivity with simultaneously substantial FAP-cleavage rates (k_{cat}) [313]. However, these substrates did not include a cytostatic payload but were rather designed for spatial localization of FAP on a cellular level.

1.4. Radiopharmaceutical and Nuclear Medicine Basics

Nuclear medicine – in some respect – can be divided into three main application fields: single-photon computed emission tomography (SPECT) or positron emission tomography (PET) for diagnosis and endoradiotherapy (ERT) for therapy. ERT is also called radionuclide therapy (RNT) or radioligand therapy (RLT). It can be further subdivided by the type of radionuclide (covalently bound organic radionuclides vs. complexation of radiometals with chelators) as well as the type of ionizing radiation (α , β^+ , β^- , γ , Auger electron emitters).

Since this work and also most of the nuclear medicine research regarding FAP deals with chelator-based radiopharmaceuticals labeled with various radiometals, the general design of a chelator-based radiopharmaceutical shall be depicted in Figure 6a. In contrast, radiotracers utilizing covalently bound radionuclides like fluorine-18 can be directly coupled to a targeting vector as shown in Figure 6b.

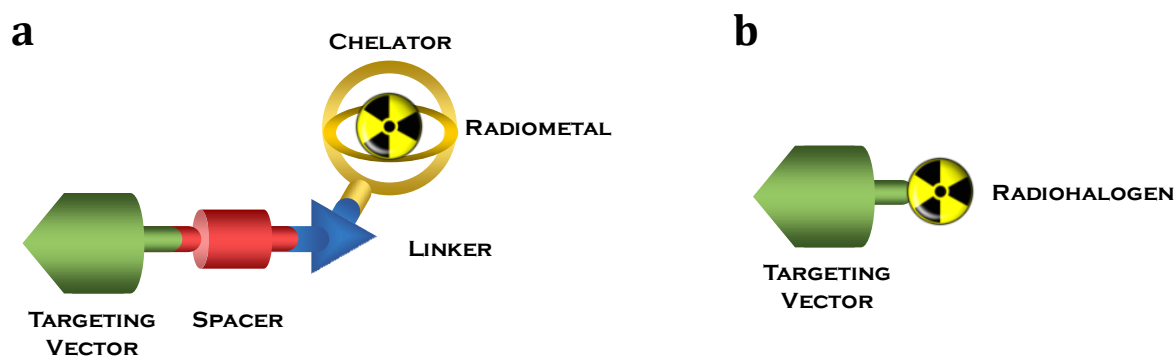


Figure 6: Schematic structure of a) a chelator-based radiopharmaceutical and b) a radiotracer with covalently bound radionuclide (modified from ROSS AND ROESCH [317]).

The radiopharmaceutical design as in Figure 6a combines a targeting vector (TV) with a bifunctional chelator via a linker, and in some cases additional spacer unit. The TV ensures high affinity and selectivity for binding to the biological target. In this case, FAP is addressed with a FAP inhibitor TV. TV and chelator are coupled via a linker unit. This is realized by turning a chelator into a bifunctional chelator. Bifunctional means that on top of the donor ligands for complexation, the chelator has an additional functional group that allows coupling to the TV. The linkage can be realized by many different coupling strategies. Amide bond formation is among the most used. Other important strategies include forming (thio)urea from iso(thio)cyanate and an amine, maleimide and thiol coupling, 1,2,3-triazole from alkyne and azide via Cu(II)-catalyzed click chemistry as well as Cu(II)-free variants that are known for example with dibenzocyclooctin (DBCO) and azide or *trans*-cyclooctene (TCO) with tetrazine etc. [318].

An additional spacer unit is introduced in many cases. The spacer increases the distance between chelator and TV to minimize the potentially negative effects of the chelator on binding properties (affinity, selectivity) as well as radiolabeling properties (steric hindrance). Spacers are often alkyl,

polyethylene glycol (PEG_n) groups or a peptide backbone that might include polar or lipophilic/aromatic groups. The spacer units are optimized with regard to pharmacological properties (lipophilicity, binding affinity etc.) [10,317,319].

The advantage in this approach is that the translation from a diagnostic to a therapeutic radiopharmaceutical can be realized with the same precursor molecule by labeling it with another radiometal. This facilitates the translation from diagnostics to therapy which is often referred to as *theranostics*. In general, the injected activity of a diagnostic tracer is just enough to achieve good molecular imaging to keep the radiation dose as low as possible whereas a therapeutic radiopharmaceutical is used in much higher activities to achieve a therapeutic effect. The decay type of the radionuclide determines the application field. Positron (β^+) emitters are needed for PET, γ emitters for SPECT and short-range α , β^- (or Auger electron) particle emitters are utilized in ERT.

1.4.1. Positron Emission Tomography (PET)

Positron emitters are proton-rich radionuclides that convert a proton of the parent nuclide X into a neutron in the daughter nuclide Y according to equation (1) where Z is the atomic number, A the mass number and N the neutron number:



Additionally, a positron β^+ and an electron neutrino ν_e is emitted energy ΔE is released. ΔE corresponds to the mass difference between reactants and products of the nuclear decay. ΔE is statistically distributed between β^+ and ν_e resulting in two important correlating parameters: the average beta energy $E_{\beta^+_{\text{avg}}}$ and the maximum beta energy $E_{\beta^+_{\text{max}}}$. The positron continuously loses kinetic energy due to inelastic collisions (thermalization) and when it has lost almost all its kinetic energy, it either recombines with an electron directly or forms a metastable positronium. The spins of electron and positron can either be antiparallel (singlet state ${}^1S_0$) and this so-called *para*-positronium has a short half-life ($t_{1/2} = 87$ ps) and results in the emission of two 511 keV photons at an angle of roughly 180° because of the conservation of momentum [320,321]. The energy of 2×511 keV photons corresponds to the invariant mass of the electron-positron pair according to the mass-energy equivalence. Alternatively, the spins can be parallel (triplet state ${}^3S_1$) and this so-called *ortho*-positronium is predominantly formed first. It results in the emission of three photons with random angel distribution which is unusable for PET. But it has a much longer half-life ($t_{1/2} = 98$ ns) and a slightly higher energy than the singlet state which is why it mostly converts to the singlet state [4,322–325].

PET scanners are equipped with a large number of scintillation detectors arranged in several rings, either rotatable partial rings or full-rings to detect the two 180° photons by opposite detectors [326]. If two photons are registered within a set interval of a few ns, if these detectors

are in the range of an acceptable angle, and if the energy of the photons is in the range of 400-650 keV, a *prompt* coincidence event is registered and allocated to the line/volume of response (LoR/VoR) [4,327]. The sum of all registered *prompt* coincidences includes not only *true* coincidences but also *random* coincidences and *scattered* coincidences due to COMPTON scattering [328]. A transmission scan before the emission scan with radiotracer can be performed for *scattering* and absorption correction to determine the attenuation factor which enables activity quantification [4,329]. Software is also implemented for correction of *random* coincidences to improve image quality. A series of slice (2D) cross sectional images is acquired and all coincidence events are gathered in a sinogram which is then reconstructed into a 3D image with appropriate software [4]. Scintillator materials should have a high photoelectric effect, high energy resolution, short decay time, and high stopping power (correlating with effective Z) which will overall result in high luminous efficacy [4,330]. The past standard thallium-doped sodium iodide NaI(Tl) scintillators has been replaced by new materials like cerium-doped lutetium oxyorthosilicate (LSO, Lu_2SiO_5) or gadolinium oxyorthosilicate (GSO, Gd_2SiO_5) [328,330].

The resolution of a PET scanner is physically limited. This is mainly caused by the mean free path length of the positron between emission and annihilation which is shorter the smaller the kinetic energy E_{β^+} . For example, the average and maximum range of gallium-68 is 3.7 and 13.6 mm, respectively. Another limitation results from the deviation of the annihilation angle (180°) due to residual kinetic energy from electron and/or positron [4,327].

The spatial resolution of PET is continuously improved by using more sensitive and robust materials and by improved software. A typical spatial resolution of a human PET scanner with detector length of 5 mm is around 5 mm [327]. The resolution can be improved to 1 – 2 mm by using smaller detectors areas, e.g. in μ PET devices for small animal studies, or by so-called block detectors where multiple small detectors are coupled to one photomultiplier [327,328,330]. Modern *time of flight* (ToF)-PET scanners have very high temporal resolution and can measure the time delay between the two coincidence 511 keV photons and thus can narrow down the location of the annihilation to a small region of the LoR/VoR. Therefore, the improved temporal also leads to an improved spatial resolution [4]. Combined PET/CT and PET/MRI scanners have been developed to improve anatomical resolution. It allows to precisely localize the radiotracer accumulation morphologically [331]. In addition, both images can be acquired in one measurement, which is more convenient for the patient as well as time- and cost-saving. In general, the instrumentation further progresses. Recent progresses are made in the reconstruction software, higher sensitivity (larger axial field) of the detectors which can shorten the time per scan or reduce the administered dose, motion correction due to breathing or cardiac patient motion [332].

1.4.2. Single-Photon Emission Computed Tomography (SPECT)

SPECT is utilizing γ -emitting radionuclides. Since only one photon is measured there is no inherent spatial resolution. Therefore, a key element of the gamma camera is the collimator made out of lead and placed in front of the detectors. It is usually designed as parallel septa/slant on a rotating gamma camera [333]. Only gamma rays that are within the acceptance angle of the collimator can get passed the collimator and to the detector. Higher resolution can be achieved by using more narrow septa but this decreases the sensitivity because less photons are registered [327,333]. Therefore, a compromise between resolution and sensitivity must always be considered. For SPECT, optimal gamma ray energies are $E_\gamma = 100 - 250$ keV according to efficiency of NaI(Tl) detectors. Scattering correlates with E_γ and is more prominent in SPECT than in PET because of lower E_γ [327]. If $E_\gamma < 100$ keV, many photons are scattered or absorbed in the patient tissue which increases background noise and lowers image quality [334]. In turn, scattering in the detector is an intrinsic effect, along with signal noise of the photomultiplier, and increases with higher E_γ [333]. If E_γ is too high, filtering the gamma rays does not work as good because the penetration of collimator septa is stronger which causes false signal registration [333]. Furthermore, scattering in the collimator septa might still lead to detection if the scattered photon is still within the energy window of the detector [333,334]. Recent advances in collimator design have optimized the setup, especially for specific tasks, e.g. fan-beam, rotating multi-segment slant-hole or pinhole collimators that are optimized to image a small organ or a specific region such as in mammography [333].

One more aspect to consider is that the NaI(Tl) detectors have a higher detection efficiency at lower E_γ . Standard SPECT is a non-quantitative method since the attenuation correction is very elaborate and only works via approximations and simplifications (e.g. constant absorption coefficient). SPECT/CT has improved diagnostic value and morphological location significantly. Recently, efforts have been made to reach absolute quantification in SPECT/CT although this has been not vastly translated into clinical routine [335,336].

SPECT is more cost-effective than PET and therefore more vastly used in routine diagnostics because of the availability of the most used SPECT radionuclide technetium-99m from $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ radionuclide generator (cf. chapter 1.4.5.) and the variety of the easy to prepare $^{99\text{m}}\text{Tc}$ -kit-type radiopharmaceuticals. $^{99\text{m}}\text{Tc}$ -SPECT imaging accounts for more than 80% of all diagnostic nuclear medicine procedures [337,338]. Besides that, iodine-131 is another very important SPECT radionuclide.

1.4.3. Endoradiotherapy (ERT) and Radioligand Therapy (RLT)

The principle of endoradiotherapy (ERT) is based on injecting radiopharmaceuticals that deposit radiation dose in the region of interest such as tumor lesions. Oncology is the most important application field. When an unfunctionalized radionuclide is used, it is usually referred to as radionuclide therapy (RNT), e.g. [^{89}Sr]SrCl₂ and [^{223}Ra]RaCl₂ for bone metastasis and [^{131}I]NaI for thyroid cancer treatment. In contrast, it is called radioligand therapy (RLT) or targeted RNT when functionalized radiopharmaceuticals with a targeting vector (TV) are used, e.g. SSTR agonists TOC/TATE, PSMA or FAP inhibitors coupled with chelators and radiolabeled with a therapeutic radionuclide. Higher activities are injected compared to diagnostic PET or SPECT to achieve a therapeutic effect. Radionuclides that emit short-range charged particles such as α particles or electrons (β^- particles or Auger electrons) are most suitable for this method [9,339]. Because of the strong interaction of these particles with matter, the range is short. Gamma rays are unsuitable for therapy because of its very long range and low LET. The linear energy transfer (LET) is the amount of energy that ionizing particles deposit per unit length and is proportional to Q^2 (Q : charge) and inversely proportional to the particle energy E_{kin} . The LET reaches its maximum at low E_{kin} (BRAGG-Peak), so at the end of the path length when the particle is stopped [7].

Alpha particles are doubly charged, have a higher distinct particle energy of usually 4 – 8 MeV and a much shorter range in biological tissue of < 100 μm than beta particles. Beta particles generally have an average particle energy of < 1 MeV with continuous energy distribution and a much longer free path length of 12 mm at maximum for yttrium-90 for example [11,334,340,341]. The LET is roughly 20 – 100 times higher for α particles (50 – 230 keV/ μm) than for beta particles (≤ 2 keV/ μm) [7,342,343]. Ionizing particles either damages cells directly or by radical formation (ROS). α particles mostly cause DNA damage via double-strand breaks that are lot more difficult for a cell to repair than single-strand breaks which are the main consequence of β^- particle irradiation along with increased ROS formation in the cell [343]. All these properties result in the fact that 1 – 20 α particles are enough to kill a cell whereas 100 – 1000 β^- particles are needed [344,345].

Presupposing that the radiopharmaceutical selectively accumulates in the tumor, targeted alpha-particle therapy (TAT) results in reduced radiation dose to the tissue surrounding the tumor. On the other hand, it must be guaranteed that α emitters get at least to the cell surface or are preferably internalized, otherwise it will not have the desired therapeutic effect. Auger emitters must get into the cell nucleus as the range is even shorter than of α particles. The challenge to ensure this specific accumulation is very high which is why no Auger electron emitters have found their way into clinical routine yet [9,339]. The advantage of β^- emitters, especially those with longer range like yttrium-90, can irradiate more distant cells that do not express the biological target due to *crossfire effect* [7,334,341]. The physical barrier of the tumor lesion, e.g. caused by

the TME, is another reason preventing a homogenous radiotracer accumulation in the tumor. On the other hand, healthy cells in neighboring tissue are also damaged, especially with long-range β^- emitters with high $E_{\beta^+_{\max}}$ (e.g. ^{90}Y) [334].

Critical organs regarding toxic radiation dose or side effects are often liver (biotransformation of radiotracer to radioactive metabolites, hepatobiliary excretion), blood vessels (radiotracer circulation), kidneys (renal excretion) and red bone marrow (hematotoxicity). Therapeutic radiopharmaceuticals are designed to have optimal targeting properties (binding affinity and selectivity) as well as pharmacokinetic properties (fast renal clearance), pharmacodynamic properties (low off-target accumulation) and high serum stability [7,11,343]. Some critical organs can be blocked by combining RNT/RLT with additional drugs like arginine/lysine cocktails for kidney protection [343]. Overall, if selective accumulation in the tumor is achieved, ERT is much less toxic to healthy tissue than conventional chemotherapy and also more selective than external beam radiotherapy [9,339].

1.4.4. Radionuclides

The physical properties of the radionuclide such as half-life $t_{1/2}$, type of radiation including its percentage and energy as well as its availability (production route) are key factors to consider when choosing the ideal radionuclide for the respective radiopharmaceutical. High specific activity is an important aspect of radionuclide production besides radiochemical yield (RCY) and purity (RCP).

Some radionuclides are used directly in their cationic or anionic form as salts. Important examples include $[^{82}\text{Rb}]\text{RbCl}$ ($t_{1/2} = 1.3$ min, PET) and $[^{201}\text{Tl}]\text{TlCl}$ ($t_{1/2} = 73$ h, SPECT) as perfusion tracers in myocardial imaging or $[^{89}\text{Sr}]\text{SrCl}_2$ ($t_{1/2} = 50.5$ d), $[^{223}\text{Ra}]\text{RaCl}_2$ ($t_{1/2} = 11.4$ d) to treat bone metastases.

Table 2 summarizes the most important radionuclides that are used in nuclear medicine as functionalized radiotracers with a TV.

Table 2: Most important radionuclides in nuclear medicine and their respective physiological properties (half-life $t_{1/2}$, type of radioactive decay and respective percentage and energy E of the particle) and main production route [10,334,346–351].

Nuclide	$t_{1/2}$	Type of radioactive decay (percentage [%])	$E_\alpha, E_{\beta^+ \text{ max}}$ or E_γ [keV]	Main production route
^{11}C	20.4 min	β^+ (99.8)	961	$^{14}\text{N}(\text{p},\alpha)^{11}\text{C}$
^{18}F	110.0 min	β^+ (96.7)	634	$^{18}\text{O}(\text{p},\text{n})^{18}\text{F}$ (or $^{20}\text{Ne}(\text{d},\alpha)^{18}\text{F}$)
^{44}Sc	4.0 h	β^+ (94.3)	1474	$^{44}\text{Ti}/^{44}\text{Sc}$ generator or $^{44}\text{Ca}(\text{p},\text{n})^{44}\text{Sc}$
^{64}Cu	12.7 h	β^- (38), β^+ (18)	579 (β^-), 653 (β^+)	$^{64}\text{Ni}(\text{p},\text{n})^{64}\text{Cu}$, $^{67}\text{Zn}(\text{p},\alpha)^{64}\text{Cu}$ etc.
^{68}Ga	67.8 min	β^+ (89.1)	1899	$^{68}\text{Ge}/^{68}\text{Ga}$ generator
^{86}Y	14.7 h	β^+ (33)	1200	$^{86}\text{Sr}(\text{p},\text{n})^{86}\text{Y}$
^{90}Y	64.0 h	β^- (100)	2280	$^{90}\text{Sr}/^{90}\text{Y}$ generator
^{89}Zr	78.4 h	β^+ (23)	902	$^{89}\text{Y}(\text{p},\text{n})^{89}\text{Zr}$
$^{99\text{m}}\text{Tc}$	6.0 h	γ_1 (99), γ_2 (1)	140.5 (γ_1) 142.7 (γ_2)	$^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ generator
^{111}In	67.3 h	γ_1 (91), γ_2 (94), EC(100)	171 (γ_1) + 245 (γ_2)	$^{112}\text{Cd}(\text{p},2\text{n})^{111}\text{In}$
^{123}I	13.0 h	γ (83)	159	$^{123}\text{Te}(\text{p},\text{n})^{123}\text{I}$ or $^{124}\text{Te}(\text{p},2\text{n})^{123}\text{I}$
^{131}I	8.02 d	β^- (89), γ (81)	606 (β^-), 364 (γ)	$^{235}\text{U}(\text{n},\text{f})^{131}\text{I}$ or $^{130}\text{Te}(\text{n},\gamma)^{131}\text{Te} \xrightarrow{\beta^-} ^{131}\text{I}$
^{177}Lu	6.65 d	β^- (100), γ_1 (6), γ_2 (10),	498 (β^-), 113 (γ_1), 208 (γ_2)	$^{176}\text{Lu}(\text{n},\gamma)^{177}\text{Lu}$ or $^{176}\text{Yb}(\text{n},\gamma)^{177}\text{Yb} \xrightarrow{\beta^-} ^{177}\text{Lu}$
^{188}Re	17.0 h	β^- (100)	2120	$^{188}\text{W}/^{188}\text{Re}$ generator
^{211}At	7.0 h	EC (58), α (42)	5982 (α)	$^{209}\text{Bi}(\alpha,2\text{n})^{211}\text{At}$
^{212}Pb	10.6 h	β^- (100) + $1\times\alpha$ and $1\times\beta^-$ of daughters	335 (β^-)*1	^{228}Th or $^{224}\text{Ra}/^{212}\text{Pb}$ generator
^{225}Ac	10.0 d	α (100) + $3\times\alpha$ and $2\times\beta^-$ of daughters (sum: α (400), β^- (200))	5000 – 6000 (α)*2	$^{229}\text{Th}/^{225}\text{Ac}$ generator

*1 α particles with ca. 6 MeV (^{212}Bi , 36%) and ca. 9 MeV (^{212}Po , 64%) are emitted subsequently.

*2 Sum of all four emitted α particles in decay chain is ca. 28 MeV [340].

Overall, the most used radionuclide in nuclear medicine is technetium-99m ($t_{1/2} = 6.0$ h). It is clinically used in roughly 80% of all cases and is provided via $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ generators ($t_{1/2}(^{99}\text{Mo}) = 66$ h) that are routinely used for one week although direct production routes have been established recently [337,338,352]. Molybdenum-99 is produced via nuclear fission from uranium-235 with a high yield of 6.1% or, less preferred, in a $^{98}\text{Mo}(\text{n},\gamma)^{99}\text{Mo}$ reaction [334,337,353]. It is fixed on an inorganic Al_2O_3 matrix as (para)molybdate ($[\text{MoO}_4]^{2-}$ or $[\text{Mo}_7\text{O}_{24}]^{6-}$) and $^{99\text{m}}\text{Tc}$ is eluted with sterile saline (0.9% NaCl solution) as $[^{99\text{m}}\text{Tc}][\text{TcO}_4]^-$ which can directly be used for application in thyroid or myocardial perfusion SPECT imaging [353,354]. The energy of the emitted gamma rays are around 140 keV (cf.

Table 2) and in the optimal range of the NaI(Tl) detector [355]. It has to be considered that after emission the long-lived ^{99g}Tc ($t_{1/2} = 2.14 \cdot 10^5$ a) is formed and accumulates on the generator matrix the longer the time since last elution. For the radiolabeling of functionalized radiotracers, $^{99m}\text{Tc(VII)}$ has to be reduced but the radiolabeling chemistry is very complex due to the many oxidation states of technetium. A reducing agent like Sn(II) salts (SnCl_2 , SnF_2) or sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) are added to the labeling solution to reduce $^{99m}\text{Tc}[\text{TcO}_4]^-$ *in situ*. Tc(V) is one of the most common oxidation states where $^{99m}\text{Tc}[\text{TcO}]^{3+}$ can be complexed by N_xS_y chelators that are often tetradentate ligands ($x + y = 4$) such as N_4 , N_3S chelators like MAG3 (see chapter 3.6.), N_2S_2 chelators like DADT, DADS or MAMA. Two bidentate ligands are also possible but less stable [334]. Weak intermediate ligands like tartrate or citrate are also added to the labeling buffer solution that can form intermediate complexes very fast to stabilize Tc(V) and prevent further reduction to colloidal $^{99m}\text{Tc}[\text{TcO}_2]$ [337,353,356–359].

Other cores like $^{99m}\text{Tc}[\text{TcO}_2]^+$ or $^{99m}\text{Tc}[\text{TcN}]^{2+}$ are also known [337]. The HYNIC approach is utilized quite a lot where a functionalized 6-hydrazinonicotinamide (HYNIC) chelates Tc(V) together with co-ligand system like different phosphines, tricine, EDDA (ethylenediaminediacetic acid), nicotinic acid, glucaric acid or glucoheptonate [360,361]. In Tc(I) radiotracers, the other important oxidation state, tricarbonyl complexes are utilized most of the times. Therefore, $^{99m}\text{Tc}[\text{TcO}_4]^-$ can be reduced to the intermediate *fac*- $^{99m}\text{Tc}[\text{Tc}(\text{CO})_3]^+$ with a commercial IsoLink® kit and afterwards the tricarbonyl complex is used for labeling of the biomolecule that is attached to a tridentate ligand [337,359].

PET is mostly used in oncologic diagnosis since the most important radiotracer 2- ^{18}F fluoro-2-deoxy-D-glucose (^{18}F -FDG) is utilized as a glucose analog to visualize the upregulated glucose metabolism in tumors [10,362]. The ideal physical properties ($t_{1/2}$, low E_{β^+}) make fluorine-18 the most popular PET radionuclide. In general, diagnostic (PET) radionuclides have comparably short half-lives which reduces the radiation dose for the patient but the physical half-life has to match the biological half-life and of the radiotracer and its pharmacokinetic profile. For small molecule radiotracer, short half-lives with radionuclides like fluorine-18 or gallium-68 are optimal. On the other hand, special strategies (synthons etc.) that enable fast radiosynthesis and subsequent administration had and have to be developed for short-lived radionuclides like carbon-11. Radionuclides with even shorter half-lives like nitrogen-13 (10.0 min) or oxygen-15 (2 min) are mostly used as perfusion tracers in the basic form of ^{15}O - H_2O or ^{13}N - NH_3 [10,362]. Radionuclides with longer half-lives are suited for immuno-PET such as zirconium-89, indium-111 etc. due to the slow pharmacokinetic profile/accumulation of mAbs [362–364].

A high β^+ percentage is beneficial for high PET detection rates but β^+ decay can only occur when $\Delta Q \geq 1022 \text{ keV}$ ($2 \times m_e$). Electron capture (EC), as shown in equation (2), where an inner shell electron is captured by the nucleus competes with β^+ decay:



The probability of EC increases in bigger atoms as the inner orbital, especially the 1s orbital, is contracted stronger. Thus, almost all PET radionuclides with high β^+ percentage have small atomic numbers. The proton-rich PET radionuclides are usually produced via proton irradiation in a cyclotron [10,362,365].

1.4.4.1. Gallium-68

Despite the relatively high atomic number ($Z = 31$), gallium-68 has a high β^+ percentage (89%) as well as a half-life ($t_{1/2} = 67.8 \text{ min}$) that is sufficient for radiolabeling and subsequent purification and suitable for the pharmacokinetics of most small molecules [366]. Gallium-68 has a $E_{\beta^+ \text{ avg}} = 740 \text{ keV}$ and $E_{\beta^+ \text{ max}} = 1899 \text{ keV}$, respectively, which is higher than of fluorine-18 but is still sufficient for PET images with good quality [366,367].

Figure 7a shows the decay of germanium-68 exclusively into the ground state of gallium-68 via EC, followed by the further decay to zinc-68, either directly into the ground state via β^+ (87.7%) and EC (8.9%) or to an excited state via β^+ (1.2%) and EC (1.8%) followed by gamma ray emission ($E_\gamma = 1077 \text{ keV}$, 3.2%) [367].

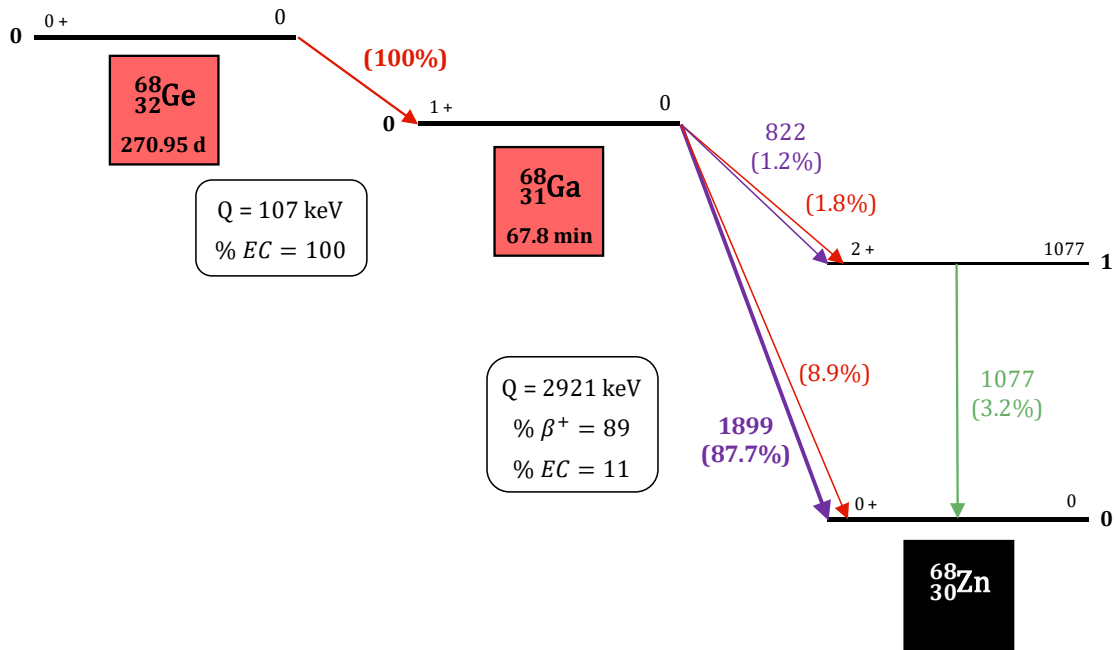


Figure 7: Decay scheme of ${}^{68}\text{Ga}$: ${}^{68}\text{Ge}$ decays exclusively into the ground state of ${}^{68}\text{Ga}$ via electron capture (EC, red arrows). ${}^{68}\text{Ga}$ then decays to stable ${}^{68}\text{Zn}$ predominantly via β^+ decay (purple arrows). The excited 2^+ state is populated to some extent which results in the emission of a gamma photon (green arrow). The numbers beside the arrow stand for $E_{\beta^+ \text{ max}}$ in keV (purple arrows) and E_γ in keV (green arrow), respectively. The emission probability is given in brackets (%).

The clinical breakthrough of gallium-68 happened with the availability of adequate $^{68}\text{Ge}/^{68}\text{Ga}$ generators in the beginning of the 2000s. Nevertheless, the direct cyclotron production via $^{68}\text{Zn}(\text{p},\text{n})^{68}\text{Ga}$ was established over the last years [368–370]. The long-lived parent nuclide germanium-68 ($t_{1/2} = 270.95$ d) is produced with the highest yields via $^{69}\text{Ga}(\text{p},2\text{n})^{68}\text{Ge}$ reaction in a cyclotron with medium high-energy protons (≈ 23 MeV) [370,371]. It is then fixed on an inorganic column matrix. Commercially produced generators with different materials such as TiO_2 , Al_2O_3 , SnO_2 , ZrO_2 , Ta_2O_3 and even organic polymers were developed [369–371].

The generator can be eluted again after 3 – 4 half-lives (≈ 3.5 – 4.5 h) with 87.5 – 93.75% of the maximum theoretical activity. This is feasible for clinical routine and usually eluted twice per day. Due to the secular equilibrium, the eluted activities are nearly constant for a long time and the generator is clinically used for at least one year. The generator is eluted with diluted hydrochloric acid, e.g. 5 mL 0.05 – 0.1 M HCl, depending on the exact generator type. Almost all germanium-68 remains on the matrix but minimal amounts are eluted as well. If the ^{68}Ge -breakthrough is $< 0.001\%$, the eluted gallium-68 can be directly used according to the European Pharmacopoeia [372]. In the case of a higher ^{68}Ge -breakthrough additional post-processing is necessary.

Metallic impurities, e.g. Ti(IV) or Sn(IV) from the matrix, Ge(IV), ubiquitous Fe(III) or Zn(II), can have a negative effect on subsequent radiolabeling as they can also build chelator complexes. Therefore, different post-processing methods were developed because fractioning of the eluate is not optimal [373]. ZHERNOSEKOV et al. developed acetone-based cationic post-processing. This was further developed by EPPARD et al. using ethanol and the procedure is shown in Figure 8 [374,375]. The eluate is passed through a cation exchange resin (CXR, AG 50W-X4) and all metal ions except germanium-68 are fixed. The remaining impurities can be separated with 1.0 mL 0.15 M hydrochloric acid (80% ethanol) and 1 mL air. Gallium-68 is obtained in very high radiochemical purity with 400 – 500 μL 0.9 M hydrochloric acid (90% ethanol) + 1 mL air in just a few minutes. The resin is regenerated with 1 mL 4 M HCl, 1 mL air and finally 1 mL water [375].

Anionic post-processing has also been developed but much higher HCl concentrations are needed which is not optimal as high buffer capacities for subsequent radiolabeling are required [376]. Alternatively, elution of gallium-68 from a strong cation exchange resin has also been investigated with 500 μL 5 M NaCl + 12.5 μL 5.5 M HCl [377].

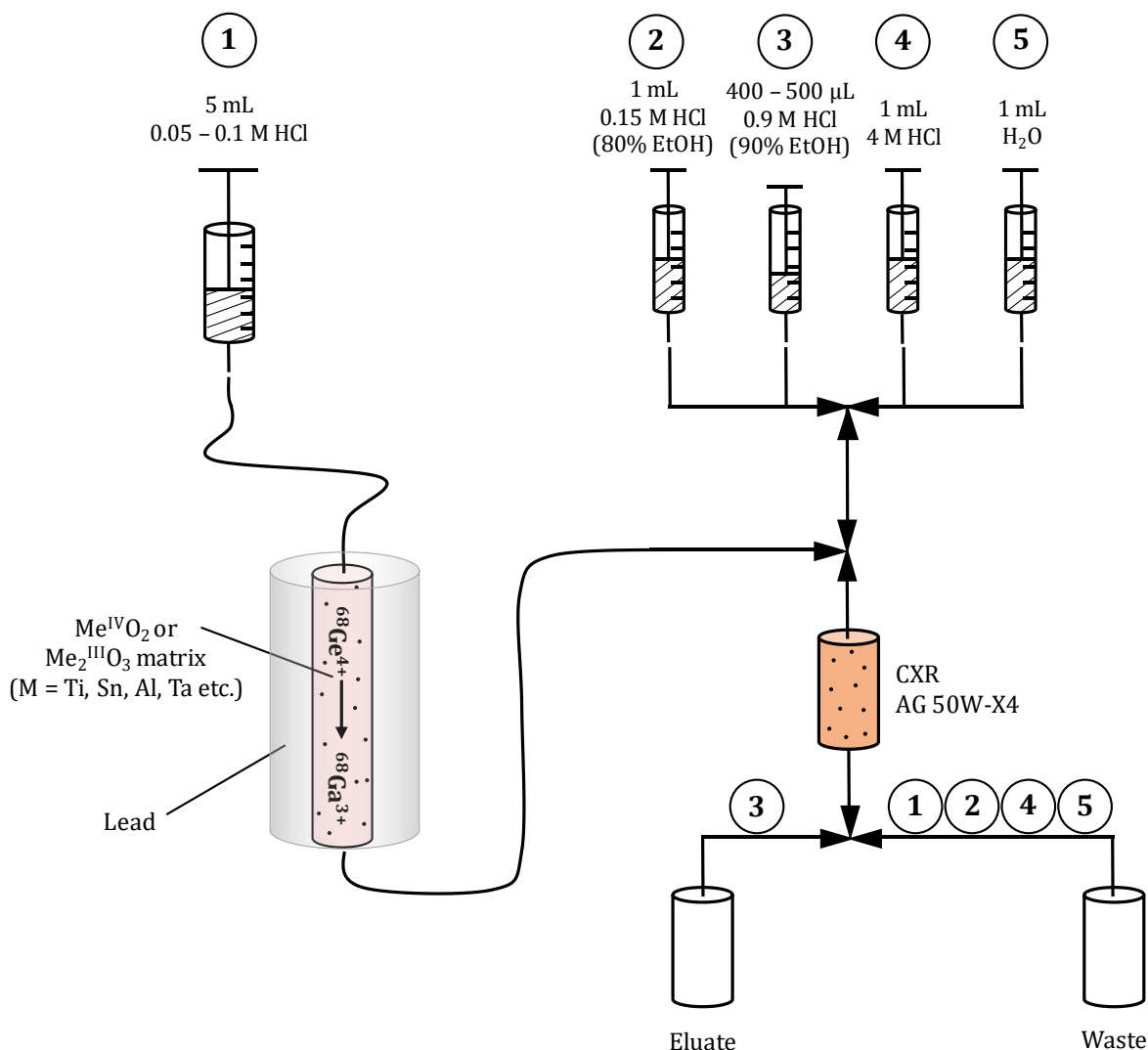


Figure 8: Schematic $^{68}\text{Ge}/^{68}\text{Ga}$ radionuclide generator with ethanol-based post-processing developed by Eppard et al. with cation exchange resin (CXR) AG 50W-X4 [375]. The order of elution is accordingly to the numbering. Air is used between each step to ensure that the respective solution is passed through completely.

Gallium is only stable as a trivalent metal in aqueous solution. The hard Lewis acid Ga^{3+} has an ionic radius of 62 pm and usually prefers six-fold coordination in a (distorted) octahedral geometry with hard ligand atoms (oxygen, nitrogen) for stable complexation [366,378]. $[\text{Ga}(\text{H}_2\text{O})_6]^{3+}$ is the dominant form in acidic conditions but at $\text{pH} > 3$ gallium can hydrolyze and colloidal $\text{Ga}(\text{OH})_3$ precipitates. In strong basic conditions, gallium is soluble again and as $[\text{Ga}(\text{OH})_4]^-$ is formed [334,366,379]. ^{68}Ga -radiolabeling is usually performed in a slightly acidic buffer system ($\text{pH} = 3.5 - 5.5$), depending on the exact chelator and precursor. In some cases like HEPES buffer it can additionally act as a weak intermediate ligand [380,381]. For example, HEPES and acetate buffer fulfills both aspects at once (pH and weak intermediate ligand) to avoid formation of colloidal gallium $\text{Ga}(\text{OH})_3$ [334,366,373]. Organic solvents like ethanol have been reported to accelerate labeling and lead to better radiochemical yields (RCY) [382,383].

1.4.4.2. Lutetium-177

Lutetium-177 ($t_{1/2} = 6.65$ d) can be broadly applied due to its established radionuclide production. Carrier-added (c.a.) $[^{177}\text{Lu}]\text{LuCl}_3$ can be produced in high quantities due to a very high cross section with thermal neutrons at nuclear reactors from highly enriched lutetium-176 in a (n,γ) reaction. However, long-lived metastable $^{177\text{m}}\text{Lu}$ ($t_{1/2} = 160.4$ d) is formed as a byproduct. An alternative indirect route has been developed: $^{176}\text{Yb}(n,\gamma)^{177\text{m}}\text{Yb} \xrightarrow{\beta^-} ^{177}\text{Lu}$ [384]. Highly enriched ytterbium-176 is irradiated with thermal neutrons in a high-flux reactor to give ytterbium-177 ($t_{1/2} = 1.91$ h) which decays to lutetium-177 via β^- decay and yields $[^{177}\text{Lu}]\text{LuCl}_3$. This non-carrier-added (n.c.a.) $[^{177}\text{Lu}]\text{LuCl}_3$ is of highest specific activity because it can be chemically separated from the irradiated ytterbium-176 [384].

Presupposing the usage of high-purity $[^{177}\text{Lu}]\text{LuCl}_3$, ^{177}Lu -labeled radiopharmaceuticals can be synthesized with very high RCP and RCY that do not require any subsequent purification [385,386]. The low average beta energy $E_{\beta^- \text{ avg}} = 130 - 140$ keV corresponds to a range of ca. 700 μm in tissue is beneficial concerning possible side effects of RLT [340,351]. Emission of gamma photons $\gamma_1 = 113$ keV (6.2%) and $\gamma_2 = 208$ keV (10.4%) enables SPECT imaging to monitor therapy. Figure 8 shows the decay scheme of lutetium-177 to stable hafnium-177 via β^- decay.

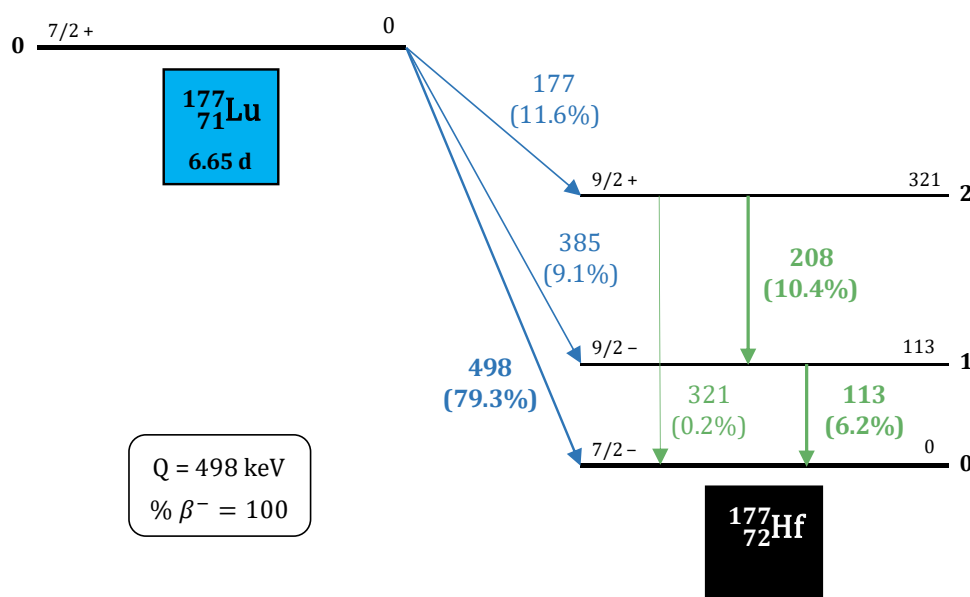


Figure 9: Decay scheme of lutetium-177 to stable hafnium-177. Blue arrows represent β^- decay with $E_{\beta^- \text{ max}}$ in keV and green arrows show subsequent gamma emission with E_γ in keV. The respective emission probability is given in brackets (%).

In aqueous solution, Lu^{3+} has 8 water molecules in the first hydration sphere oscillating between two-capped trigonal prism and square antiprism [387]. The coordination number varies between 6 and 12, e.g. a square antiprismatic crystal structure was determined in the complex with unfunctionalized DOTA [388]. Functionalized DOTA- or DOTAGA-based precursors can be labeled

with lutetium-177 very well because the ion radius and the cavity size of DOTA match [319,322,334,389–396].

The same type of chelators (cf. chapter 1.4.5) are used for gallium-68 which lays the foundation for $^{68}\text{Ga}/^{177}\text{Lu}$ -based theranostics. Although the theranostic pair is not matched in the sense of being different chemical elements, it is used extensively in nuclear medicine because gallium-68 is permanently available in clinics with $^{68}\text{Ge}/^{68}\text{Ga}$ generator and quantitative ^{68}Ga -PET scans pre- and post-treatment provide useful diagnostic information for treatment planning and assessment [390–394]. The first therapeutic ^{177}Lu -labeled radiopharmaceuticals [^{177}Lu]Lu-PSMA-617 (Pluvicto®) and [^{177}Lu]Lu-DOTA-TATE (Luthathera®) were approved by the FDA and the EMA in recent years.

The β^- emitters can be subdivided into short, medium and long range β^- emitters according to their E_{β^-} . As mentioned above, short-range radionuclides like lutetium-177 are advantageous for small lesions and (micro)metastases because the surrounding tissue is less exposed to radiation. In contrast, long-range radionuclides like yttrium-90 bigger lesions more effectively due to higher *crossfire effect*. Generally, Y^{3+} and Lu^{3+} have very similar properties, e.g. ion radii (88 vs. 86 pm) and labeling properties. Therefore, the same chelators are used for both radionuclides (cf. chapter 1.4.5). In contrast to lutetium-177, yttrium-90 ($t_{1/2} = 64.0$ h) is available via a $^{90}\text{Sr}/^{90}\text{Y}$ generator. Due to the high-energy beta particles, radiolysis is more critical with yttrium-90 and radical scavengers like ascorbic acid or gentisic acid (2,5-dihydroxybenzoic acid) are often added to a ^{90}Y -radiopharmaceutical formulation [397,398].

1.4.4.3. Actinium-225

Actinium-225 ($t_{1/2} = 10.0$ d) has a complex decay scheme and emits a series of α particles among with β^- particles (Figure 10). The resulting high radiation dose per actinium-225 radionuclide forms the foundation of its high therapeutic potential.

The recoil energy after the first α transformation will lead to dissociation of francium-221 from ^{225}Ac -labeled radiopharmaceutical. Once the daughter nuclides are released, they can redistribute in the body as they are not linked to the TV anymore. The first three α particles are emitted in quick succession as the half-lives of francium-221 and astatine-217 are short. Bismuth-213 ($t_{1/2} = 45.6$ min) has a longer half-life which can be critical since Bi(III) is known to accumulate in the kidneys and can lead to dose-limiting radiotoxicity [399]. Studies were carried out mainly using antibodies where encapsulating the ^{225}Ac -payload could minimize off-target accumulation of bismuth-213 [400,401]. Small molecules labeled with actinium-225 might behave differently when they are internalized into the cell as it might slow down the washout of bismuth-213. Using bismuth-213 directly was seen as an alternative but the anti-tumor effect seemed to be much less compared to actinium-225 [402].

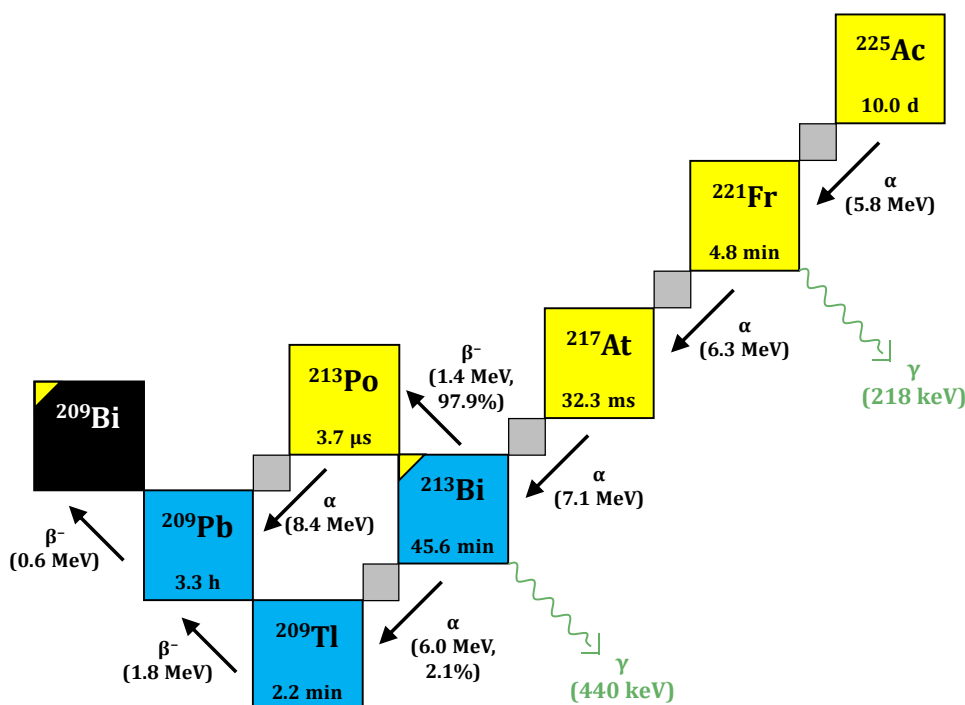


Figure 10: Decay chain of actinium-225. Four α particles and two β^- are emitted in total to get to bismuth-209. Francium-221 and bismuth-213 emit gamma rays that are useful for analytics.

Availability of actinium-225, however, is an important issue to date that limits ^{225}Ac -TAT. Currently, the actinium-225 is mainly available via $^{229}\text{Th}/^{225}\text{Ac}$ generator but these sources are limited to around 60 – 70 GBq per year which allows for only a few thousand treatment cycles per year even with optimized generator elution cycle [334,403,404]. The thorium-229 was isolated from an old uranium-233 stockpile and it is not possible to isolate more thorium-229 from this due to political reasons (potential weapon use of uranium-233 etc.) [403,405]. Therefore, new production routes are currently investigated due to the increasing demand of actinium-225 for TAT and include the spallation of thorium-232 ($^{232}\text{Th}(p,x)^{225}\text{Ac}$) by high-energy proton irradiation (> 100 MeV), photonuclear reaction ($^{226}\text{Ra}(\gamma,n)^{225}\text{Ra} \xrightarrow{\beta^-} ^{225}\text{Ac}$) or production via cyclotron ($^{226}\text{Ra}(p,2n)^{225}\text{Ac}$) with medium energy protons (10 – 25 MeV) amongst others [406–408].

Actinium-225 is chemically similar to lanthanides and the oxidation state Ac(III) is the most stable. Ac^{3+} has the largest ion radius of all trivalent radiometals (112 pm) which makes it the least acidic and it seems to not be hydrolyzed at $\text{pH} < 9$ [409]. Due to its size high coordination numbers are preferred. The Ac -aquo complex was determined to be eleven-fold coordinated in the inner hydration sphere [410]. DOTA-containing precursors could be radiolabeled with actinium-225 in numerous examples [345,409,411–415]. This combination is not a perfect match due to the large ionic radius of Ac^{3+} . This results in weaker electrostatic interaction with the donor ligands and the insufficient number of donor ligands in chelators like DOTA leads to less stable complexes that might not be completely stable *in vivo* [409,416,417].

The gamma emissions of francium-221 ($E_\gamma = 218$ keV) and bismuth-213 ($E_\gamma = 440$ keV, cf. Figure 10) are important for the radiopharmaceutical analytics such as TLC and gamma spectroscopy but cannot be utilized in SPECT imaging because the administered activity amounts are not high enough [334,418]. Due to the matching half-lives, indium-111 ($t_{1/2} = 67.3$ h) might be an interesting theranostic counterpart for SPECT imaging. Gallium-68 is also an option for radiopharmaceuticals with fast pharmacokinetics. For example, first clinical trials (phase I) have started with [^{225}Ac]Ac-DOTA-TOC or -TATE for neuroendocrine tumors or [^{225}Ac]Ac-PSMA-617, a prostate specific membrane antigen (PSMA) inhibitor, usually for patients resistant to previous ^{177}Lu -RLT [411–413,419–421]. ^{225}Ac -labeled mAbs like the PSMA-targeting J591 are also (pre)clinically studied [422,423].

1.4.5. Chelators

Multidentate ligands (chelators) are utilized to attach trivalent radiometals such as gallium-68, yttrium-90, lutetium-177 and actinium-225 to a TV because they are more stable than monodentate complexes due to increased entropy (*chelate effect*) [379,424]. Optimum stability is thus achieved when all coordination sites can be covered by the chelator. For the abovementioned trivalent radiometals, the coordination number is at least six. According to the HSAB theory the trivalent radiometals, especially the smaller ones like gallium-68, are hard Lewis acids and therefore prefer hard Lewis bases like oxygen or nitrogen containing functional groups as electron donor groups (ligands). Ideally, a chelator can be radiolabeled under mild conditions fast and the complex is stable at physiological pH and *in vivo*. An important indicator is stability in human serum. The protein transferrin, which is present in the blood, is responsible for the transport of iron in the body and has two binding sites for Fe(III) [366]. It also forms stable complexes with chemically similar Ga(III) ($\log K = 20.3$). Similar applies for other trivalent radiometals [366]. To prevent transchelation, the chelator-based radiopharmaceutical must have a higher stability constant than transferrin or be kinetically inert.

Chelators can be categorized into acyclic and macrocyclic ones. All the following examples are bifunctional chelators which means that they have a functional group where the chelator can be by coupling it to the TV for further functionalization.

Radiolabeling with acyclic chelators is usually quick and works very well at room temperature. Acyclic chelators are less kinetically inhibited, which is why problems with *in vivo* stability can occur [379,425].

Figure 11a shows the structures of the acyclic chelators diethylenetriaminepentaacetic acid (DTPA), *N,N'*-bis[(2-hydroxy-5-propanic acid)benzyl]ethylenediamine-*N,N'*-diacetic acid (HBED-CC), and deferoxamine (DFO). DTPA is derived from EDTA (ethylenediaminetetraacetic acid) and was one of the first chelators used in radiochemistry, and the N_3O_5 core makes it suitable for

metals requiring six-site coordination (^{68}Ga , ^{89}Zr) as well as metals with more coordination sites (e.g. ^{111}In , ^{177}Lu) [379,426]. The O_6 ligand DFO is preferably utilized with the hard Lewis acid $^{89}\text{Zr}(\text{IV})$ and in the context of ^{89}Zr -based immuno-PET, but is also suitable for other radiometals such as gallium-68 [364,379,427]. HBED-CC has two aromatic moieties and was developed as a chelator that is more lipophilic. It forms stable complexes with gallium-68 although investigations with ^{68}Ga [Ga]-HBED-CC-PSMA-11 revealed that it forms several unfavorable diastereomers and enantiomers [428,429].

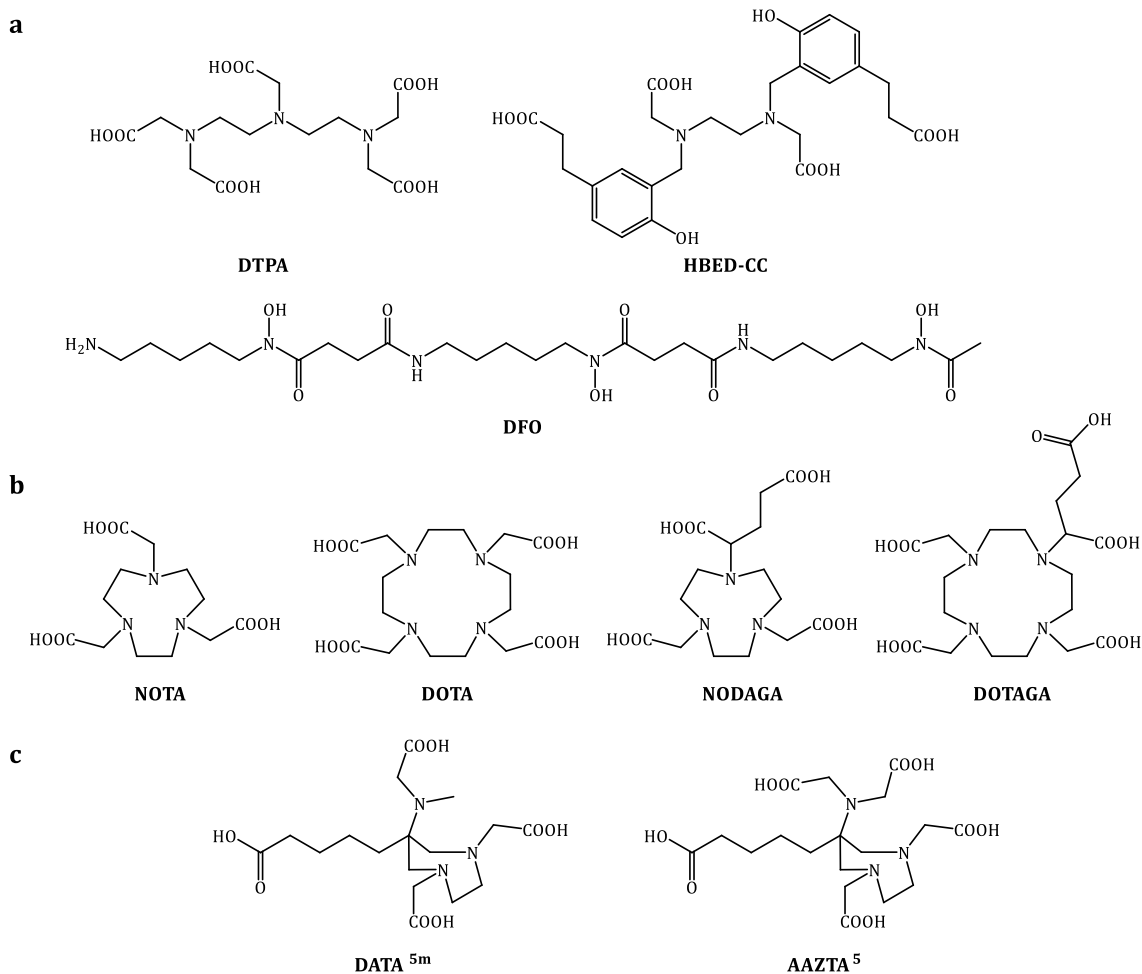


Figure 11: Selected important examples of a) acyclic, b) macrocyclic and c) hybrid chelators for trivalent radiometals: a) the acyclic chelators DTPA, HBED-CC and DFO; b) the macrocyclic chelators NOTA, DOTA, NODAGA, DOTAGA and c) the hybrid chelators DATA^{5m} and AAZTA⁵.

Macrocyclic chelators (cf. Figure 11b) are arranged all around the central ion and form a cage-like complex. In general, macrocyclic complexes are thermodynamically more stable than acyclic chelators with similar geometry and donor ligands and the dissociation of the ligand is kinetically inhibited. The chelate effect (entropy) and the additional preorganized structure of macrocyclic chelators contribute to the stability and complex formation and is referred to as the *macrocyclic effect* [429]. However, kinetic inhibition also plays a role in the complex formation which is why elevated temperatures are often required to achieve quantitative and fast complexation.

Ga-complexes with 1,4,7-triazacyclononane-1,4,7-triacetic acid (NOTA) are more stable than with 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) because the ionic radius of the Ga(III) matches the inner radius of NOTA very well, whereas DOTA is slightly too large [430]. However, NOTA is only a hexadentate ligand which is problematic when it is supposed to be functionalized because then only five donor groups are left which is not optimal for stable complex formation with Ga(III) and other trivalent metals. Therefore, NODAGA (1,4,7-triazacyclononane-1-glutaric acid-4,7-diacetic acid) was developed which contains a side chain with an additional carboxyl group for functionalization. Similarly, DOTAGA (1,4,7,10-tetraazacyclododecane-1-glutaric acid-4,7,10-triacetic acid) was developed as a DOTA derivative with an additional carboxyl group. DOTA is an octadentate ligand, or heptadentate if functionalized at one carboxyl group, and thus has enough donor groups for complexation of lutetium-177. DOTA is the gold standard as a chelator in theranostic approaches with gallium-68 and lutetium-177 [7,12,322,373,389,391,392,395,431]. Many variants of DOTA exist that differ in the way the chelator can be coupled. This includes alkyne or azide groups for coupling via click chemistry, iso(thio)cyanates to form (thio)ureas, *N*-hydroxysuccinimide (NHS) active esters for facilitated amide coupling, DOTA derivatives with partial or complete substitution of the carboxylic acid to amide (e.g. DOTAM) or phosphonate groups (e.g. DOTPI) [430,432]. Many derivatives were developed to optimize labeling, chemical and/or *in vivo* properties for NOTA and other common macrocyclic chelator systems as well. For example, the chelator TRAP (1,4,7-triazacyclononane-1,4,7-tris[methyl(2-carboxyethyl)-phosphinic acid] with its phosphonate groups could be robustly labeled with gallium-68 in a wide pH range [379,433]. Other groups have developed novel chelators that enable radiolabeling at neutral pH, e.g. THP (tris(hydroxypyridinone))-based ligands) [434–436].

²²⁵Ac-chelator optimization led to the development of Macropa, an 18-membered macrocycle with 10 donor ligand atoms that allows effective radiolabeling at room temperature of very stable complexes. Macropa was recently conjugated to the somatostatin agonist TATE to be used in the first radiopharmaceutical [²²⁵Ac]Ac-Macropa-TATE [417,437].

Hybrid chelators (cf. Figure 11c) like AAZTA⁵ (5-[[6-(*N,N'*-diacetic acid)amino]-1,4-diacetic acid-1,4-diazepane]-pentanoic acid) or DATA^{5m} (5-[[6-(*N*-acetic acid)(*N'*-methyl)amino]-1,4-diacetic acid-1,4-diazepane]pentanoic acid) have been developed to unite the advantages of acyclic and macrocyclic chelators [379,438–440]. Structurally, hybrid chelators take an intermediate position between acyclic and macrocyclic chelators. AAZTA⁵ has a rigid cyclic 1,4-diazepane backbone, but also a tertiary amine moiety with two acetate groups that can be seen as an acyclic moiety [379]. In comparison to AAZTA⁵, one acetate group is substituted by a methyl group in DATA^{5m} and it is only hexadentate. The pentanoic acid residue of both chelators enables coupling to a TV [439].

AAZTA⁵ and DATA^{5m} fuses the favorable properties of acyclic and macrocyclic chelators, as they combine fast complexation kinetics under mild conditions (room temperature) with high stability *in vivo* [438,439,441–443]. They show high stabilities in human serum and are therefore promising chelators for radiopharmaceuticals. The heptadentate AAZTA⁵ forms very stable complexes with radiometals such as gallium-68, scandium-44, indium-111, and lutetium-177, whereas DATA^{5m} is limited to gallium-68. Quantitative radiolabeling can often be achieved after a few minutes at room temperature [379,438,439,441–443].

1.5. *FAPi-based Radiopharmaceuticals*

The selective expression pattern of FAP makes it a very exciting oncological target for broad application in nuclear medicine as many epithelial cancer types can be addressed with the same radiopharmaceutical. Quite a few radiolabeled monoclonal antibodies (mAbs) have been developed but the following chapter will focus on small molecule FAP inhibitors.

Unlike with the FAP inhibitors discussed in chapter 1.3.4., the anti-tumor efficacy of FAP-targeting radiopharmaceuticals comes from the ionizing radiation and not from inhibiting the protease. Therefore, the expression pattern is the crucial factor and the biological role of FAP in cancer is secondary.

In fact, the first developed FAP-targeting radiopharmaceuticals were labeled mAbs such as [¹³¹I]-mAbF19 [285]. Satisfactory biodistribution, high accumulation in the colorectal carcinoma lesions and limited accumulation in healthy tissue and interestingly also in incision/op scar tissue was demonstrated via scintigraphy. Sibrotuzumab did not show FAP inhibition or direct cytotoxicity and phase I and early phase II studies demonstrated that Sibrotuzumab was well tolerated but was not efficacious [285,444,445].

1.5.1. *Diagnostic FAPi-based Radiotracers*

The first small molecule FAPi-based radiopharmaceutical was [¹²⁵I]-MIP-1232 (4-[¹²⁵I]Iodobenzoyl-Gly-boroPro, cf. Figure 12a) and was used for atherosclerotic plaques investigations [446]. The IC_{50} value of the boronic acid derivative was determined to be 0.6 nM and showed decent PREP/FAP selectivity ($SI = 32$). The radiotracer showed high binding to FAP-positive SK-Mel-187 melanoma xenograft and low binding to NCI-169 xenografts with low FAP levels in *in vitro* autoradiography. But no selectivity was observed regarding plaques versus normal arterial tissue [446].

In 2018, the group of UWE HABERKORN from the Heidelberg University was the first to couple a highly-selective FAPi, a functionalized version of difluorinated UAMC-1110 or its non-fluorinated counterpart (cf. Table 1) [302,447] to the bifunctional chelator DOTA via different linker groups [448,449]. The chelator DOTA was introduced for subsequent radiolabeling with radiometals such as gallium-68 for application in diagnostic ⁶⁸Ga-PET. Their strategy was to insert heterocyclic linkers like piperidine or piperazine and identified FAPI-04 as the leading candidate that utilized the difluorinated UAMC-1110 in contrast to the non-fluorinated FAPI-02 and a piperazine linker (cf. Figure 12b).

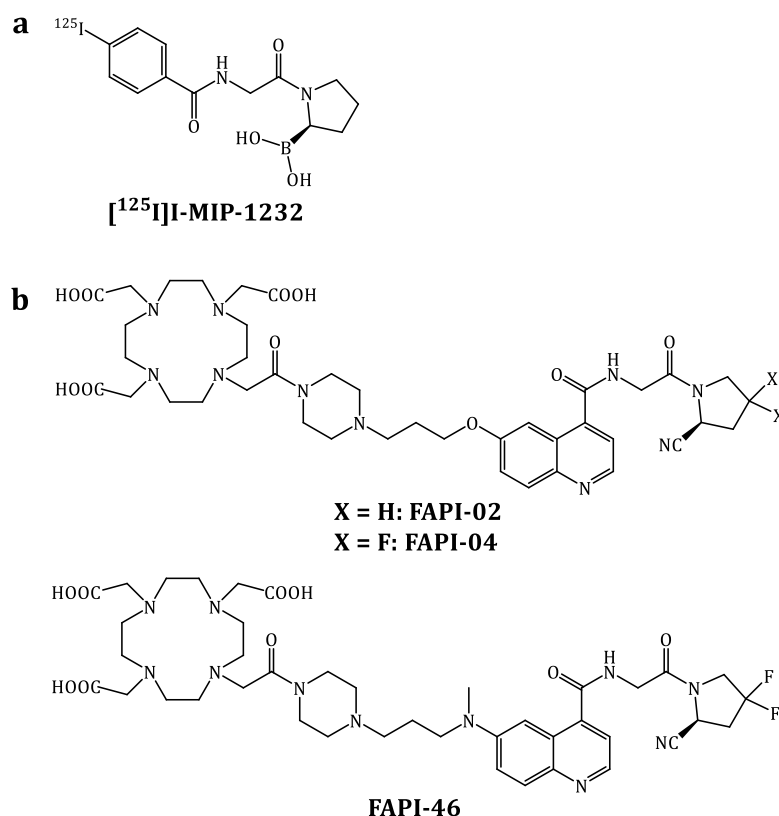


Figure 12: Structures of the first FAPi-based radiotracers a) [¹²⁵I]I-MIP-1232 as well as b) FAPI-02 (X = H), FAPI-04 (X = F) and c) FAPI-46 from the Heidelberg group.

In vitro cell studies revealed that ¹⁷⁷Lu-labeled FAPI-04 almost completely internalized into HT-1080 cells after one hour and a biological half-life of 3.0 h compared to 1.7 h for FAPI-02 [449]. *Ex vivo* biodistribution studies with FAP-positive HT-1080 xenografts showed higher tumor accumulation, especially at later time points compared to FAPI-02 and good tumor-to-blood ratio which was confirmed in first murine μ PET imaging studies. [⁶⁸Ga]Ga-FAPI-04 showed fast and high tumor uptake and renal clearance which gave PET images with high tumor-to-background ratio (TBR) as early as 10 minutes post-injection (10 min p.i.) in human PET studies [448,449]. Original dosimetry and biodistribution studies as well as the first bigger study demonstrated a favorable pharmacokinetic (PK) profile and favorable TBR compared to [¹⁸F]F-FDG in the liver, oral mucosa, and especially in the brain [450]. It established the FAPI-PET imaging protocol where the PET scan is routinely carried out one hour p.i. for optimal image quality [450,451]. Figure 13 shows the SNMMI 2019 Image of The Year [451].

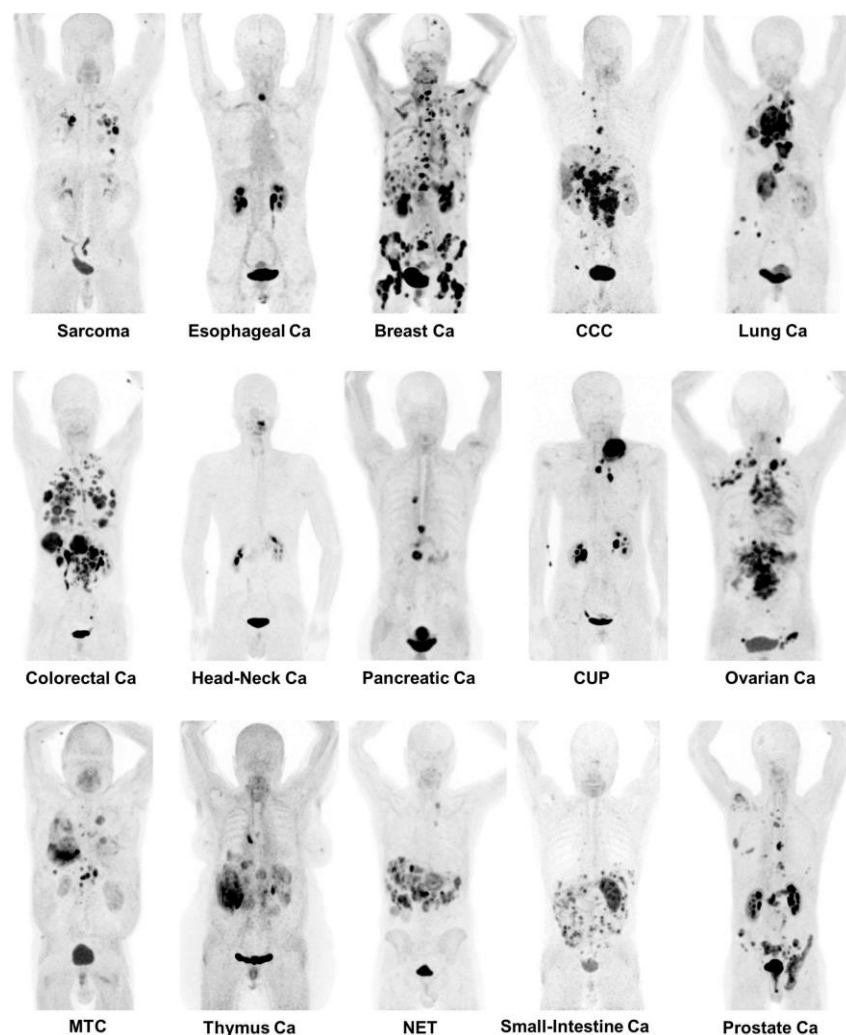


Figure 13: SNMMI 2019 Image of The Year: $[^{68}\text{Ga}]\text{Ga-FAPI-04}$ was evaluated in 28 different cancer types of which 15 examples are depicted. This figure was originally published in *Journal of Nuclear Medicine (JNM)* by KRATOCHWIL et al. $^{68}\text{Ga-FAPI PET/CT: Tracer Uptake in 28 Different Kinds of Cancer}$. *J Nucl Med.* **2019**, 60, 801–805 [451]. © 2019 SNMMI (“JNM allows reuse of excerpted original material such ... figures...” for “non-commercial” use e.g. “academic theses/dissertations”: <https://jnm.snmjournals.org/page/permissions>, accessed on May 14, 2023).

Among the 28 different cancer types investigated by KRATOCHWIL et al. [451] many showed intense accumulation of $[^{68}\text{Ga}]\text{Ga-FAPI-04}$ in the tumor lesions including sarcoma, lung, breast, prostate, ovarian, head-neck, pancreatic, hepatocellular and colorectal cancer among others. Numerous studies and meta-analyses have since explored $[^{68}\text{Ga}]\text{Ga-FAPI-04}$ and further verified that $[^{68}\text{Ga}]\text{Ga-FAPI-04}$ PET/CT is superior to $[^{18}\text{F}]\text{F-FDG}$ in many cases [452–461]. It has been also investigated in PET/MRI studies that drew similar conclusions [461–463].

It was tried to optimize tumor retention with the second generation compounds of the HABERKORN group [464]. The leading compound FAPI-46 (cf. Figure 12c) showed similar cell internalization and improved binding compared to FAPI-04 *in vitro*. Improved TBR (blood, liver, muscle) and higher tumor uptake but only slight and tumor retention was observed in murine and human ^{68}Ga -PET studies [464,465].

The group of FRANK ROESCH (JGU Mainz) developed the FAPi ligands DOTA.SA.FAPi, DATA^{5m}.SA.FAPi and AAZTA⁵.SA.FAPi using a squaramide (SA) linker derived from squaric acid for coupling of the respective chelator and the targeting vector FAPi-NH₂ (cf. Figure 14a – d) [466,467]. FAPi-NH₂ is a derivative of UAMC-1110 with an additional 4-aminobutoxy group at 6-position of the quinoline moiety for linkage.

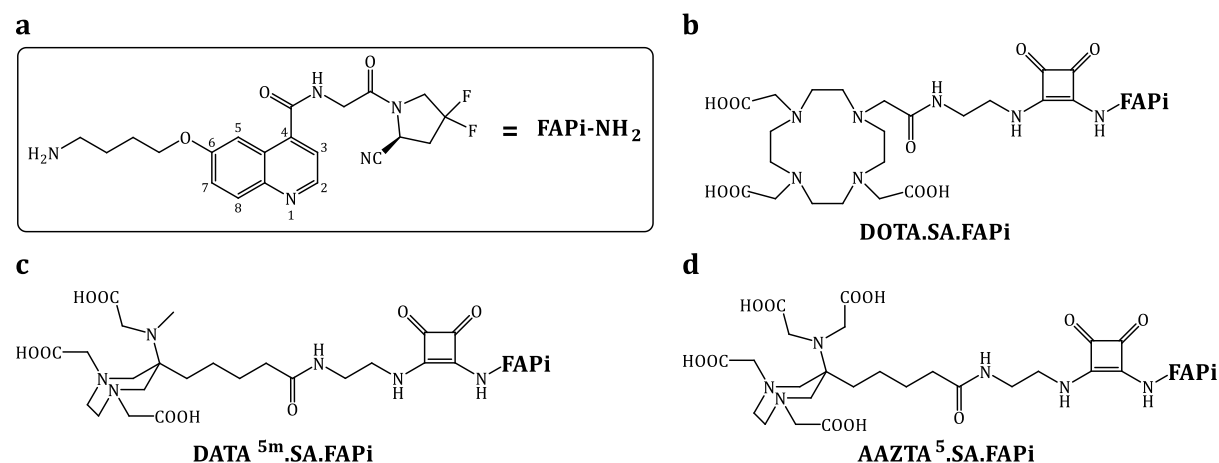


Figure 14: Structures of a) the targeting vector FAPi-NH₂ derived from UAMC-1110 and the monomeric FAPi ligands of the Mainz group b) DOTA.SA.FAPi, c) DATA^{5m}.SA.FAPi and d) AAZTA⁵.SA.FAPi.

SA has already been used in several radiopharmaceutical applications like PSMA inhibitor-based radiotracers and is characterized by a convenient coupling chemistry [468–471]. Two amines can be coupled to SA stepwise/asymmetrically under pH-controlled mild reaction conditions where the first amidation is usually carried out at neutral pH in aqueous solution or with small amounts of organic base (e.g. TEA, DIPEA) in organic solvents whereas the second amidation is carried out at pH $\approx$ 9 or in excess of organic base [468–471]. The reaction is characterized by low side product and byproduct formation as compared to amide/peptide coupling and the monoamidated intermediate can be isolated.

Binding affinity studies demonstrated high FAP affinity and selectivity over PREP and DPP4 (*SI* > 1000, respectively) for all compounds DOTA-, DATA^{5m} and AAZTA⁵.SA.FAPi. Murine and human PET studies demonstrated a PK profile for [⁶⁸Ga]Ga-DOTA.SA.FAPi similar to [⁶⁸Ga]Ga-FAPi-04 or -FAPi-46 with tumor uptake but short tumor retention. High-contrast PET images could also be achieved as early as 10 min p.i. in first breast and lung cancer patient studies as shown in Figure 15. Since there is some blood pool activity in some cases (see lung cancer patient in Figure 15) imaging after 1 h p.i. proved to be the optimal time point for [⁶⁸Ga]Ga-DOTA.SA.FAPi PET imaging [472,473].

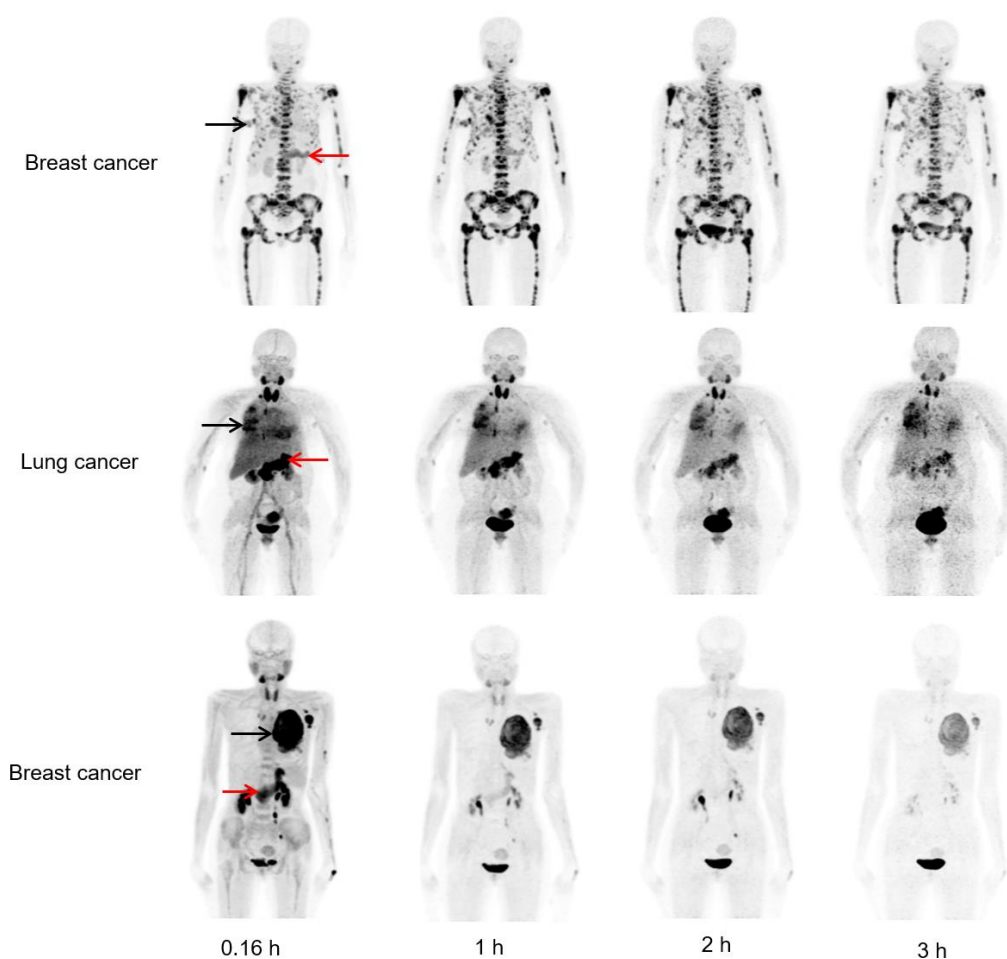


*Figure 15: Biodistribution of $[^{68}\text{Ga}]\text{Ga-DOTA.SA.FAPi}$ in breast and lung cancer patients at different time points. Black arrows indicate accumulation in the primary tumor lesion and red arrows some physiological pancreas uptake. This figure was originally published in *European Journal of Nuclear Medicine and Molecular Imaging (EJNMMI)* by BALLAL et al. Biodistribution, pharmacokinetics, dosimetry of $[^{68}\text{Ga}]\text{Ga-DOTA.SA.FAPi}$, and the head-to-head comparison with $[^{18}\text{F}]\text{F-FDG}$ PET/CT in patients with various cancers. *Eur J Nucl Med Mol Imaging*. **2021**, 48, 1915–1931 [473]. © 2021 Springer (“Reproduction of figures or tables from any article is permitted free of charge and without formal written permission from the publisher or the copyright holder...”: <https://www.springeropen.com/about/policies/reprints-and-permissions>, accessed on May 14, 2023).*

Recently, head-to-head comparisons with $[^{18}\text{F}]\text{F-FDG}$ in large patient cohorts of breast cancer and radioiodine-resistant follicular-cell derived thyroid cancer were published [474,475]. Higher tumor uptake was found compared to $[^{18}\text{F}]\text{F-FDG}$ and PET/CT scans led to improved detection of metastases in lymph nodes, brain, liver and colon among others. $[^{68}\text{Ga}]\text{Ga-DOTA}^{\text{Sm}}.\text{SA.FAPi}$ demonstrated to be a suitable radiotracer in various types of carcinomas [476]. Additionally, the derivative $\text{AAZTA}^{\text{Sm}}.\text{SA.FAPi}$ showed promising radiolabeling properties with scandium-44 for a potential future application in $^{44}\text{Sc-FAPi-PET}$ [467].

Overall, both the Heidelberg and Mainz ^{68}Ga -labeled FAPi-radiotracers show excellent imaging properties despite some uptake in the pancreas seen in some DOTA- and $\text{DATA}^{\text{Sm}}.\text{SA.FAPi}$ studies, possibly due to physiological FAP expression in the pancreas (cf. chapter 1.3.1.).

Other groups also developed FAPi radiotracers that were designed for radiolabeling with gallium-68 and other trivalent radiometals that used slightly different FAPi TVs (cf. Figure 16).

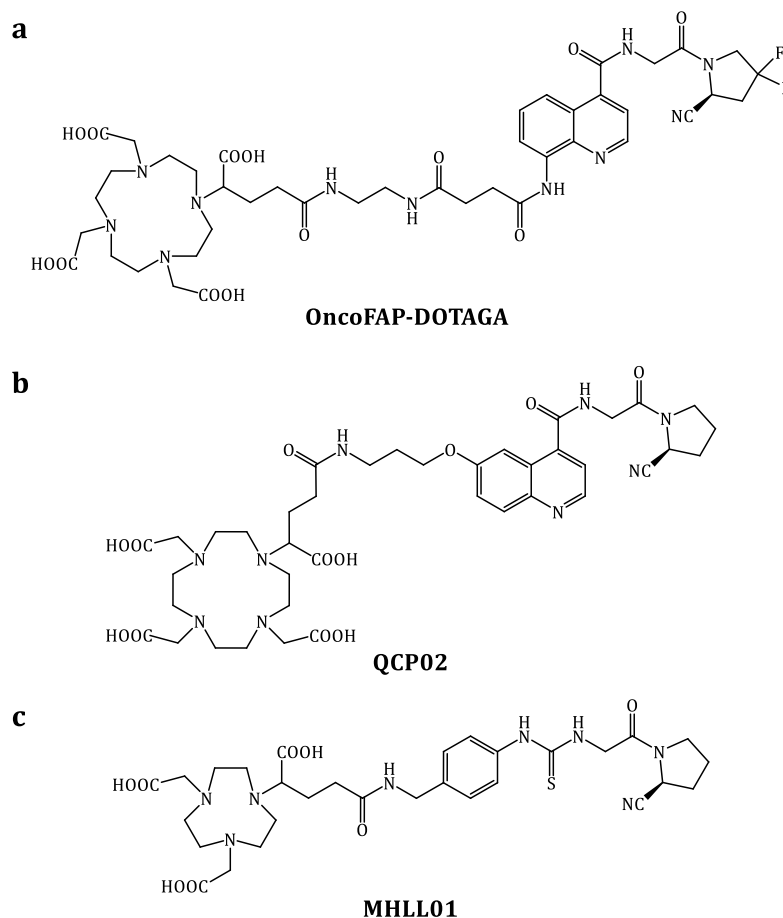


Figure 16: Structures of further FAPi ligands for ^{68}Ga -PET and other trivalent radiometals: a) OncoFAP-DOTAGA, b) QCP02 and c) MHLL01.

The R&D department of Philochem AG developed FAP inhibitor OncoFAP where quinoline moiety is substituted at 8-position by an amine that is coupled to succinic acid. The free carboxylic acid of succinic acid is used for further linkage to the chelator DOTAGA [315]. The IC_{50} value of OncoFAP for humanFAP was determined to be 16.8 nM ($K_D = 0.68$ nM). Selectivity of [^{68}Ga]Ga-OncoFAP-DOTAGA over PREP and DPP8 ($SI > 1000$, respectively) was reported in a following study but no DPP4 selectivity although a high DPP4 selectivity seems likely [477]. First clinical application of [^{68}Ga]Ga-OncoFAP-DOTAGA in HT-1080.hFAP tumor-bearing mice and as well as subsequent first human PET studies in 12 patients with diverse cancer types indicated superiority to [^{68}Ga]Ga-FAPi-46 with substantially higher tumor uptake, e.g. up to 20 %ID/g [^{68}Ga]Ga-OncoFAP-DOTAGA in several patients and cancer types combined with higher TBR, e.g. tumor-to-blood-ratio [477].

[^{111}In]QCP02 with a DOTAGA chelator directly attached to non-fluorinated FAPi-02-like TV was reported to bind to six different FAP-expressing cancer cell lines *in vitro*. K_i value of 16.2 nM and high DPP4 selectivity was reported, but no PREP selectivity. Furthermore, xenograft studies of

mice with two types of cancer cell lines (FAP-positive U87 and FAP-negative PC3) showed significantly higher accumulation of [^{111}In]QCP02 in the FAP-positive U87 lesion compared to the FAP-negative PC3 lesion [478].

[^{68}Ga]Ga-MHLL1 with NODAGA chelator and a probably less selective Gly-cyanoPro TV was investigated in small animal studies for potential application in myocardial infarction imaging [479]. Although selective accumulation in FAP-positive cells was reported, no significant difference was found in the infarcted vs. non-infarcted myocardium.

Most FAP-targeting radiotracers have been focused on using trivalent radiometals which is plausible due to the potential theranostic application and the good availability of gallium-68 in many PET centers. On the other hand, diagnostic nuclear medicine will strongly benefit from suitable ^{18}F - or $^{99\text{m}}\text{Tc}$ -labeled FAPi radiotracers.

Fluorine-18 can be produced and radiolabeled at centralized production facilities in large quantities. The first ^{18}F -FAPi radiotracer [^{18}F]FGlc-FAPi (cf. Figure 17a) by Toms et al. [480] was based on a glucose with 1-azide and 6-tosylate substitution. The glucose precursor was first labeled with [^{18}F]F $^-$ and subsequently coupled to the TV with alkyne linker in a second step via Cu(II)-catalyzed click chemistry. Despite high tumor uptake, [^{18}F]FGlc-FAPi showed longer tumor retention but also longer blood pool circulation and hepatobiliary excretion.

Several new compounds were synthesized by substituting the DOTA chelator of FAPI-02/04 with a NOTA chelator which were radiolabeled with fluorine-18 via chelation of [^{18}F]AlF $^{2+}$ and also with gallium-68 in subsequent studies [481,482]. The polar chelators led to a renal instead of hepatobiliary excretion [481]. Low background led to good TBR at 1 h p.i. for precise detection of primary tumors as well as lymph nodes and distant metastases for [^{68}Ga]Ga- and [^{18}F]AlF-FAPI-74 [482]. Surprisingly, the non-fluorinated FAPI-74 proved to be slightly superior to the difluorinated FAPI-42 in the original preclinical studies (cf. Figure 17b) [481]. Nevertheless, [^{18}F]AlF-FAPI-42 was investigated in several studies already and the radiotracer seems to be on par with [^{68}Ga]Ga-FAPI-04 and [^{18}F]F-FDG [483–485]. An additional polar spacer unit, a combination of PEG $_2$ with a short glycosylated peptide sequence, was introduced in the compound NOTA-FAPT (cf. Figure 17c) between NOTA chelator and TV + piperazine linker moiety (analogous to FAPI-04) and further minimized hepatobiliary excretion [486].

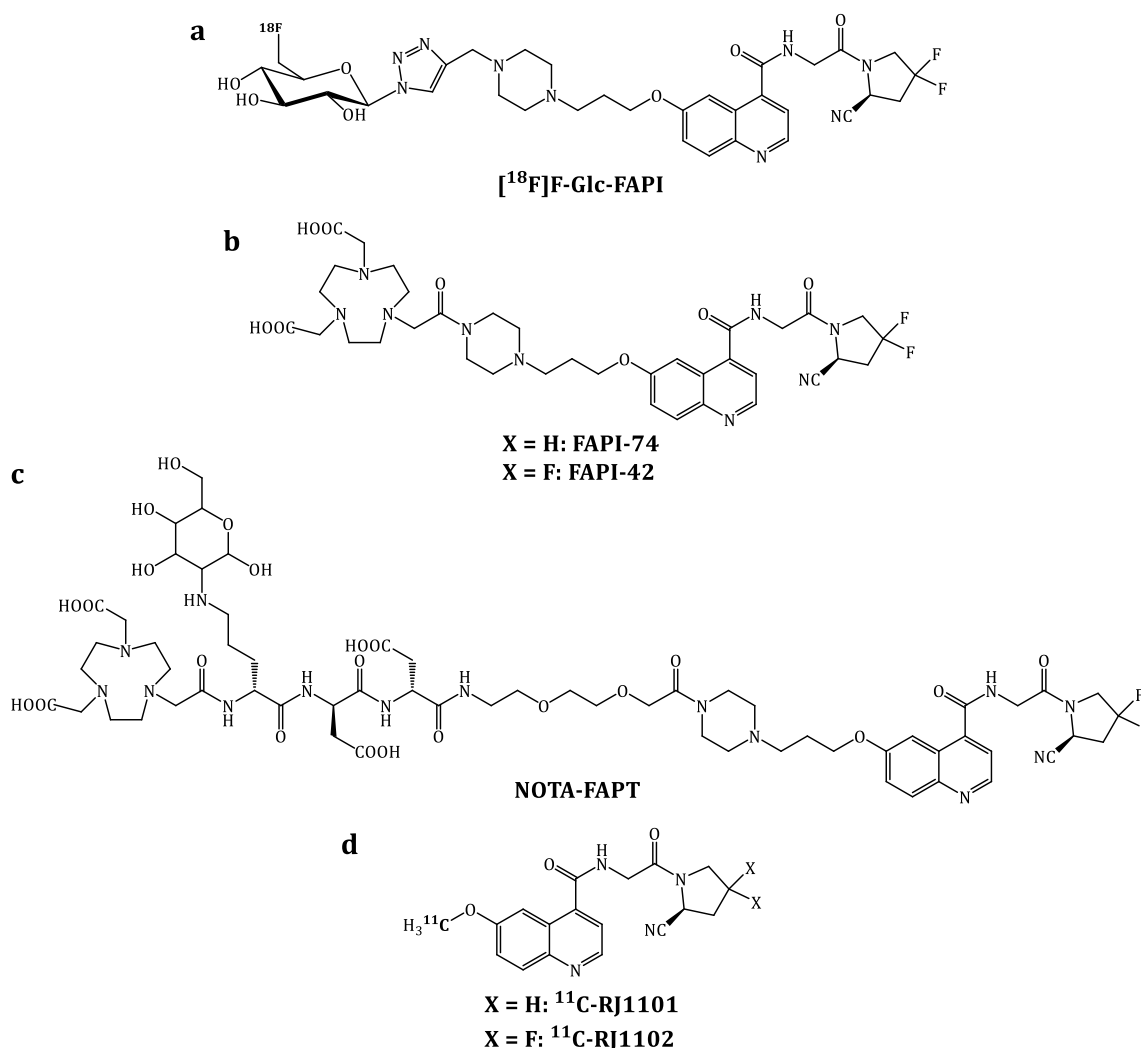


Figure 17: Further FAPi-PET radiotracers: a) [¹⁸F]F-Glc-FAPI; b) FAPI-42 (X = H) and FAPI-74 (X = F) as well as c) NOTA-FAPT for ¹⁸F- or ⁶⁸Ga-radiolabeling; d) ¹¹C-RJ1101 (X = H) and ¹¹C-RJ1102 (X = F).

UAMC-1110 has also been modified to enable radiolabeling with carbon-11 by methylation with [¹¹C]CH₃I of 6-hydroxy-UAMC-1110 and its non-fluorinated counterpart to yield ¹¹C-JR1101 (X = H) ¹¹C-JR1102 and (X = F, 6-[¹¹C]methoxy-UAMC-1110) as depicted in Figure 17d, respectively [487]. Both ¹¹C-labeled radiotracers were studied in xenograft models with multiple glioma cells (U87MG) but the lipophilic compounds were excreted hepatobiliary and resulted in high liver and intestine uptake.

Despite the dominating role of ^{99m}Tc-SPECT imaging there is no universal oncological radiotracer like [¹⁸F]F-FDG for PET. ^{99m}Tc-FAPi SPECT might fill that gap and although the clinical demand is very high, the research on ^{99m}Tc-FAPi radiotracers is limited. The HABERKORN group developed the first series of FAPi ligands based on the FAPI-04 structure but substituted the chelator for labeling with the tricarbonyl intermediate *fac*-[^{99m}Tc][Tc(CO)₃]⁺ [488]. Since the first compound FAPI-19 showed no tumor uptake but high liver uptake and biliary excretion, hydrophilic units were added to improve pharmacological properties which led to the lead candidate [^{99m}Tc]Tc-FAPI-34 (cf.

Figure 18a). The radiotracer had high FAP affinity, internalized into the cell almost completely and accumulated significantly in the tumor lesions in murine and human SPECT studies.

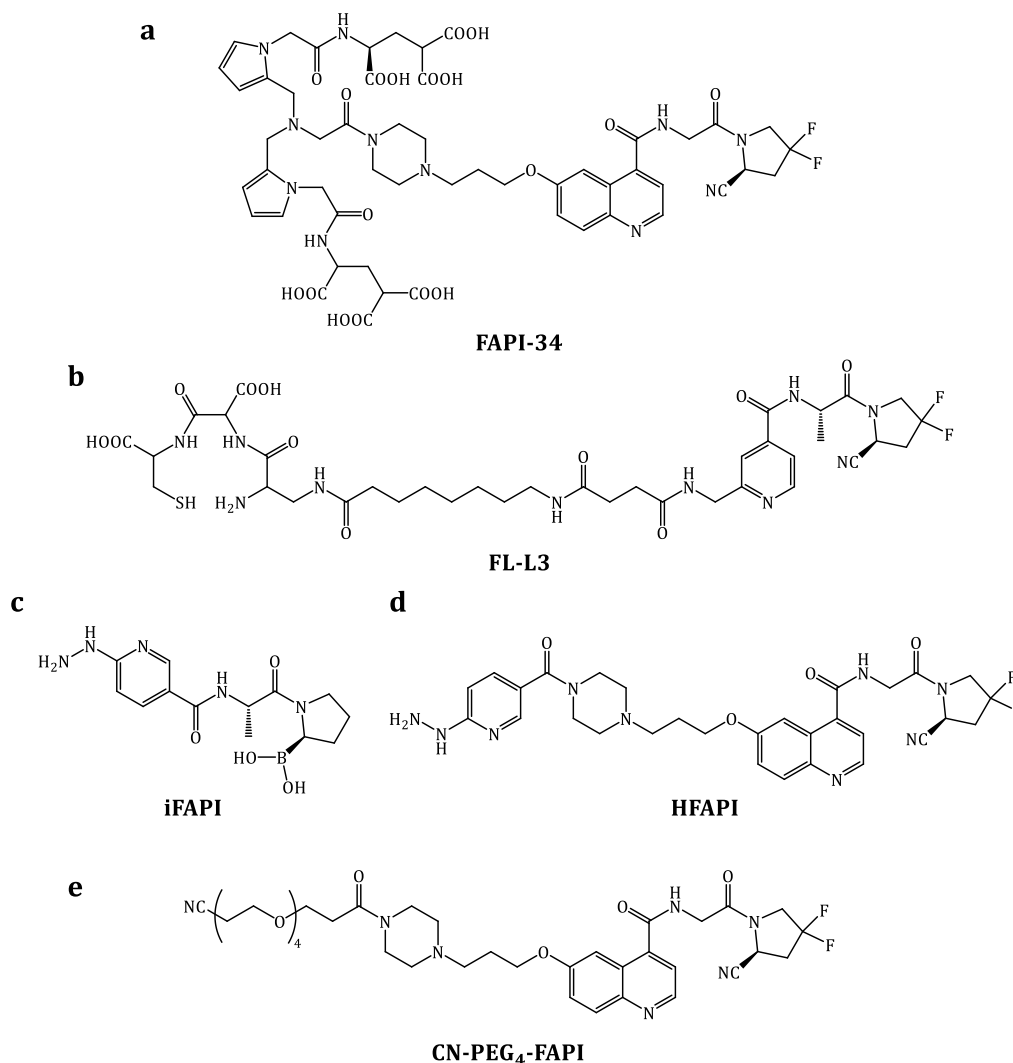


Figure 18: FAPi ligands for ^{99m}Tc -SPECT imaging: a) FAPI-34, b) FL-L3, c) iFAPi and d) HFAPi.

A ^{99m}Tc radiotracer based on the FAPi ligand FL-L3 (cf. Figure 18b) with N_3S chelator utilized a different TV with 4-pyridinoyl instead 4-quinolinoyl aromatic moiety [489]. The HYNIC strategy was pursued by two groups. TRUJILLO-BENITEZ et al. coupled the 2-hydrazino-nicotinic acid to a D-Ala-boroPro TV (cf. Figure 18c) which is probably not optimal regarding protease selectivity (cf. chapter 1.3.4.) [490,491]. Recently, JIANG et al. developed a HYNIC derivative of FAPI-04/FAPI-34 (cf. Figure 18d) [492]. It has been studied (pre)clinically and showed some physiological distribution at early time points in the liver, pancreas etc. but indicated superior metastasis finding compared to contrast-enhanced CT [492–494]. Furthermore, a radiotracer with hexadentate coordination of $\text{Tc}(\text{I})$ with six cyano ligands was proposed [495]. Each cyano ligand CN-PEG₄-FAPi (cf. Figure 18e) was coupled to FAPI-04-analogous TV and piperazine linker moiety via a PEG₄ spacer. It is questionable if these cyano ligands act as donor ligands since the FAPI-04-TV also has a cyano group [495].

1.5.2. Emerging FAPi-based Radiotheranostics

FAPi-based radiotracers have already proven to be very suitable for diagnostic PET and the number of applications is increasing every year since the precursors become better available and more clinics start using these radiotracers for oncological molecular imaging.

From the beginning with the emergence of the first suitable FAPi-based radiotracers by the Heidelberg group (FAPi-04 etc.), the strategy of using DOTA-based ligands had a theranostic translation in mind. But these compounds like FAPi-04 suffered from short biological half-life only a few hours. This does not allow for an efficient RLT with trivalent radiometals like lutetium-177 ($t_{1/2} = 6.65$ d), actinium-225 ($t_{1/2} = 10.0$ d) and even not for yttrium-90 ($t_{1/2} = 64.0$ h) although some attempts were made. Therapeutic variants like [^{90}Y]Y- or [^{225}Ac]Ac-FAPi-04 showed short tumor residence time with rapid washout from tumor lesions that can already be seen via ^{68}Ga -PET scans (25% average washout from 1 h p.i. to 3 h p.i.) [449,450,496]. Radiation dose is not deposited efficiently in the tumor for optimal anti-tumor effect.

Several approaches can be pursued to optimize FAP-targeted RLT or TAT. Two important strategies shall be covered in the following chapter. Other recently emerging strategies and developments will be discussed in detail in the chapter 4 “Discussion and Summary”.

Firstly, pharmacological properties can be modified and optimized by variations of the linker and spacer units of the radiotracers. This was tried with the second-generation compounds from the Heidelberg group. But even the leading candidate FAPi-46 did not possess significantly prolonged the tumor retention to fully match the physical half-lives of lutetium-177 or actinium-225. Nevertheless, several therapeutic *in vivo* studies (murine and human) of [^{90}Y]Y- and [^{177}Lu]Lu-FAPi-46 are published and indicate some therapeutic efficacy although the PK profile is not optimal for RLT [497–502].

Secondly, the combination of relatively short-lived therapeutic radionuclides (^{188}Re , ^{211}At) with the abovementioned FAPi ligand FAPi-34 or derivatized versions of FAPi-04 or FAPi-46 was investigated. This strategy led to radiopharmaceuticals with better matching physical half-life of the radionuclide and biological half-life of the radiotracer *in vivo*.

[^{211}At]At-FAPi-04 (cf. Figure 19a) showed better anti-tumor efficacy than previous [^{131}I]I-FAPi-04 studies that could be further improved significantly by intratumoral injection but hepatobiliary excretion issues were observed due to high lipophilicity [503,504]. Although the supply of astatine-211 ($t_{1/2} = 7.0$ h) is currently very limited, it could be an interesting alternative for RLT with modified, more polar versions of FAPi-04 or FAPi-46 since the shorter physical half-life of astatine-211 fits the PK properties of these FAPi ligands.

Due to the similar chemistry of technetium and rhenium, a $^{99m}\text{Tc}/^{188}\text{Re}$ -theranostic approach with FAPI-34 and the relatively short-lived rhenium-188 ($t_{1/2} = 17.0$ h) is interesting [488]. But the challenging production of the parent radionuclide tungsten-188 ($t_{1/2} = 69.8$ d) in a double neutron capture $^{186}\text{W}(2n,\gamma)^{188}\text{W}$ reaction is only possible at large high-flux reactors which limits the $^{188}\text{W}/^{188}\text{Re}$ generator availability [505]. Despite the proposal in the original paper from LINDNER et al. [488] no (pre)clinical data regarding [^{188}Re]Re-FAPI-34 have been published.

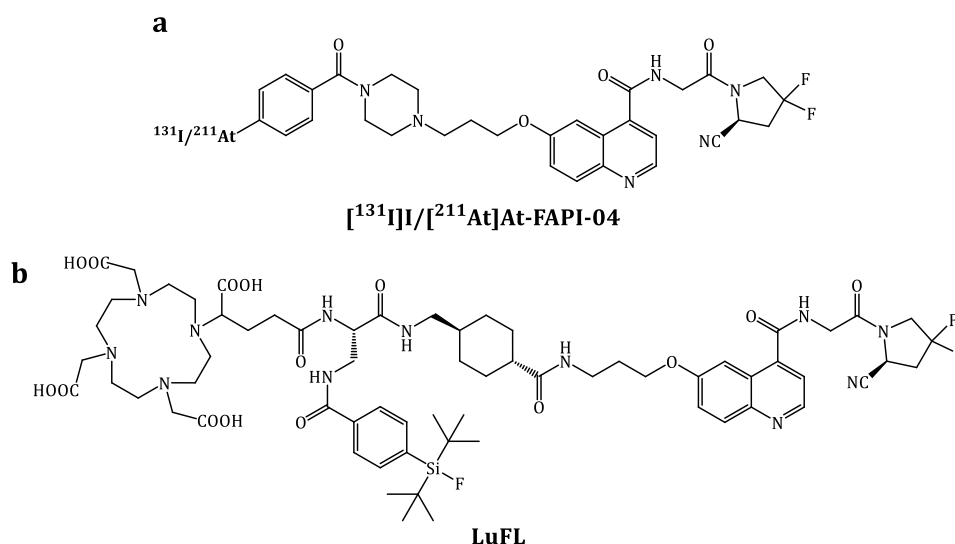


Figure 19: Structures of a) [^{131}I]- and [^{211}At]At-FAPI-04; b) the radiohybrid LuFL.

Recently, the first published radiohybrid FAPI radiotracer was published by YANG et al. [506]. Radiohybrid means that there are two labeling moieties in one molecule. In this case, a DOTAGA chelator for radiolabeling with trivalent radiometals like lutetium-177 and a silicon-fluoride-acceptor (SiFA) moiety for radiolabeling with fluorine-18 in an isotopic exchange reaction was used. The compound LuFL (cf. Figure 19b) is further characterized by an UAMC-1110 like TV and cyclohexyl-containing spacer unit and the two labeling moieties are coupled via 2,3-diaminopropionic acid (Dpr). The ^{nat}Lu -complex was used for ^{18}F -PET imaging because in this case the molecular structure will be exactly like the ^{177}Lu -labeled therapeutic counterpart. This molecular design is the first to combine fluorine-18 and lutetium-177 as a matched theranostic pair [506].

The third strategy is to synthesize homodimers with two identical targeting vectors (TVs). Previous studies on PSMA inhibitors indicated that this could lead to prolonged tumor retention for FAPI-based radiotracers as well [507]. In 2021, MOON et al. [305] from our group was the first to publish (homo)dimeric FAPI-based radiotracers. DOTA.(SA.FAPI) $_2$ and DOTAGA.(SA.FAPI) $_2$ (cf. Figure 20) contain two TVs where FAPI-NH $_2$ is coupled to the central DOTAGA chelator via squaramide (SA) linkers [305].

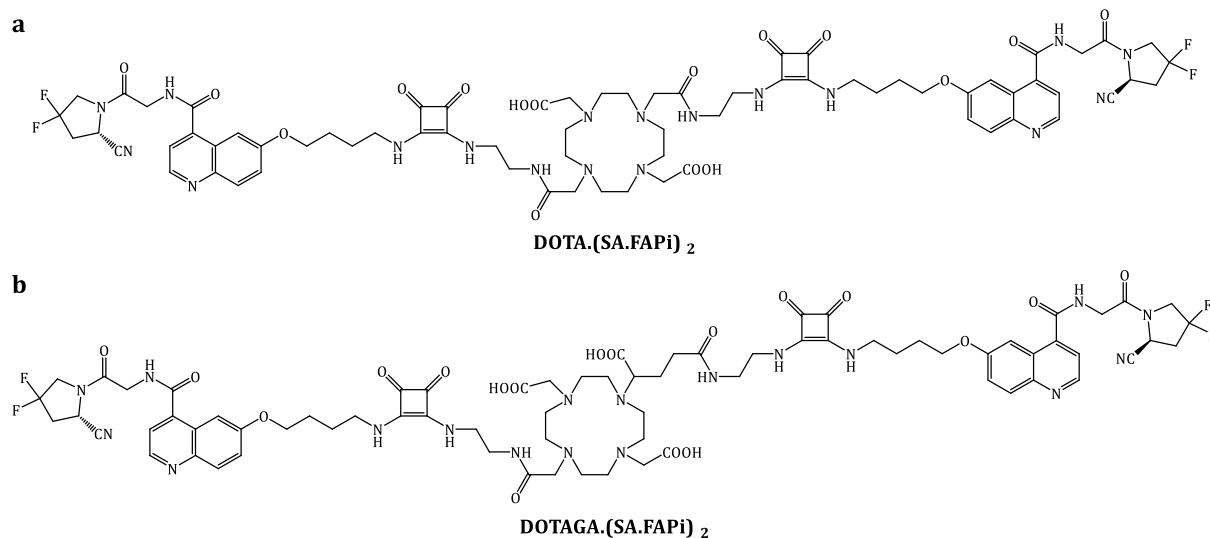


Figure 20: Structures of the first FAPi homodimers a) DOTA.SA.FAPi₂ and b) DOTAGA.(SA.FAPi)₂.

[⁶⁸Ga]Ga-DOTAGA.(SA.FAPi)₂ showed better radiolabeling kinetics than [⁶⁸Ga]Ga-DOTA.(SA.FAPi)₂. Furthermore, the additional carboxylic acid of DOTAGA is beneficial for radiolabeling of trivalent radiometals with larger ion radii like lutetium-177 [305].

[⁶⁸Ga]Ga- and [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂ was subsequently investigated *in vivo*. The tumor retention time of [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂ was improved drastically and lasted at least one week. This was demonstrated in a comparative study with [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂ and the SA-based monomer [¹⁷⁷Lu]Lu-DOTA.SA.FAPi [472].

The safety and therapeutic efficacy [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂ was investigated in several cancer types and mainly in radioiodine-refractory differentiated thyroid cancer (RR-DTC) and found to be very promising with an average effective half-life of 88 hours (interquartile range: 86.6 – 99 h) [472,508]. DOTAGA.(SA.FAPi)₂ showed slower pharmacokinetics compared to DOTA.SA.FAPi which was not optimal for ⁶⁸Ga-PET imaging at early time points due to higher blood pool activity [472,509]. Therefore, [⁶⁸Ga]Ga-DOTA.SA.FAPi is routinely used as the diagnostic PET counterpart [508]. The rather slow predominantly hepatobiliary excretion of [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂ resulted in relatively high radiation dose to the liver and the colon, in particular, which was found to be the critical organ. In general, the colon tolerates radiation doses well. The limit in external beam radiotherapy is high (38 Gy) and BALLAL et al. [508] only observed grade I diarrhea which lasted for 24 hours in 1/15 patients in the study on RR-DTC. On the other hand, the absorbed dose to the tumor lesions was significantly higher and the radiation dose to most other organs (salivary glands, bone marrow, kidneys) was lower than for the FDA- and EMA-approved [¹⁷⁷Lu]Lu-PSMA-617. No serious grade III/IV adverse events occurred. Figure 21 shows exemplary [⁶⁸Ga]Ga-DOTA.SA.FAPi PET/CT scans of a patient before and after treatment (A and D) with two cycles of [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂. Figure 21B and C show planar whole-body scan 24 h and 168 h p.i. of [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂, respectively [508]. The tumor

uptake at several lesions including the primary neck lesion is still clearly visible after one week. The patient partially responded to the treatment on a biochemical level as the thyroglobulin level, a clinical established tumor marker for thyroid cancer, decreased from > 300 000 to 6586 ng/mL as well as a clear molecular imaging response with smaller tumor lesions.

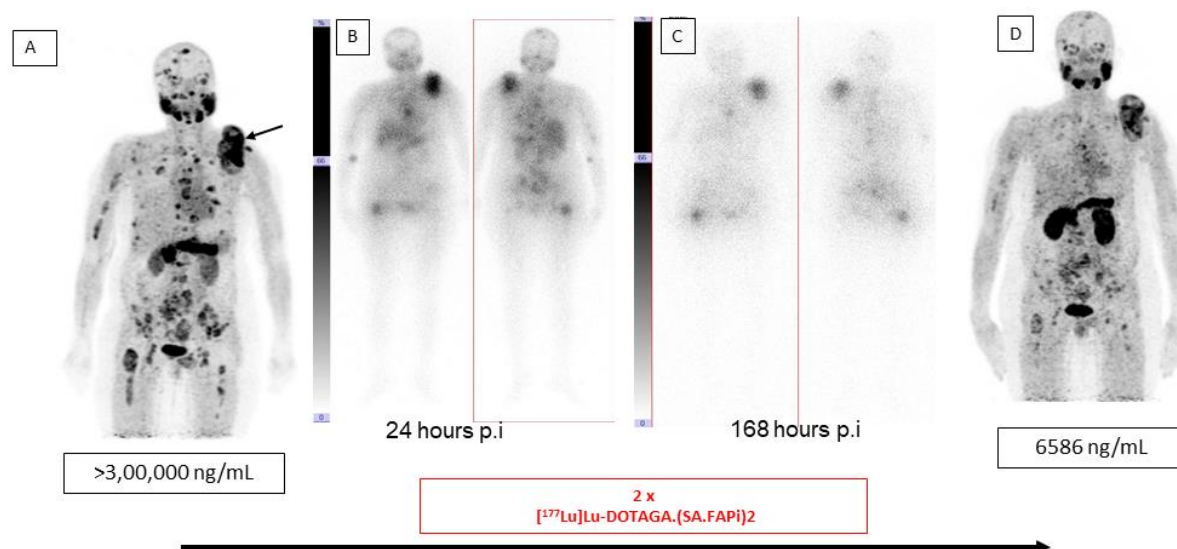


Figure 21: A 50-year old women a follicular variant of papillary carcinoma failed radioiodine therapy, Soranib and Lenvatinib. Baseline (A) and post-treatment (B) $[^{68}\text{Ga}]\text{Ga-DOTA.SA.FAPi}$ PET/CT scan with thyroglobulin level decreasing from > 300 000 ng/mL to 6586 ng/mL. B and C show planar whole-body scans of $[^{177}\text{Lu}]\text{Lu-DOTAGA}(\text{SA.FAPi})_2$ 24 h and 168 p.i., respectively. This figure was originally published in *Thyroid* by BALLAL et al. Novel Fibroblast Activation Protein Inhibitor-Based Targeted Theranostics for Radioiodine-refractory Differentiated Thyroid Cancer Patients: A Pilot Study. *Thyroid*. **2022**, 32(1), 65–77 [508]. Included with written permission from © 2022 Wolters Kluwer Health, Inc. received on May 14 2023).

Partial response (4/15) and stable disease (3/15) was observed for the RR-DTC patients [508]. Prolonged overall survival (data under review) upon treatment with $[^{177}\text{Lu}]\text{Lu-DOTAGA}(\text{SA.FAPi})_2$ indicate the therapeutic potential of RLT with FAPi homodimers.

Several groups have adopted this approach or have come up with other strategies like adding albumin-binding units or using cyclic peptides to improve tumor retention and published first results recently. This will be presented extensively in chapter 4 “Discussion and Summary” and compared to the results of this work.

2. Problem and Objective

During the last few years, FAP-targeting radiotracers have emerged in the field of nuclear medicine thanks to the development of the very selective high-affinity FAP inhibitor UAMC-1110. Diagnostic FAPi- and chelator-based radiotracers for ^{68}Ga -PET imaging like ^{68}Ga -FAPi-04 and ^{68}Ga -FAPi-46 have already proven to be highly beneficial in the diagnosis of various different cancer types and superior or at least on par with ^{18}F -FDG in most of them [451,452,454,456–458,460,461,474,510,511].

The idea and advantage of using precursors with DOTA or similar chelators is that these precursors can be radiolabeled with different trivalent radiometals which theoretically permits a rather straightforward theranostic translation. Therefore, the same precursor can be used for different applications. For example, PET with gallium-68 or scandium-44, SPECT with indium-111, FAP-targeted RLT with lutetium-177 or yttrium-90 and even FAP-targeted alpha particle therapy (FAP-TAT) with actinium-225.

There are great expectations that FAP might become “The Next Billion Dollar Nuclear Theranostics Target” [512] after the clinical translation and EMA- and FDA-approval of similar theranostic pairs like ^{68}Ga - and ^{177}Lu -DOTA-TATE for neuroendocrine tumors as well as ^{68}Ga -PSMA-11 and ^{177}Lu -PSMA-617 for prostate cancer. The original idea was that the diagnostic DOTA-based FAPi radiotracers can be translated straight into therapeutic counterparts by simply exchanging the radionuclide. Surprisingly, and very different to analogous approaches with DOTA-conjugated PSMA inhibitors like PSMA-617 for use in prostate cancer, all the currently established FAPi ligands (FAPi-04, FAPi-46 etc.) fail in this respect. Obviously, all these monomers are not optimal for RLT because the biological half-life does not match the physical half-life of the established therapeutic radionuclides mentioned above.

In contrast, the first homodimers with two TVs by MOON et al. [305] achieved significantly prolonged tumor retention and promising results in first clinical applications. Nevertheless, these compounds may still need further optimization, for example regarding pharmacological and radiolabeling properties. Next-generation homodimeric FAPi radiotheranostics definitely have the potential to become pan-tumor drugs.

The aim of this work was to synthesize novel FAPi-based compounds for improved theranostics in many endothelial cancer types that contain an extensive TME. This work is divided into five projects and an overview of the different projects is depicted in Figure 22.

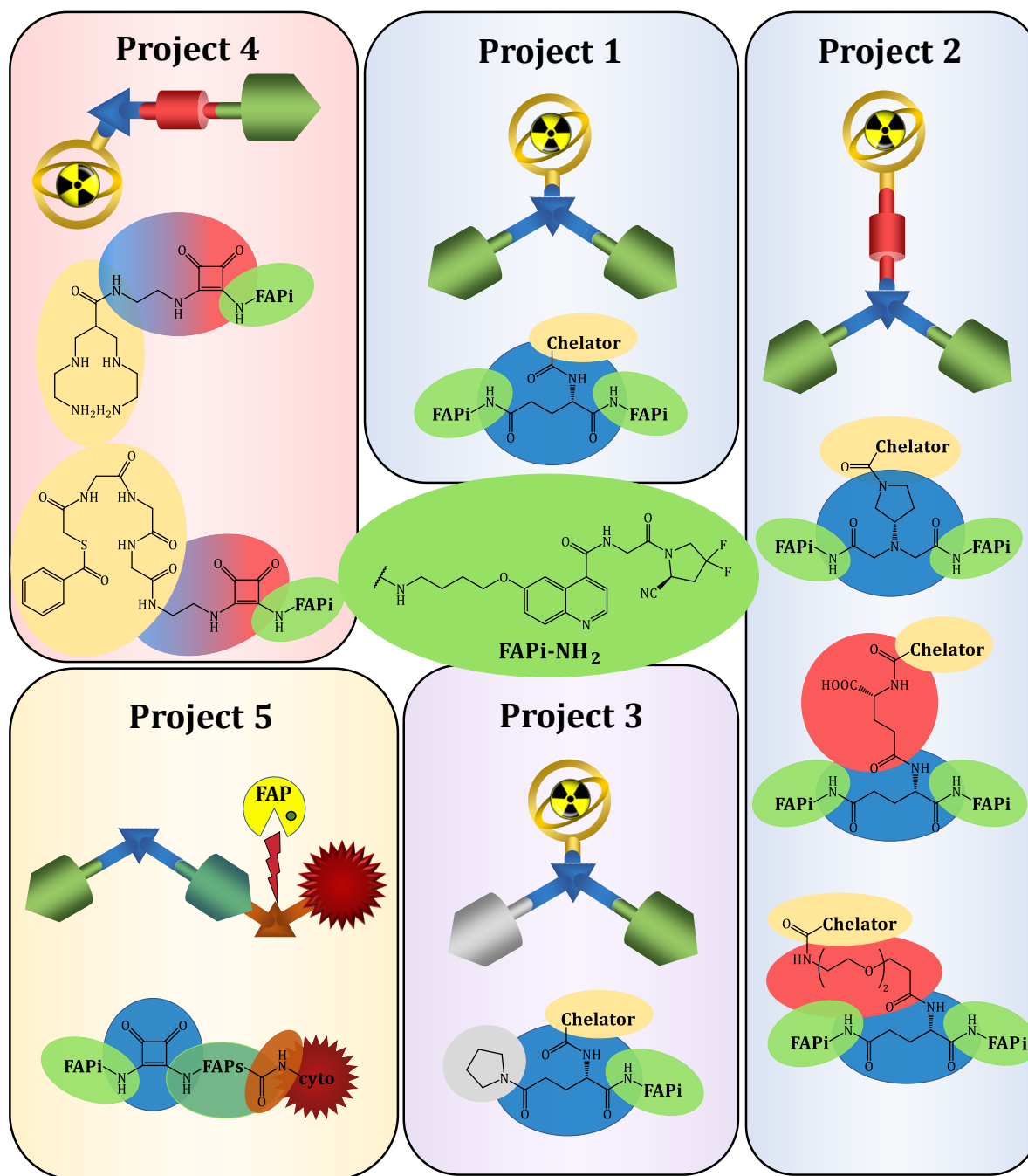


Figure 22: Overview of the five projects including their respective general schematic and chemical structures. The different subunits of the radiotracers are: radiometal-chelator complex (yellow), linker or central linker unit (blue), spacer (red), FAPi targeting vector (green) and inert group (grey). For the on homodimeric FAPi radiotracers of project 1 and 2, the chelators DO3A and DOTAGA were used. The radiotracers are mainly designed for improved FAP-targeted radioligand therapy with trivalent radiometals (^{68}Ga , ^{177}Lu , ^{225}Ac , ^{90}Y).

An additional spacer unit or a different central linker unit was used for project 2. DO3A, DOTAGA and DATA^{5m} were used for the FAPi monomers in project 3. The monomers are structurally derived from the homodimers in project 1 and 2 where one FAPi TV is substituted by an inert group (grey). The radiotracers in project 4 are designed for the use in ^{99m}Tc -SPECT and the chelators N4 and MAG3 are used. Project 5 focuses on FAP-targeted drug delivery with compounds that have a FAPi TV subunit and a FAP substrate moiety (FAPs, turquoise) that is coupled to cytostatic (dark red) via a FAP-cleavable amide bond (brown). After FAP-cleavage of the prodrug the cytostatic shall be released in its active form into the TME.

Projects 1 – 3 deal with D03A- and DOTAGA-based radiotracers for complexation of trivalent radiometals. Project 1 and 2 focuses on FAPi homodimers for efficacious and effective FAP-targeted RLT with lutetium-177/yttrium-90 and also FAP-TAT with actinium-225. In contrast to project 1, an additional spacer unit between chelator and central linker unit or a different central linker unit was used. Of course, suitable diagnostic counterparts are needed so that molecular imaging via ^{68}Ga -PET can go hand in hand with ^{177}Lu -, ^{90}Y -RLT or ^{225}Ac -FAP-TAT. Therefore, the aim of project 3 is to develop matching diagnostic counterparts to the FAPi homodimers from project 1. First and foremost, the FAPi homodimers are optimized with regard to maximizing the radiation dose that is deposited in the tumor lesion by prolonged tumor retention with the therapeutic ^{177}Lu -, ^{90}Y - or ^{225}Ac -labeled radiopharmaceutical. In contrast, ^{68}Ga -PET radiotracers are supposed to show fast (renal) clearance to achieve high-contrast PET images at early time points. The optimized FAPi monomers of project 3 shall enable a more accurate treatment assessment via ^{68}Ga -PET pre- and post-treatment with FAPi homodimers. This is supposed to improve theranostic interplay between ^{68}Ga -FAPi-PET and FAP-targeted ^{177}Lu - or ^{90}Y -RLT or ^{225}Ac -FAP-TAT.

Surprisingly little research has been put into the development of $^{99\text{m}}\text{Tc}$ -radiotracers for SPECT although it is the workhorse in nuclear medicine with a big diagnostic potential that comes along with it. Therefore, project 4 focuses on synthesizing new $^{99\text{m}}\text{Tc}$ -FAPi radiotracers for application in SPECT imaging in many different cancer types. The $^{99\text{m}}\text{Tc}$ -specific chelators N4 and MAG3 are coupled to the FAPi-TV via squaric acid to yield a squaramide linker/spacer (SA). The SA-strategy has already proven to be successful with the first-generation monomeric FAPi radiotracers [^{68}Ga]Ga-DOTA- and [^{68}Ga]Ga-DATA^{5m}.SA.FAPi from MOON et al. [466,474–476,509].

In addition, there are various expectations regarding drug delivery to the TME by targeting FAP with FAP inhibitors and/or FAP substrates as small molecule vectors. A new approach was pursued with project 5 by combining a FAP inhibitor (FAPi) and a FAP substrate (FAPs) moiety along with a cytostatic drug in one molecule. In this approach, FAP plays two essential roles at the same time. Firstly, it acts as the oncological target for the FAPi-TV. Secondly, it is supposed to cleave the prodrug to release the cytostatic in its active form. In this case, the post-proline amide bond that couples FAPs and the cytostatic is supposed to be cleaved by FAP.

Each project covers the organic synthesis of the respective chelator-based precursor molecules. Many of the different moieties in this work have amino and carboxyl groups for amide coupling in which established protecting groups from peptides synthesis come into play. It was also chosen to enable flexible and straightforward exchange of different units/moieties. Subsequently, radiolabeling properties are investigated and reaction conditions are optimized. The complex

stability in different media and lipophilicity of each radiolabeled complex are explored. Furthermore, the affinity for FAP and the selectivity over the related proteases PREP, DPP4, DPP8 and DPP9 was determined for every compound by measuring the IC_{50} values for each protease. These *in vitro* data are obtained in collaboration with the groups of PIETER VAN DER VEKEN and INGRID DE MEESTER from the University of Antwerp (Belgium). Projects 1 and 3 further include first *in vivo* studies that were realized in collaboration with the nuclear medicine group of CHANDRASEKHAR BAL from the All India Institute of Medical Sciences (AIIMS, New Delhi, India) together with his experienced physicians SANJANA BALLAL and MADHAV PRASAD YADAV.

Novel Strategy for Homodimeric FAPi-based Radiotracers to Achieve Improved Application in Radiotheranostics (Project 1 and 2)

The linear homodimer DOTAGA.(SA.FAPi)₂ showed favorable properties like increased uptake and prolonged retention in the tumor lesions compared to the monomer DOTA.SA.FAPi [472]. Yet predominantly hepatobiliary excretion of [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂ was observed in planar whole-body imaging and resulted in liver and colon uptake and radiation dose to these organs. Although no serious adverse events occurred because the colon usually tolerates even higher radiation doses quite well, the limit of 38 Gy will be reached with a cumulative dose of around 30 GBq [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂ [508]. Besides, DOTAGA.(SA.FAPi)₂ did not show satisfactory radiolabeling properties with actinium-225 most probably because of steric hindrance of the chelator.

The aim of project 1 and 2 is to synthesize radiopharmaceuticals with improved pharmacological properties, faster (and preferably) renal excretion pattern, while maintaining the high and prolonged tumor uptake and secondly improve radiolabeling properties to enable efficient ²²⁵Ac-radiolabeling for potential FAP-TAT application.

A novel strategy for homodimeric FAPi-based radiotracers was developed and based on a new molecular design. The most significant difference compared to linear homodimer design of DOTAGA.(SA.FAPi)₂ is that the chelator not in the center of the molecule but at terminal *exo*-position. Furthermore, the design is no longer restricted to (DOTA-based) chelators but can be expanded to any bifunctional chelator or a suitable labeling unit (LU) for radiolabeling of radiohalogens (¹⁸F etc.) and other covalently bound radionuclides. The molecular design of these central linker-based homodimers can be schematically illustrated by the general structure in Figure 23, shown in two variants with a general labeling unit (LU) in Figure 23a and a chelator with radiometal in Figure 23b.

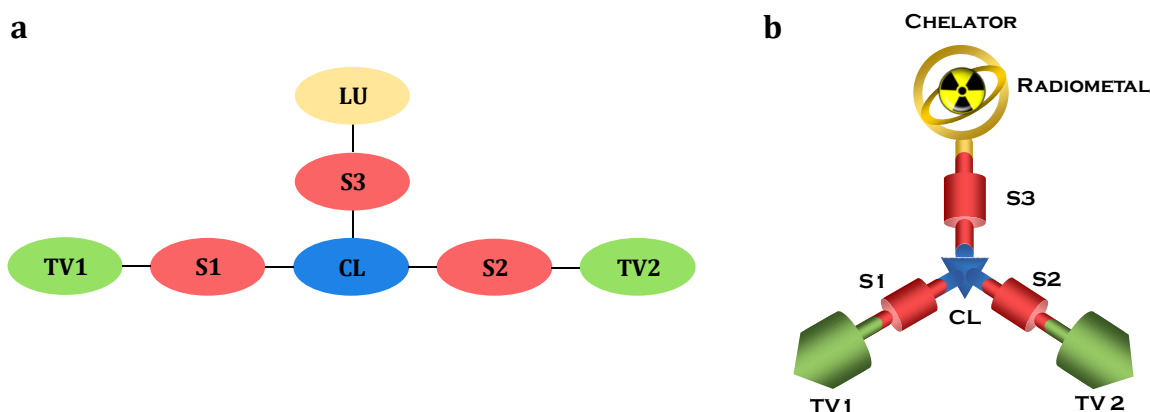


Figure 23: General structure of novel homodimeric FAPi-based radiotracers: TV = targeting vector (e.g. FAPi); S = spacer; CL = central linker unit and a) with general LU = labeling unit that is b) radiometal complexed by a chelator within the context of this work.

TV1 and TV2 are targeting vectors, S1 – S3 are spacer units, CL is a central linker unit and LU a labeling unit for complexation of a radionuclide/radiometal with a bifunctional chelator or to covalently bind an organic radionuclide. Every unit can be chosen individually. For example, TV1 and TV2 can be identical (homodimer, e.g. two FAPi TVs) or different (heterodimer, e.g. one FAP and one PSMA inhibitor). Spacer units can be all different, all the same, partially or totally absent etc. All these structure variations are extensively defined in the patent WO/2022/258637 (“Trislinker-conjugated dimeric labelling precursors and radiotracers derived therefrom”) created by the inventors FRANK ROESCH, EUY SUNG MOON, TILMANN GRUS, MARCEL MARTIN and leadingly drafted by MARCEL MARTIN [513].

This new molecular design allows to choose the labeling unit (e.g. bifunctional chelator) completely freely because it is at terminal *exo*-position similar to a monomeric radiotracer design. Therefore, the optimal labeling unit can be optimally chosen for the respective problem independently from the rest of the molecule. Additionally, the spacer units can be freely chosen to optimize binding affinity/selectivity or finetune pharmacological properties (PK/PD profile of the radiotracer).

TV1 and TV2 can be chosen independently from targeting vector structures including examples of cyclic peptides targeting SSTR in neuroendocrine tumors like Octreotid (D-Phe-cyclo[Cys-Phe-D-Trp-Lys-Thr-Cys]Thr(ol), TOC (D-Phe-cyclo[Cys-Tyr-D-Trp-Lys-Thr-Cys]Thr(ol), NOC (D-Phe-cyclo[Cys-1-Nal-D-Trp-Lys-Thr-Cys]Thr(ol) or TATE (D-Phe-cyclo[Cys-Tyr-D-Trp-Lys-Thr-Cys]Thr. Other examples are PSMA inhibitors for prostate cancer or bisphosphonates for bone metastasis and FAP inhibitors as the key targeting vectors of this work as shown in Figure 24 [513].

Chemical structures of four different peptides:

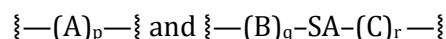
- (a) A linear peptide with a wavy line at the N-terminus, representing a peptide with a free N-terminus.
- (b) A cyclic peptide with a wavy line at the C-terminus, representing a peptide with a free C-terminus.
- (c) A linear peptide with a wavy line at the N-terminus, representing a peptide with a free N-terminus.
- (d) A linear peptide with a wavy line at the N-terminus, representing a peptide with a free N-terminus.

$n = 1, 2, 3, 4, 5, 6, 7, 8, 9, 10$
 $Z = \text{H, OH, NH}_2, \text{Cl}$

[illegible]

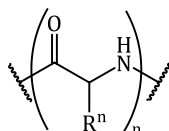
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S1, S2 and S3 can be chosen independently from the following substructures with SA = squaric acid/squaramide:



The linker groups A, B and C can be chosen independently from the group including amide, alkyl $\{-CH_2-\}$, ethylene glycol $\{-CH_2CH_2O-\}$, triazole, (thio)urea, maleimide, amino acid $\{-CH_2-CH(COOH)-NH-\}$ and amines $\{-(CH_2)_sNH-\}$ with $s = 1, 2, 3, 4, 5, 6, 7, 8, 9$ or 10 ; where $p, q, r = \{0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20\}$.

Furthermore, S1 – S3 can be further selected from (poly)peptide structures:



Where the side chain R^n ($n = 1, 2, 3$) can be chosen independently from all the proteinogenic α -amino acids also including all the corresponding D-amino acids [513].

The central linker unit (CL) is preferably chosen from one of the examples in Figure 25 including some amino acids with three functional groups.

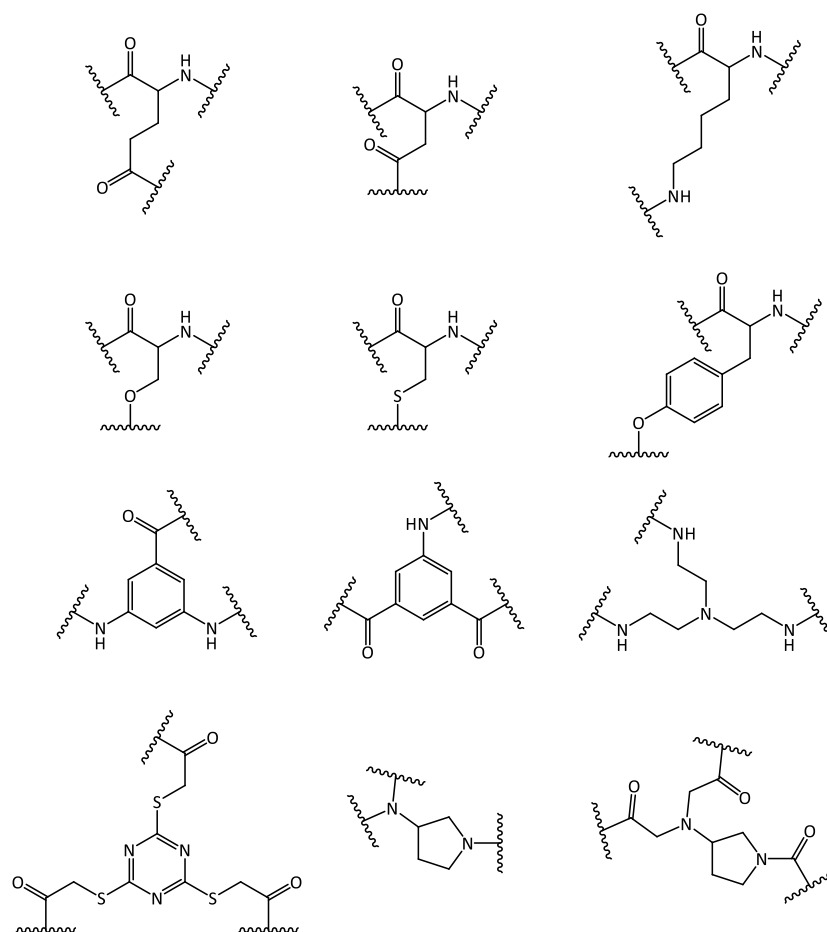


Figure 25: Selected examples of possible central linker units (CL) including some amino acids with additional functional group at the side chain: top row (from left to right): glutamic acid (Glu), aspartic acid (Asp), lysine (Lys); second row (from left to right): serine (Ser), cysteine (Cys), tyrosine (Tyr).

The labeling unit (LU) includes not only bifunctional chelators but can further be a covalently bound precursor molecule with a leaving group X for example, that can then be substituted in the radiolabeling with organic radionuclides like fluorine-18, different isotopes of iodine (iodine-123/124/125/131) or astatine-211. X can be chosen from halogens (^{19}F , Cl, Br, I), tosylate, mesylate, brosylate, nosylate, triflate, 2-(*N*-morpholino)ethanesulfonic acid nonaflate or other similar leaving groups. Alternatively, ^{18}F -synthons like 2- ^{18}F Fluoroethyl tosylate and azide can be synthesized in a first step and coupled via nucleophilic substitution with a reactive nucleophilic group (e.g. $-\text{OH}$, $-\text{NHR}$) and in a click reaction with an alkyne, respectively [513].

Bifunctional chelators are the main focus of this work. Therefore, several examples are shown in Figure 26. It has to be noted that many more different variations, especially in the linking group exist commercially for established chelators as DOTA, e.g. *p*-NCS-Bn, DBCO, BCN (bicyclo[6.1.0]nonyne) or terminal azide or alkyne groups for click reactions and are part and is all covered/included in the patent as well [513].

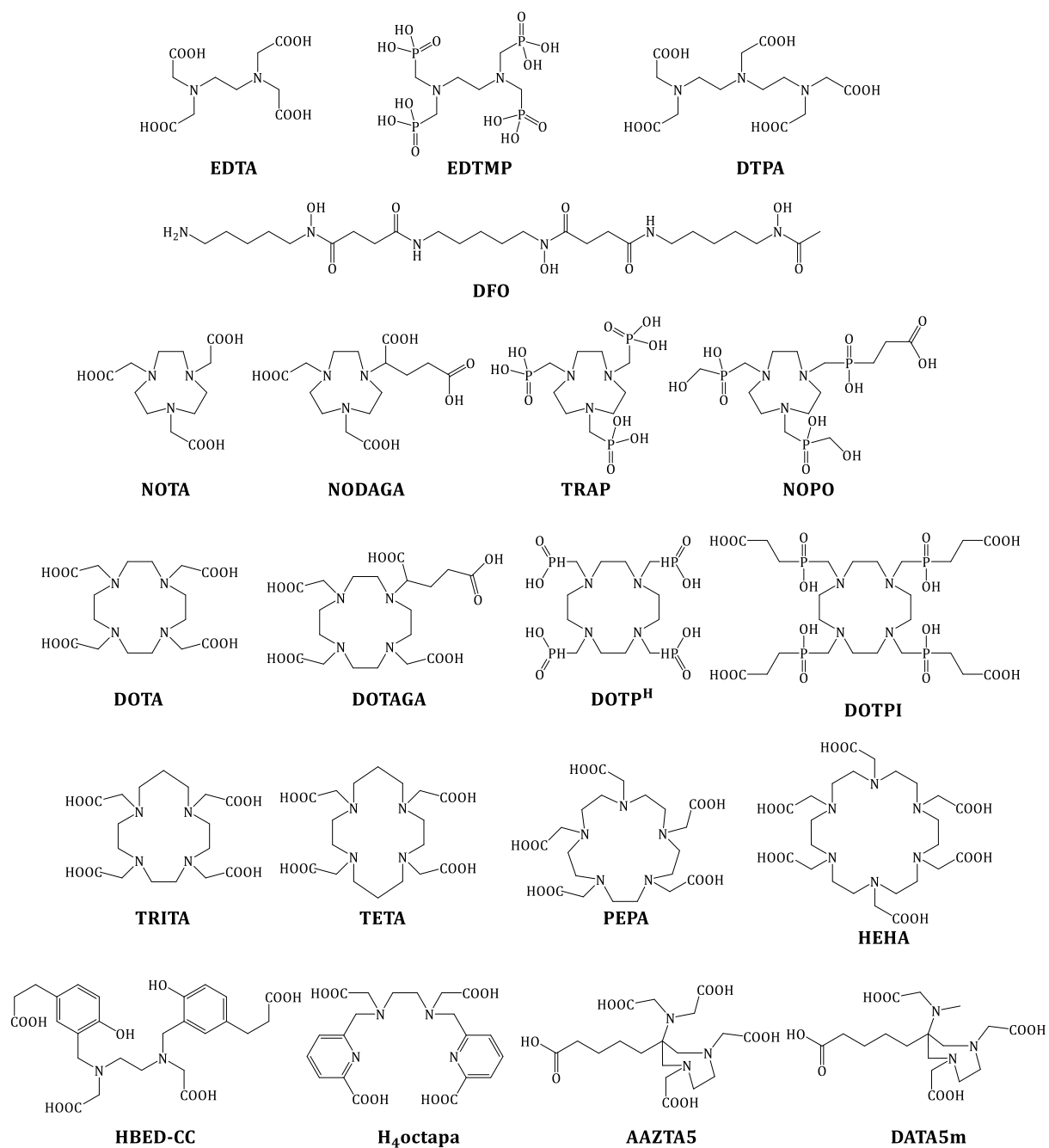


Figure 26: Selected bifunctional chelators as potential labeling unit (LU).

Project 1 – Novel Generation of FAP Inhibitor-Based Homodimers for Improved Application in Radiotheranostics – Preclinical Investigations

The strategy presented in the previous section offers the possibility of thousands of new homodimeric FAPi radiotracers with all its options for each of the different moieties (targeting vectors TV1 and TV2, central linker CL, spacer S1 – S3 and labeling unit LU). The almost endless possibilities go far beyond what can be realized within the context of this thesis. Therefore, only selected structures were synthesized in this project and in project 2.

FAPi-NH₂ (cf. Figure 22) was chosen as a targeting vector because UAMC-1110 and the FAPi radiotracers that utilized UAMC-1110 as a TV have proven to be highly suitable for FAP-targeting. In this project, glutamic acid (Glu) was used as the central linker unit (CL). One advantage is that its two carboxylic acid groups and one amino group are ideally suited to link three moieties (two TVs and a bifunctional chelator). Many combinations of partially protected Glu units with diverse protecting groups are commercially available that allow to adapt the synthetic strategy with regard to further derivatization (project 2). Furthermore, the use of Glu might have beneficial effects on hydrophilicity compared to the lipophilic squaramide used in DOTAGA.(SA.FAPi)₂. Hydrophilicity is believed to substantially influence pharmacological properties. In particular in FAPi-based compounds, a high hydrophilicity seems to be crucial to avoid liver uptake and hepatobiliary excretion.

The FAPi-TVs are supposed to be directly coupled to Glu without the use of any additional spacer units. It has to be noted that the term “spacer” is not clearly defined and the 4-aminobutoxy residue of FAPi-NH₂ could already be considered as a short spacer unit, but within this work the TV was defined as FAPi-NH₂ including the 4-aminobutoxy residue.

Finally, DO3A and DOTAGA were chosen as chelators. Since the chelator is at terminal *exo*-position it is only coupled to the rest of the molecule by one functional group and not two as in linear homodimers like DOTAGA.(SA.FAPi)₂. This is reminiscent of monomeric radiotracers and shall lead to improved monomer-like labeling properties because of minimized steric hindrance. Additionally, when using the chelator DOTAGA, it will have an additional free carboxyl/carboxylate donor ligand and thus a coordination number of 8 instead of 7. This is not only supposed to lead to improved ²²⁵Ac-radiolabeling and more stable complexes but also to higher hydrophilicity.

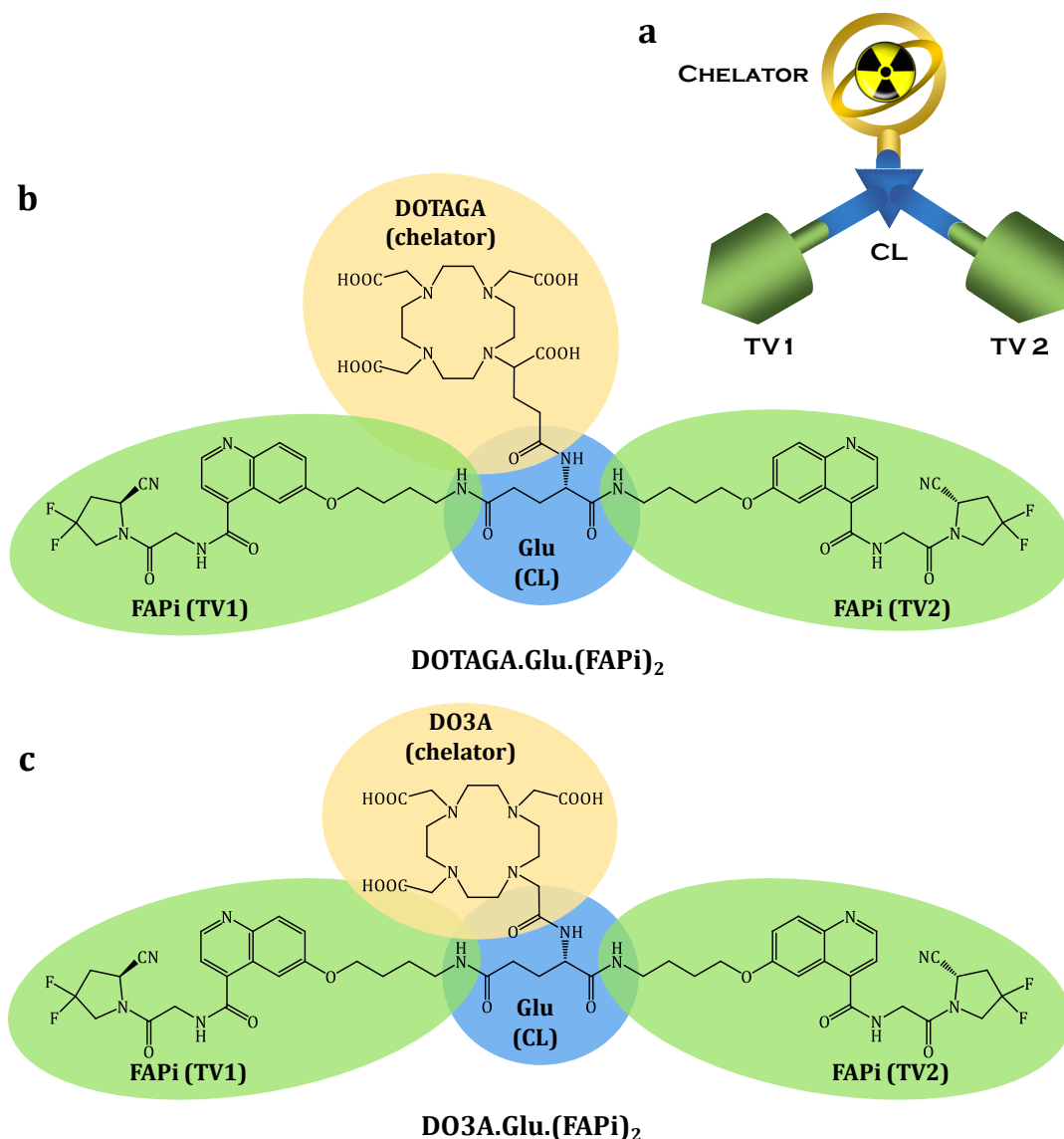


Figure 27: a) General structure of the FAPI homodimers from project 1: b) DOTAGA.Glu.(FAPI)₂ and c) DO3A.Glu.(FAPI)₂.

FAPI-NH₂ is first coupled to *N*-Boc-protected Glu twice before the chelator DO3A or DOTAGA is introduced. After organic synthesis of the precursors DO3A.Glu.(FAPI)₂ and DOTAGA.Glu.(FAPI)₂, the radiolabeling is optimized by investigating the reaction kinetics of different labeling conditions for the radiometals gallium-68, lutetium-177, actinium-225 and yttrium-90. The complex stability of the radiolabeled products is determined in human serum (HS) and phosphate-buffered saline (PBS). Furthermore, the log*D*_{7.4} value will be determined via shake-flask method to quantify lipophilicity.

In addition, *IC*₅₀ values for FAP and the competitive proteases PREP, DPP4, DPP8 and DPP9 were measured in *in vitro* studies to determine FAP affinity and selectivity of the new compounds. Finally, first proof-of-concept patient studies are carried out where tumor uptake, retention and pharmacokinetics of [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPI)₂ are directly compared to [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPI)₂ in the same patient. The published manuscript can be found in chapter 3.2.

Project 1 – Novel Generation of FAP Inhibitor-Based Homodimers for Improved Application in Radiotheranostics – Clinical Applications

The favorable radiolabeling, *in vitro* properties and first promising proof-of-concept patient study of DOTAGA.Glu.(FAPi)₂ encourages further clinical studies on ¹⁷⁷Lu-FAPi dimer RLT with [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂.

Project 1 – Glioblastoma Case Report

It has been reported that the TME of neuroepithelial cancers such as glioblastoma (GBM) strongly expresses FAP in many cases. Some results suggest that there also might be direct FAP expression on glioma cells although this is not fully validated yet [201,210–212].

Furthermore, FAP expression seems to be correlated with prognosis of GBM and aggressiveness of glioma cells which indicates the contributing role of FAP in GBM growth. Therefore, potential patients are scanned first with [⁶⁸Ga]Ga-DOTA.SA.FAPi to assess FAP expression and potential radiation dose to tumor lesions with subsequent FAPi dimer RLT.

A first proof-of-concept glioblastoma case report on treatment response of RLT with two cycles of [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ is presented in chapter 3.3.

Project 2 – FAP Inhibitor-Based Homodimers with Different Linker and Spacer Units for Application in Radioligand Therapy

In contrast to project 1, this work introduces two different chemical variations in FAPi homodimers, (i) in the central linker structure or (ii) an additional spacer unit ("S3", cf. Figure 28a). The molecules are designed in a branched, triangular structure like DOTAGA.Glu.(FAPi)₂. Long tumor residence times shall be ensured by using two FAPi TVs. DOTAGA and DO3A are again used as chelators. DOTAGA deserves special attention since DOTAGA.Glu.(FAPi)₂ showed slightly better radiolabeling and *in vitro* properties than DO3A.Glu.(FAPi)₂ in the previous study [514]. The effect of the different linker and spacer units on radiolabeling properties and *in vitro* affinities are investigated. The aim is to see if further optimization can be achieved that will ultimately have a positive effect on the pharmacological properties *in vivo* with regard to FAP-RLT.

The two compounds DO3A.NPyr.(FAPi)₂ and DOTAGA.NPyr.(FAPi)₂ are shown in Figure 28b and c. The central linker unit Glu is substituted by a heterocyclic 3-aminopyrrolidine (NPyr) with two additional acetic acid groups that enable coupling to the FAPi TVs analogous to Glu. Heterocyclic linkers like piperazine have been used extensively in FAPI-04 and FAPI-46 and the compounds derived therefrom which showed high FAP affinity [449,515].

Additional spacer units are introduced between the central linker Glu and the respective chelator in the other new molecules. There are a number of reasons why this might be beneficial. Firstly, more space and flexibility between the chelator and the central linker unit will further reduce the possibility of steric hindrance during radiolabeling. Secondly, it will also increase the space between chelator and the FAPi TVs. This might have beneficial effects on FAP affinity as the chelator will have more distance to the active site of FAP and interfere less in binding to FAP. The selectivity over PREP and/or the DPPs is probably affected by the alterations. Thirdly, the hydrophilicity and potential other factors might alter the PK and PD properties of the radiotracers. Another factor that might be influenced is the internalization and externalization rates. Decreased externalization from CAFs might be one of the reasons of prolonged tumor retention and thus a desirable effect if this proves to be true.

A second Glu in DOTAGA.Glu₂.(FAPi)₂ and DO3A.Glu₂.(FAPi)₂ (cf. Figure 28d and e) is coupled only at two of the three functional groups, the γ carboxylic acid with the central linker Glu and the α amino group to the chelator. The other carboxyl group remains unsubstituted ("free"). This spacer was mainly chosen to increase the hydrophilicity of the molecules even further compared to the compounds of project 1.

Furthermore, a diethylene glycol (PEG₂) spacer is introduced which is further functionalized and has an amino group and a carboxyl group at both ends to enable analogous coupling like with the previously mentioned Glu spacer. This is the longest spacer and was mainly chosen to further

increase the distance between the TVs and the chelator in DOTAGA.PEG₂.(FAPi)₂ (cf. Figure 28f) without negatively influencing the hydrophilic character of the new FAPi homodimers.

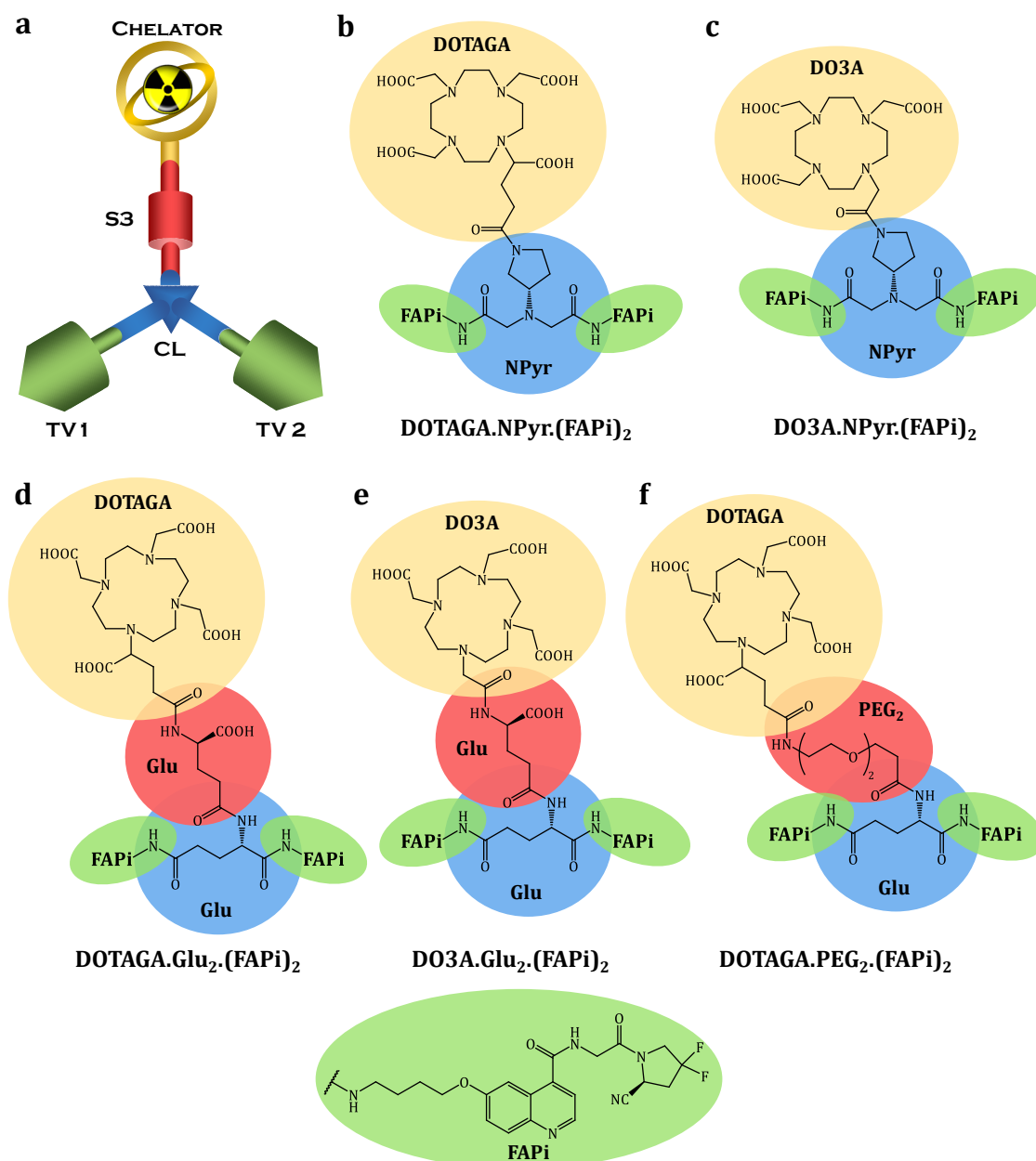


Figure 28: a) General structure of the FAPi homodimers from project 2: b) DOTAGA.NPyr.(FAPi)₂, c) DO3A.NPyr.(FAPi)₂, d) DOTAGA.Glu₂.(FAPi)₂, e) DO3A.Glu₂.(FAPi)₂ and f) DOTAGA.PEG₂.(FAPi)₂.

The organic synthesis is supposed to start with the coupling of the linker and spacer units before the two FAPi units and finally the chelator are coupled to the rest of the molecule.

Radiolabeling with gallium-68 and lutetium-177 and the respective complex stability in HS and PBS and lipophilicity is investigated subsequently. The influence of the different linker and spacer units on binding to FAP and the related proteases is tested *in vitro* and is compared to the previously synthesized FAPi-based homodimers, especially to DOTAGA.Glu.(FAPi)₂, which showed the best properties of all FAPi homodimers so far. The respective manuscript is found in chapter 3.4.

Project 3 – Novel FAP Inhibitor-Based Monomers as Diagnostic PET/CT Counterpart for FAPi Dimer Radioligand Therapy

[⁶⁸Ga]Ga-DOTA.SA.FAPi is still used as the diagnostic counterpart in ⁶⁸Ga-PET guiding RLT with [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂ because of the slow pharmacokinetics of [⁶⁸Ga]Ga-DOTAGA.(SA.FAPi)₂ that showed relatively high blood pool activity one hour p.i. (and even 3 hours p.i.) [472]. Later time points than one hour p.i. are not really convenient due to the half-life of gallium-68 and the clinical routine in general. [⁶⁸Ga]Ga-DOTAGA.Glu.(FAPi)₂ is also not optimal for ⁶⁸Ga-PET imaging at early time points since there is still some blood circulation one hour p.i. (data unpublished). The dimers are optimized for RLT and to bring the highest radiation dose possible to the tumor lesions and not for ⁶⁸Ga-PET application. The theranostic pair of [⁶⁸Ga]Ga-DOTA.SA.FAPi and [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ is not perfect either due to the structural differences as the monomer carries a SA-linker unit and the dimer a Glu central linker unit. Therefore, new FAPi monomers were designed that are structurally closer to DOTAGA.Glu.(FAPi)₂ as a better fitting diagnostic counterpart.

One FAPi TV was substituted by a pyrrolidine heterocycle which is supposed to be an inert group (cf. Figure 29a). Again, the heterocyclic pyrrolidine was chosen because it is reminiscent of the heterocyclic linkers of FAPI-04 and FAPI-46 [449,464]. In addition to DO3A and DOTAGA, the hybrid chelator DATA^{5m} was used as well. Radiolabeling of DATA^{5m} with gallium-68 can be carried out at room temperature and is potentially suitable for kit-type labeling. The structures of the three compounds DOTAGA.Glu(Pyr).FAPi, DO3A.Glu(Pyr).FAPi and DATA^{5m}.Glu(Pyr).FAPi are depicted in Figure 29b – d.

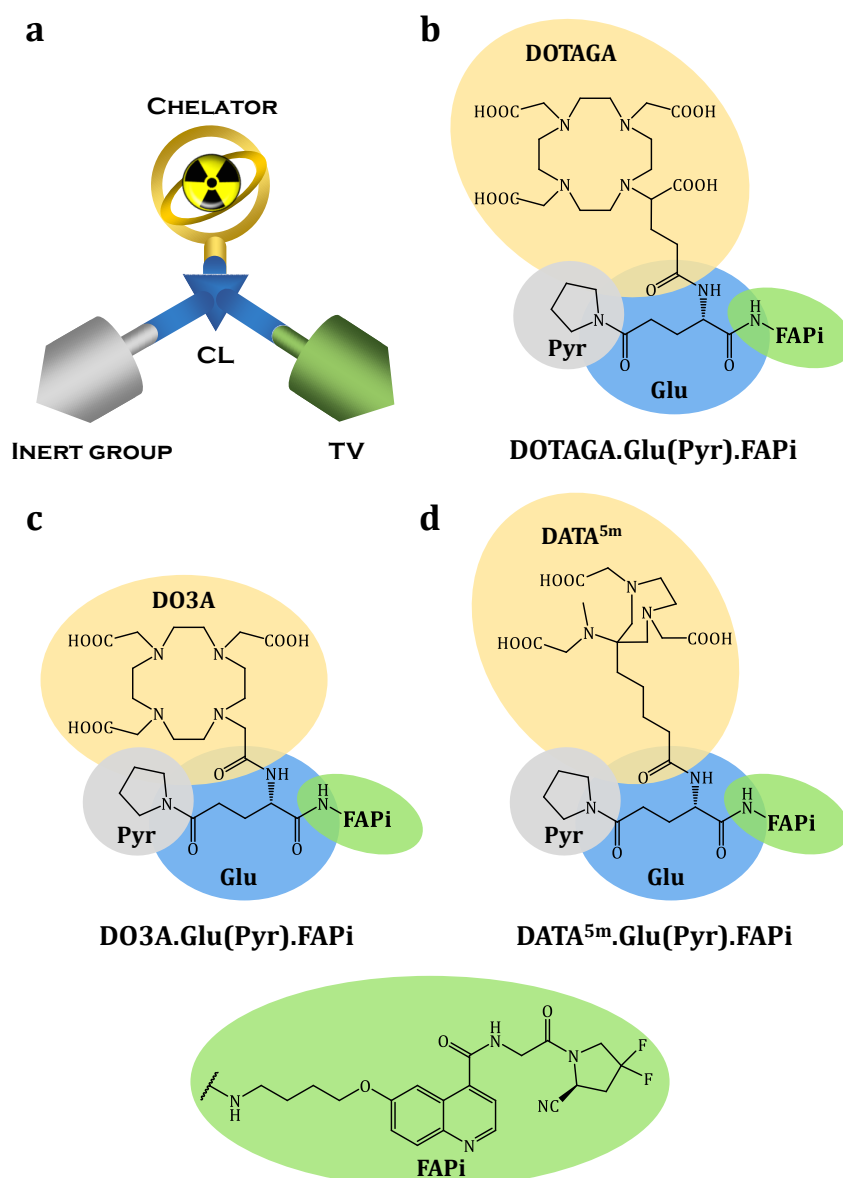


Figure 29: a) General structure of the FAPI monomers with inert group (grey) from project 3: b) DOTAGA.Glu(Pyr).FAPi, c) DO3A.Glu(Pyr).FAPi and d) DATA^{5m}.Glu(Pyr).FAPi.

Organic synthesis is carried out by first coupling pyrrolidine and asymmetrically protected Boc-Glu-OBzl with its γ carboxyl group. This is followed by FAPi TV and finally by chelator coupling with protecting group cleavages in between. Afterwards, reaction kinetics of radiolabeling with gallium-68 is compared in different buffer systems and with various precursor amounts to optimize reaction conditions. Again, the complexes shall be stable in HS and PBS and the hydrophilicity should be high and close to DOTAGA.Glu.(FAPi)₂ ideally. Precursors and ^{nat}Ga-complexes are tested for their affinity and *IC*₅₀ values for all the important related proteases and the *in vitro* results are compared to DOTA.SA.FAPi and DOTAGA.Glu.(FAPi)₂ in particular. The *in vivo* performance of [⁶⁸Ga]Ga-DO3A.Glu(Pyr).FAPi and [⁶⁸Ga]Ga-DOTAGA.Glu(Pyr).FAPi are finally investigated in a first proof-of-principle human PET/CT. The manuscript is found in chapter 3.5.

Project 4 – ^{99m}Tc-FAPi-Based Radiotracers for Application in SPECT Imaging of Various Cancers

There are few publications on FAPi radiotracers for SPECT imaging. Technetium-99m is the most used radionuclide in SPECT and nuclear medicine in general even without a pan-tumor oncologic ^{99m}Tc-radiotracer that would be comparable to [¹⁸F]F-FDG in PET imaging. So, the demand for a universally usable oncologic SPECT radiotracer is very high.

Novel ^{99m}Tc-labeled FAPi-SPECT radiotracers were developed and studied. The established selective high-affinity FAP inhibitor TV FAPi-NH₂ (derived from UAMC-1110) is coupled to two different chelators via squaric acid (SA). Two amines can be asymmetrically coupled with via squaric acid in pH-controlled reaction and forms a stable squaramide with high yields, low or no side product formation under mild conditions. The first amidation step is usually carried out neutral pH whereas the second amidation needs basic conditions (pH ≥ 9) [468,471]. SA has already shown to be a suitable linker for FAPi-PET imaging as [⁶⁸Ga]Ga-DOTA.SA.FAPi has been extensively studied in over 100 patients already [466,475]. The radiotracers [^{99m}Tc]Tc-MAG3.SA.FAPi and [^{99m}Tc]Tc-N4.SA.FAPi of this project are similar in this respect but of course other types of chelators are needed for stable ^{99m}Tc-complexes. For example, Lindner et al. [488] used Tc(I) tricarbonyl complexes that are used in a two-step process where *fac*-[^{99m}Tc][Tc(CO)₃]⁺ is produced from [^{99m}Tc][TcO₄]⁻ in the first step before the precursor can be labeled.

This project instead uses the N_xS_y chelators to obtain stable Tc(V) complexes with the [^{99m}Tc][TcO]³⁺ core by *in situ* reduction of [^{99m}Tc][TcO₄]⁻ with tin(II)chloride in a direct one step procedure. MAG3 is an established N₃S chelator and thiol group is protected with a *S*-benzoyl group (Bz) like in *S*-Bz-MAG3 which is used as a precursor for [^{99m}Tc]Tc-MAG3, a radiotracer for renal SPECT imaging. Previous works in our group (unpublished) have failed to synthesize *S*-Bz-MAG3.SA.FAPi in synthesis similar to DOTA.SA.FAPi due to the instability of the Bz protecting group in basic conditions (second step of SA coupling). Different protecting groups like the acid-labile trityl group did also not lead to a successful synthesis. Therefore, the order of coupling is completely changed. The chelator is not synthesized completely and then coupled to the rest of the molecule. On the contrary, the 2-(Benzoylthio)acetic acid is coupled last to avoid subsequent thioester cleavage. The tetramine chelator N4 is used as a sulfur-free alternative.

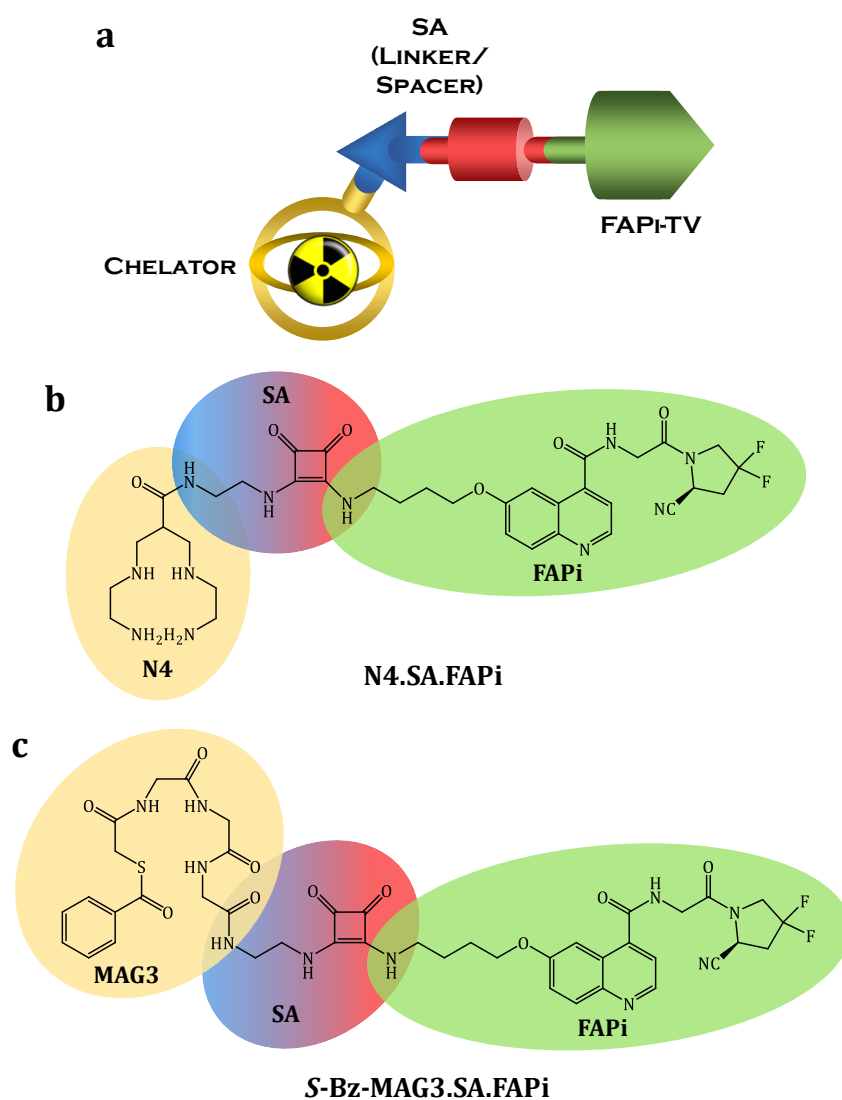


Figure 30: a) General structure of the ^{99m}Tc -FAPi monomers from project 4: b) N4.SA.FAPi and c) S-Bz-MAG3.SA.FAPi.

For ^{99m}Tc -radiolabeling different reagents are needed. Tin(II)chloride is used as reducing agent, the use of different buffer systems (ammonium acetate, phosphate etc.) and different weak exchange ligands (tartrate, citrate) are possible. Complex stability in different media is tested again, as well as affinity studies that will demonstrate binding properties to the different proteases *in vitro*. All together these results will show if $[^{99m}\text{Tc}]\text{Tc}$ -MAG3.SA.FAPi and $[^{99m}\text{Tc}]\text{Tc}$ -N4.SA.FAPi are potential candidates for further *in vivo* SPECT studies.

Chapter 3.6. shows the corresponding manuscript.

Project 5 – FAP-Targeted Drug Delivery with Different Cytostatic Prodrugs Based on a Selective Small-Molecule FAP Substrate Targeting Vector

The last project focuses on FAP inhibitors that are supposed to be combined with a FAP substrate in one molecule. The FAP substrate targeting moiety is further coupled to a cytostatic drug to improve FAP-targeted drug delivery. The strategy is to utilize FAP in two ways: (i) ensuring selective accumulation of the cytostatic prodrug by using the high-affinity FAP inhibitor UAMC-1110/FAPi-NH₂ and (ii) releasing the cytostatic by FAP-cleavage of the prodrug at the post-proline amide bond by using a highly-selective FAP substrate targeting moiety (FAPs).

In past publications, FAP-cleavable prodrugs were synthesized with substrate vectors that lacked selectivity versus PREP, or no data on PREP selectivity were reported. Nevertheless, most of these studies still showed at least some anti-tumor efficacy [246,312,314–316]. DE DECKER et al. [313] reported the first synthetic small molecule FAP substrates that are highly selective over PREP and the DPPs. These FAP substrates are structurally related to UAMC-1110 where nitrile warhead is substituted with a FAP-cleavable post-proline amide bond. Different fluorogenic residues were coupled to it to investigate cleavage.

In this project, FAPs is used and derived from the DE DECKER et al. [313] compounds but contains an additional 4-aminobutoxy substitution at 6-position of the quinoline moiety for further coupling. This is analogous to the 4-aminobutoxy substitution at 6-position of the quinoline moiety that separates FAPi-NH₂ from UAMC-1110. The terminal amino group of the 4-aminobutoxy chain can be coupled to FAPi-NH₂ via squaric acid (SA). At the other end of the molecule, a cytostatic prodrug with an amino group is coupled to form the post-proline amide bond. The general and exact structures of this project are shown in Figure 31.

The aim is to achieve selective accumulation of the FAP-prodrug in the TME followed by an efficient release of the active cytostatic drug in the TME/tumor lesion. The FAP substrate targeting moiety FAPs is coupled to the cytostatic drugs doxorubicin (Dox), monomethyl auristatin E (MMAE) and rucaparib. This is supposed to result in improved therapeutic response with simultaneously reduced side effects compared to the systemic of the cytostatic drugs.

Dox is an DNA-intercalating anthracycline that inhibits topoisomerase II with often critical cardiotoxicity when administered systemically, MMAE is a antimitotic agent that inhibits tubulin polymerization and is so toxic that it is not used systemically. Rucaparib is a poly (ADP-ribose) polymerase (PARP) inhibitor. PARP is an enzyme that initiates DNA repair of single-strand breaks. PARP inhibitors are mostly combined with other types of treatment such as radiotherapy [516,517].

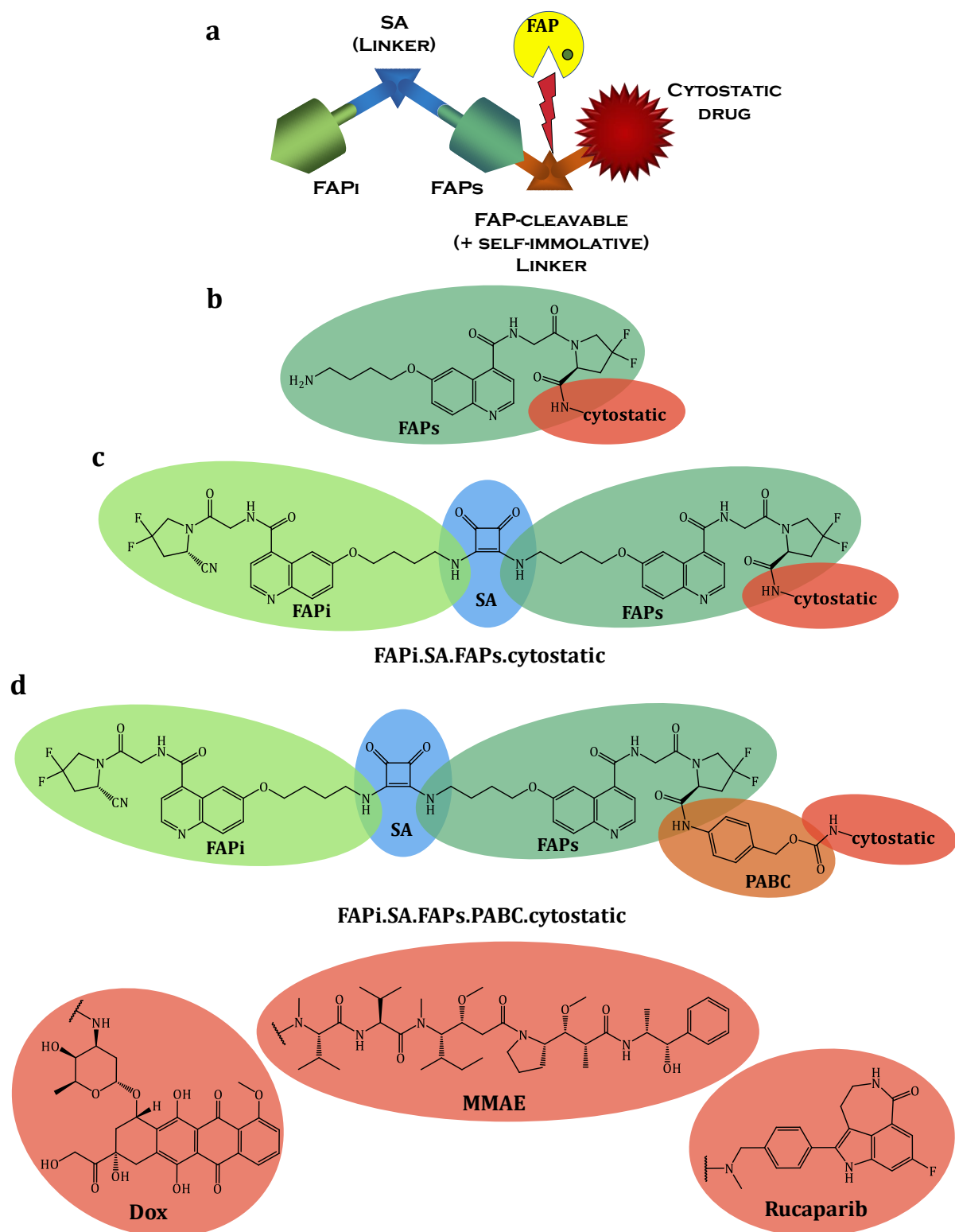


Figure 31: a) General structure of the FAP-targeting drug delivery system of project 5: b) FAPs.cytostatic, c) FAPi.SA.FAPs.cytostatic and d) FAPi.SA.FAPs.PABC.cytostatic with the cytostatic drugs doxorubicin (Dox), monomethyl auristatin E (MMAE) and rucaparib (bottom).

FAPs with its 4-aminobutoxy chain has to be synthesized from scratch first. Subsequently, it is coupled with the three different cytostatic drugs. Then, the cleavage by FAP and PREP of the compounds FAPs.cytostatic (cf. Figure 31b) is supposed to be investigated *in vitro* to see if efficiency and selectivity only by FAP and not by PREP is achieved.

In a second step, the compounds are further derivatized and coupled with FAPi via SA (cf. Figure 31c). Since the leading candidates of DE DECKER et al. [313] all had an aromatic amine at the post-proline position, an additional self-immolative linker unit like *p*-aminobenzyl carbamate (PABC) is introduced. PABC undergoes 1,6-elimination and the cytostatic drug is released from FAPi.SA.FAPs.PABC.cytostatic (cf. Figure 31d) in the same form as from FAPi.SA.FAPs.cytostatic without the self-immolative linker.

This project differs from the other projects in the sense that it does not deal with radiopharmaceuticals. No further *in vitro* studies of the second-generation compounds (cf. Figure 31c and d) or translation into *in vivo* studies could be realized within the time frame of this work. Nevertheless, substantial amount of work was put into this project and the manuscript containing the preliminary results of this project can be found in the Appendix (chapter 7.1.).

Review - Radiometal-Theranostics: The First 20 Years

This work further includes a **Review** about the development of theranostics based on trivalent radiometals in **chapter 3.1**. A historical look has been taken on the development of the diagnostic and therapeutic interplay in nuclear medicine over the last decades.

In the beginning, the review gives a general introduction into the theranostic concept and how it is utilized in the clinical routine of nuclear medicine.

Furthermore, it provides an overview of the key milestones achieved since the 1990s when the first matched radiometal pair $^{86}\text{Y}/^{90}\text{Y}$ emerged. The 2000s were characterized by new developments regarding oncological targets like somatostatin receptor (SSTR) agonists that are strongly expressed in neuroendocrine tumors. The SSTRs are addressed by octreotide derivatives like TOC or TATE and found its way into clinical routine in the 2000s along with the rise of the theranostic pair $^{68}\text{Ga}/^{177}\text{Lu}$.

New oncological targets emerged in the 2010s including prostate specific membrane antigen (PSMA) in prostate cancer addressed by PSMA inhibitor-based radiotracers, hydroxyapatite in bone metastases addressed by bisphosphonates and CAFs in endothelial cancers addressed by FAP inhibitor-based radiotracers.

Furthermore, the review gives an outlook on radionuclides and other oncological targets and targeting structures that are emerging and have the potential to find its way into the clinical routine in the years to come.

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3. Manuscripts

3.1. Radiometal-Theranostics: The First 20 Years*

Frank Roesch and Marcel Martin

Journal of Radioanalytical and Nuclear Chemistry **2023**, 332(5), 1557–1576, <https://doi.org/10.1007/s10967-022-08624-3>. (published on 24th November 2022)

3.2. Novel Generation of FAP Inhibitor-Based Homodimers for Improved Application in Radiotheranostics

Marcel Martin, Sanjana Ballal, Madhav P. Yadav, Chandrasekhar Bal, Yentl Van Rymentant, Joni De Loose, Emile Verhulst, Ingrid De Meester, Pieter Van Der Veken and Frank Roesch

Cancers **2023**, 15(6), 1889. <https://doi.org/10.3390/cancers15061889>. (published on 21th March 2023)

3.3. [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ radionuclide therapy: A new treatment option for patients with glioblastoma multiforme

Sanjana Ballal^{1*}, Madhav P. Yadav^{1*}, Shobhana Raju¹, Frank Roesch², Marcel Martin², Madhavi Tripathi¹, Chandrasekhar Bal^{1#}

Submitted to *Nuclear Medicine and Molecular Imaging* (Springer) on 7 April 2023.
(under review)

3.4. FAP Inhibitor-Based Homodimers with Different Linker and Spacer Units for Application in Radioligand Therapy

Marcel Martin, Yentl Van Rymenant, Joni De Loose, Emile Verhulst, Ingrid De Meester, Pieter Van Der Veken, Sarah Stephan, Kevin Machel and Frank Roesch

In preparation for submission.

3.5. Novel FAP Inhibitor-Based Monomers as Diagnostic PET/CT Counterpart for FAPi Dimer Radioligand Therapy

Marcel Martin, Sanjana Ballal, Madhav P. Yadav, Chandrasekhar Bal, Yentl Van Rymenant, Joni De Loose, Emile Verhulst, Ingrid De Meester, Pieter Van Der Veken, and Frank Roesch

In preparation for submission.

3.6. ^{99m}Tc -FAPi-Based Radiotracers for Application in SPECT Imaging of Various Cancers

Marcel Martin, Lukas Greifenstein, Yentl Van Rymenant, Joni De Loose, Emile Verhulst, Ingrid De Meester, Pieter Van Der Veken, Deniz Göral, Kevin Machel and Frank Roesch

In preparation for submission.

Author Contributions

There are various publications and manuscripts each having different authors that were involved in the research projects and form the basis of this dissertation. The literature research and the interpretation of these results reaching from tumor microenvironment to FAP-targeting radiopharmaceuticals was carried out by MARCEL MARTIN, the author of this dissertation. Starting from this, MARCEL MARTIN identified problems of the current state of research and explored possibilities and drafted molecular designs and chemical concepts to synthesize new optimized compounds that address the existing problems with the literature-known compounds. Subsequently, the organic synthesis route was designed, planned and the respective steps were carried out by MARCEL MARTIN including the analytics of the synthesized compounds. The same applies for all the radiochemical studies and data included in this work: the radiolabeling studies were designed/planned, carried out, analyzed and interpreted by MARCEL MARTIN.

Furthermore, MARCEL MARTIN was involved in the design and planning of the *in vitro* and patient studies, and contributed to the interpretation of the results substantially as well.

All first author manuscripts were solely written by MARCEL MARTIN. Contributions from MARCEL MARTIN varied between the various publications and manuscripts, some of the research was carried out in close collaboration with the additional authors. Before the respective publication or manuscript, a description of contributions will show the different responsibilities in detail.

3.1. Radiometal-Theranostics: The First 20 Years*

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The following **Summary** and **Author Contributions** sections do not belong to the publication. The actual publication begins after that and was not altered in any way.

Summary

In the last decades, great progress was achieved in the field of nuclear medicine and radiopharmaceutical chemistry in the interplay between diagnosis and therapy. The term theranostics stands for this optimization in a personalized medicine approach. It includes the aim to find the best treatment for every patient individually. In the context of nuclear medicine this means to find the right therapy with the optimal radiopharmaceutical for each individual patient with the optimal radiation dose at the right time. This is supported by a diagnostic counterpart that enables optimal treatment planning (dosimetry) as well as therapy control.

In the case of radiometals, the translation from diagnostic to therapeutic radiopharmaceutical can be realized by radiolabeling the same chelator-based precursor with different radionuclides. This approach is convenient since there are many different trivalent radiometals for diagnosis and therapy. Gallium-68, yttrium-86 and -90, indium-111, lutetium-177 and actinium-225 are among the most important but many other radiometals could be added to this list including scandium-44 and -47, gallium-67, zirconium-89, terbium-161, and bismuth-213 that can be all labeled with the same or very similar chelators. On top, there are other theranostic pairs that are labeled with different chelators like copper-64 and -67, technetium-99m and rhenium-186 and -188 as well as lead-203 and -212 that are not part of this review.

At first, this review gives a general overview over the four principle molecular designs of radiotracers: A) direct injection of the radionuclide, B) radionuclide-chelator complex, C) covalently coupled radionuclide to a targeting vector (TV) which is restricted to organic radionuclides and D) radionuclide complexed by a bifunctional chelator that is attached to a TV via linker with an optional additional spacer unit.

The clinical routine of a theranostic application in nuclear medicine can be divided into five steps: 1. Quantitative diagnostic PET/CT with the diagnostic counterpart of the theranostic pair, 2. Structural from diagnostic to therapeutic radiopharmaceutical (choice of the right therapeutic radionuclide etc.), 3. Pretherapeutic dosimetry via PET/CT, 4. Therapy according to pretherapeutic dosimetry and 5. Monitoring post-therapy (analogous to 1. with diagnostic PET/CT).

An historical look back reveals that the first ever applied radionuclide therapy (RNT) with [¹³¹I]NaI can also be seen as the birth of theranostics since there are iodine isotopes as matching diagnostic counterparts as iodine-124 for PET and iodine-123 for SPECT despite the fact that iodine-131 itself can also be used for SPECT imaging. The first matching theranostic trivalent radiometal pair emerged in the 1990s with the first ⁸⁶Y/⁹⁰Y-radiopharmaceuticals instead of the previously used mismatched ¹¹¹In-SPECT-guided ⁹⁰Y-RNT [21–32]. One of the main applications was the treatment of somatostatin receptor 2 SSTR2-expressing of neuroendocrine tumors that were targeted with octreotide derivatives like the agonist TOC in [⁹⁰Y]Y-DOTA-TOC [29–31,34].

This morphed into the preferred use of [⁶⁸Ga]Ga- and [¹⁷⁷Lu]Lu-DOTA-TOC and -DOTA-TATE and the 2000s due to the better availability via ⁶⁸Ge/⁶⁸Ga generator and the new indirect ¹⁷⁷Lu-production route. More radiopharmaceuticals that target different oncological targets came into increased clinical use in the 2010s. This includes PSMA inhibitors such as [⁶⁸Ga]Ga-PSMA-11 and [¹⁷⁷Lu]Lu-PSMA-617 for the treatment of prostate cancer [59–65], bisphosphonates like BPAMD for bone metastases [7–9] and FAP inhibitors to address CAFs in the TME like FAPI-04, FAPI-46 or DOTA.SA.FAPi [80–84].

Finally, new developments regarding radionuclides and new oncological targets and its respective targeting vectors are discussed. This includes SSTR2 antagonists LM-3/4, bombesin (BBN) type II receptor ligands (prostate cancer), CXCR4 ligands, and RGD-containing cyclic peptides to target $\alpha_v\beta_3$ integrin (as well as other integrins) [87–95]. The emergence of homo- and hetero-dimers and -multimers also leads to new radiopharmaceutical designs with more than one identical TV (homo) or several different TVs (hetero) to improve tumor retention and the deposited radiation dose in the tumor lesion. Interesting examples of multimers with central chelators include the SarbisPSMA with a special sarcophagine (Sar) chelator for copper-chelation, homodimeric FAP inhibitors like DOTAGA.(SA.FAPi)₂, tri- and tetrameric RGD compounds with newly developed chelators like TRAP(RGD)₃ or DOTPI(RGD)₄, or using several BBN-derived peptides in one molecule [102–110]. Heterodimers have been published in various combinations, e.g. BBN-RGD, FAP-PSMA, etc. [113–118]. Additionally, human serum albumin (HSA) binding moiety have also been introduced in combination with a classical TV such as TOC/TATE, PSMA or FAP inhibitors to increase retention and optimize the radiation dose delivered to the tumor per activity amount [121–123]. But it also prolongs blood circulation roughly in the same order of magnitude. Hence, the selectivity is not substantially improved.

Author Contributions

Frank Roesch mainly conceptualized and structured this review but was supported by MARCEL MARTIN. Frank Roesch was mainly responsible for drafting the first part of the review (Introduction, the 1990s and the 2000s) whereas MARCEL MARTIN was mainly responsible for drafting the second part of the review (the 2010s and Outlook). MARCEL MARTIN further finalized and revised the manuscript and also took care of the literature management.

Radiometal-Theranostics: The First 20 Years*

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Abstract

This review describes the basic principles of radiometal-theranostics and its dawn based on the development of the positron-emitting ⁸⁶Y and ⁸⁶Y-labeled radiopharmaceuticals to quantify biodistribution and dosimetry of ⁹⁰Y-labeled analogue therapeutics. The nuclear and inorganic development of ⁸⁶Y (including nuclear and cross section data, irradiation, radiochemical separation and recovery) led to preclinical and clinical evaluation of ⁸⁶Y-labeled citrate and EDTMP complexes and yielded organ radiation doses in terms of mGy/MBq ⁹⁰Y. The approach was extended to [^{86/90}Y]Y-DOTA-TOC, yielding again yielded organ radiation doses in terms of mGy/MBq ⁹⁰Y.

The review further discusses the consequences of this early development in terms of further radiometals that were used (⁶⁸Ga, ¹⁷⁷Lu etc.), more chelators that were developed, new biological targets that were addressed (SSTR, PSMA, FAP, etc.) and subsequent generations of radiometal-theranostics that resulted out of that.

Keywords

theranostics, radiometals, positron emission tomography (PET), radionuclide therapy (RNT), endoradiotherapy (ERT); peptide receptor radionuclide therapy (PRRT); radiopharmaceuticals; radiotracers

*The Hevesy Medal and a certificate was presented Professor RÖSCH at the at the HMA-2022 Ceremony during the 19th Radiochemical Conference (RadChem-2022) in Mariánské Lázně, Czechia, May 15-20, 2022. This article summarizes the lecture given at the conference.

1. Introduction

The term "theranostics" overlaps with terms such as "personalized medicine" or "precision oncology" insofar as medical decisions, procedures and/or products are tailored to the individual patient. This includes technologies to produce customized pharmaceutical products that contain individual dosages for one or more active ingredients. Diagnostic tests used to select appropriate therapies are referred to as "companion diagnostics".

In the context of radiopharmacy and molecular imaging, the concept is particularly succinct: the ultimate object is successful therapy with appropriate short-range particle emitters (α or β^-), crucially built on therapy-deciding and -accompanying diagnosis with photon emitters.

With the goal of patient-specific treatment, potent radiopharmaceuticals are used in a diagnostic (ideally using positron emitters for quantitative PET/CT) and a therapeutic variant. The diagnostic radiopharmaceutical is used to verify tumor indication and staging for an individual patient. Conversely, it verifies that the radiopharmaceutical at hand is a target-specific and selective molecule: the ideally suited for a specific patient. Precise diagnostic imaging also allows quantitative dosimetric information (expressed in terms of maximum radiation dose to the target tissue and tolerable dose to healthy organs) for the therapeutic variant of the theranostic agent. This is applied with optimal radioactivity of the appropriate radiopharmaceutical in the individual patient. Finally, post-therapeutic imaging with the diagnostic variant of the theranostic is part of the assessment of treatment success for the individual patient.

In the context of radiopharmacy and nuclear medicine, radiopharmaceuticals available in both a diagnostic (usually for PET/CT) and a therapeutic form are the central instruments. The transition from the diagnostic to the therapeutic derivative is made by substituting the diagnostic radionuclide by its therapeutic counterpart - where the oncological target should be identical and the pharmacological properties of the two variants should not differ. In cases where the theranostic pair is from nuclides of different elements ("mismatched pair", e.g. gallium-68 and lutetium-177) the properties are not identical but very similar so the *in vivo* behaviour should not differ significantly. Radiotheranostics, by definition, share the same physiology in that they address the same oncologic target. Fig. 1 gives an overview over four typical molecular design options from a radiochemical/radiopharmaceutical perspective.

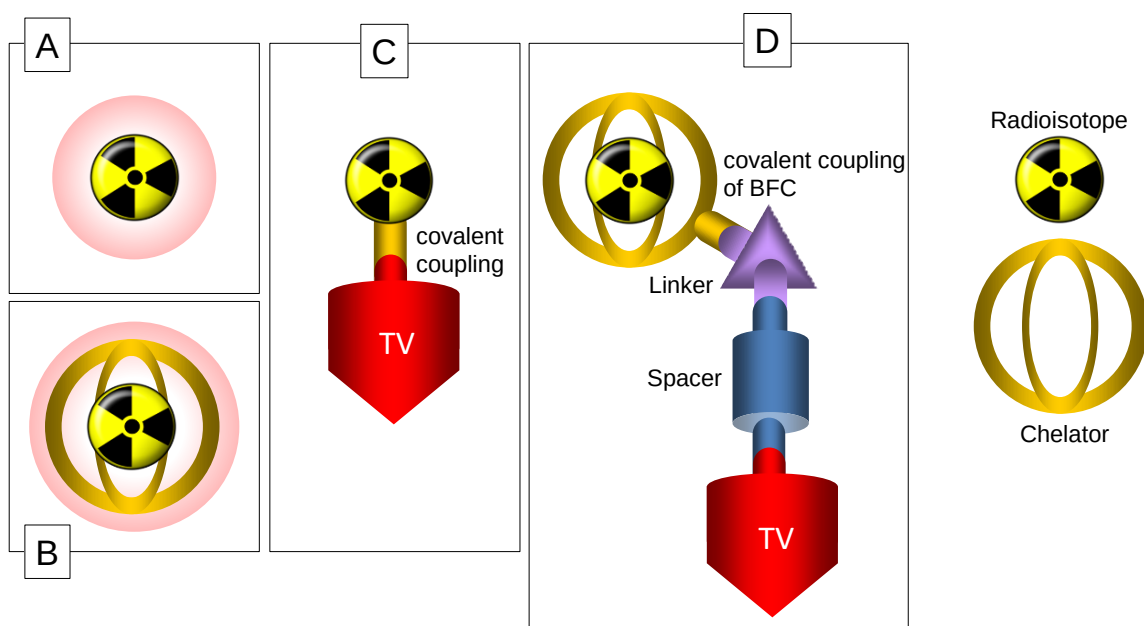


Fig. 1 Principal options of the molecular designs of Radiotheranostics – A: direct injection of radionuclide, B: injection of complexed radiometal, C: covalent coupling of a radionuclide to a targeting vector (red), D: radiometal complexed by bifunctional chelator (BFC) which is covalently bound by a linker to the targeting vector and a possible spacer

Option A (Fig. 1A) represents the specificity of individual radioisotopes to already have an oncologically relevant function in its ionic form. This includes, above all, the historically first implementation of the theranostic concept - it dates back more than 80 years. *SAUL HERTZ* realized the concept of thyroid diagnostics and therapy in the form of a single radionuclide, which offers both diagnostically and therapeutically relevant radiation: ^{131}I [1, 2].

Additional "chemistry" is not required - the physiological function of the isotope in the form of iodide [^{131}I] I^- is sufficient. Another example of option A is the use of the calcium mimetics ^{89}Sr or ^{223}Ra for the therapy of bone metastases; here, the cations Sr^{2+} or Ra^{2+} already have an effect due to their affinity for the hydroxyapatite structure of the bones too and are applied as chloride salts [^{89}Sr] SrCl_2 and [^{223}Ra] RaCl_2 , respectively [3–5]. Diagnostic analogues such as ^{18}F as [^{18}F] F^- , $^{99\text{m}}\text{Tc}$ -bisphosphonates or modern ^{68}Ga -labeled bisphosphonates are all also effective "bone seekers" [6–11].

Option B (Fig. 1B) involves the rather rare clinically realized design of radiometallic-labeled complexes for therapy, where the physiological role is not from the metal but from a property of the ligand. Typical representatives are $^{186/188}\text{Re}$ -HDEHP complexes [12, 13].

Options C and D represent a paradigm shift. The transport function for the radioisotope to a concrete oncological target is taken over by a defined targeting vector (proteins such as

antibodies, proteins as receptors, messenger analogues, inhibitors, amino acids, but also inorganic species). Now, neither the radioisotope nor the radiometallic ligand complex should have any physiological significance, but on the contrary should be physiologically inert to the greatest possible extent.

Option C applies to radiohalogens, especially ^{131}I . Clinically relevant cases are ^{131}I -MIBG and ^{131}I -labeled tyrosine derivatives for the therapy of glioblastoma and other diseases, respectively [14–17]. The radioiodine is introduced into the targeting vector in a covalent bond in such a way that the physiological role of guanidine or tyrosine is not diminished significantly. In the ideal concept of radiotheranostics, the substitution of ^{123}I or ^{124}I for the therapeutic isotope ^{131}I is sufficient to generate the potent diagnostic variant. Regardless of the type of iodine isotope, the pharmacokinetics of the theranostic remain absolutely identical.

Option D concerns radiometals. Unlike aliphatic or aromatic halogenation, the radiometal cannot be directly covalently coupled to a targeting vector, but requires a bifunctional chelating moiety. Docking such a relatively large chemical moiety to a small targeting vector can lead to major pharmacological interference (binding affinities, lipophilicity, etc.). Therefore, in terms of medicinal chemistry, optimizations of the chelator, as well as in linker and spacer units, play a major role [18].

In nuclear medicine, the clinical routine of a theranostic application (its logical and logistical processes) can be represented in five stages.

I. Quantitative diagnostics (PET/CT) with the diagnostic derivative of the theranostic agent

First, disease and disease state are evaluated for an individual patient using an appropriate diagnostic radiopharmaceutical. Conversely, or simultaneously with this, it is verified that the radiopharmaceutical at hand is a target-specific molecule: the ideally suited for that individual patient.

II. Structural translation of the diagnostic into a therapeutic radiopharmaceutical

This central pillar of theranostics is the task of radiopharmaceutical chemistry. How to replace the positron emitter of the PET/CT form of the theranostic with a beta or alpha emitter without compromising the specificity of the radiopharmaceutical? This task is subdivided into sub-areas: (1) What is the appropriate combination of the diagnostic/therapeutic radionuclide? From this, (2) what are the chemical properties of the chosen theranostic radionuclide and how this must affect (3) the structure of the theranostic in terms of stable radiolabeling. Finally, (4) the

appropriate structure must be characterized *in vitro* and in preclinical studies to demonstrate broad agreement in pharmacological properties with the diagnostic derivative of the theranostic.

III. Pretherapeutic dosimetry

Diagnostic imaging with the PET/CT form of the theranostic agent can be converted into quantitative dosimetric information in the form of time-activity correlations. In experimentally obtained time-activity correlations of the diagnostic derivative of the theranostic agent for healthy organs and lesions, the emission profile and physical half-life of the therapeutic radionuclide are now substituted. This is used to determine the optimal radioactivity (expressed in terms of maximum radiation dose to the target tissue and tolerable dose to healthy organs) of the appropriate radiotheranostic on the individual patient, cf. Fig. 2.

IV. Therapy

The therapeutic derivative of the theranostic is ideally applied at the activity level determined from the pretherapeutic dosimetry. This application can be repeated after appropriate times.

V. Post-therapeutic monitoring

Usually, the outcome of therapy is followed by quantitative imaging with the diagnostic derivative of the theranostic agent. Ideally, lesion size and SUV values will have decreased compared to pre-therapy.

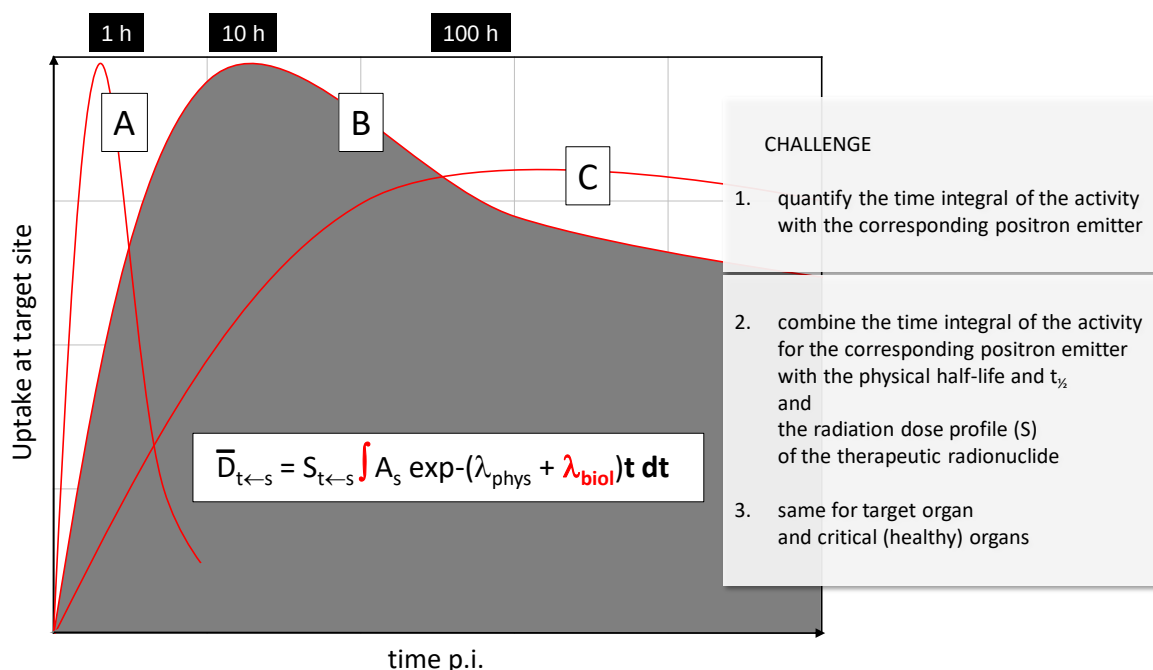


Fig. 2 Simplified illustration on the determination of radiation doses based on quantitative uptake kinetics obtained by the diagnostic PET tracer of the theranostic agent. A, B and C describe different pharmacology of the theranostic. The integral (e.g. the grey area for B) gives the cumulative radiation dose D , which is obtained from the kinetics, i.e. the biological and physical half-lives expressed by λ_{biol} and λ_{phys} , respectively. S is the S -factor representing transformation energies of a given radionuclides and A is the activity injected

2. The 1990s: $^{86}\text{Y}/^{90}\text{Y}$ matched isotope pair and prototype of radiometal-based theranostics

Nuclear chemistry and physics know more than 3000 radioactive isotopes - in contrast, the number routinely used in the field of diagnostic as well as therapeutic nuclear medicine is small. This is due to the fact that important selection criteria such as type and energy of the emissions, physical half-life and, above all, availability have to be fulfilled together.

For PET, these would be the short-lived "organic" radionuclides ^{11}C , ^{13}N , ^{15}O , halogens such as ^{18}F , ^{124}I , and radiometals such as ^{82}Rb , ^{64}Cu , ^{68}Ga , ^{86}Y and others. In the field of therapeutics, ^{131}I , ^{89}Sr , ^{90}Y , ^{153}Sm , ^{177}Lu , ^{161}Tb , $^{186/188}\text{Re}$, ^{225}Ac and others are used. Realistically (i.e., if one does not want to speculate on further "exotic" nuclides whose transformation properties appear promising but whose availability for clinical routine is currently only wishful thinking) very few ideal combinations result from this selection.

2.1 Radioiodide [$^{*}\text{I}$] $^{-}$

Historically, the most important combination is, in fact, only one radionuclide - which offers both diagnostically and therapeutically relevant radiation. Radioiodine ^{131}I is one of the best-known radionuclides that can be used directly in its anionic form as iodide [^{131}I] $^{-}$. Like nonradioactive iodide, it is accumulated in the thyroid gland. After administration, radioiodide is rapidly taken up by the thyroid via the sodium iodide symporter (NIS), and excess radioiodide is excreted renally. For administration, they are all formulated as sodium iodide in slightly basic aqueous solutions. They can be taken orally or administered by injection. Scintigraphic images of the thyroid gland allow identification of various diseases and dysfunctions of the organ, such as local foci of hyperthyroidism or hypothyroidism. Because of their long half-life, these iodine isotopes are usually provided by outside vendors for routine clinical use. For example, [^{131}I] NaI is usually supplied in capsule form with radioactivity already calibrated for specific patients. Several radioisotopes of iodine are suitable for molecular imaging and also enable PET (^{124}I) or SPECT (^{123}I , ^{131}I).

However, the spectrum of theranostically significant targeting vectors utilizing radioiodine is limited. A major challenge with radioiodinated compounds is the stability *in vivo* due to the weak C-I bond. Deiodination can be catalyzed by deiodinases, different enzymes like iodothyronine deiodinase (DIO1-3) for example, or proteolytic cleavage of peptides that contain iodine-substituted tyrosine units [19]. CAVINA et al. gave an extensive review discussing the stability of radioiodinated pharmaceuticals depending on their molecular structures [20].

2.2 $^{86}\text{Y}/^{90}\text{Y}$

In the early 1990s, several laboratories began to consider using a SPECT radionuclide as a surrogate for a therapeutic radionuclide, e.g. ^{111}In ($t_{1/2} = 2.8$ d), a trivalent metal, as a surrogate for ^{90}Y , another trivalent metal [21, 22]. The use of various other metallic radionuclides has also been discussed [21, 22].

While the β^- -emitting therapeutic radionuclide ^{90}Y ($t_{1/2} = 2.7$ d) had been available for a long time via the $^{90}\text{Sr}/^{90}\text{Y}$ generator system and was used for various therapies, there was no possibility of quantifiable imaging with ^{90}Y -labeled therapeutics at that time - the patient remained a "black box". Therefore, the β^+ -emitter ^{86}Y had to be first developed for medical use [23–25]. This included the evaluation and optimization of radionuclide production [23, 24], radiolabeling and its analytic as well as first pilot *in vivo* studies to learn about the *in vivo* quantification via PET [25]. With a half-life of $t_{1/2} = 14.7$ h and a fraction of 32% positron emission it was suitable to reproduce the pharmacology of analogous ^{90}Y -pharmaceuticals well [26–32]. RÖSCH et al. [33] gives a retrospective overview of the various aspects of the radiochemical and nuclear development work including nuclear data, cyclotron irradiation, chemical processing, quality control, etc. of ^{86}Y and ^{86}Y -labeled pharmaceuticals, the methodology established to quantify the molecular imaging of ^{86}Y -labeled compounds in the form of multiple and long-term PET recordings, and finally, application examples that highlighted the ultimate goal of radiotheranostics, namely to determine the radiation dose of the ^{90}Y -labeled compound in mGy or mSv per MBq of ^{90}Y injected, based on the long-term PET measurements with ^{86}Y analogues (Fig. 3). Of historical note, in addition to the methodological aspect, is the specific demonstration of the approach for the case of $[^{90}\text{Y}]\text{Y-DOTA-TOC}$ [29, 30]. Sandoz initially exercised the $[^{86}\text{Y}]\text{Y-DOTA-TOC}$ program as a diagnostic arm of $[^{90}\text{Y}]\text{Y-DOTA-TOC}$ before this entered the clinic. This was then realized first in baboons (Fig. 4) [29, 30] and later in patients (Fig. 5) [31, 34].

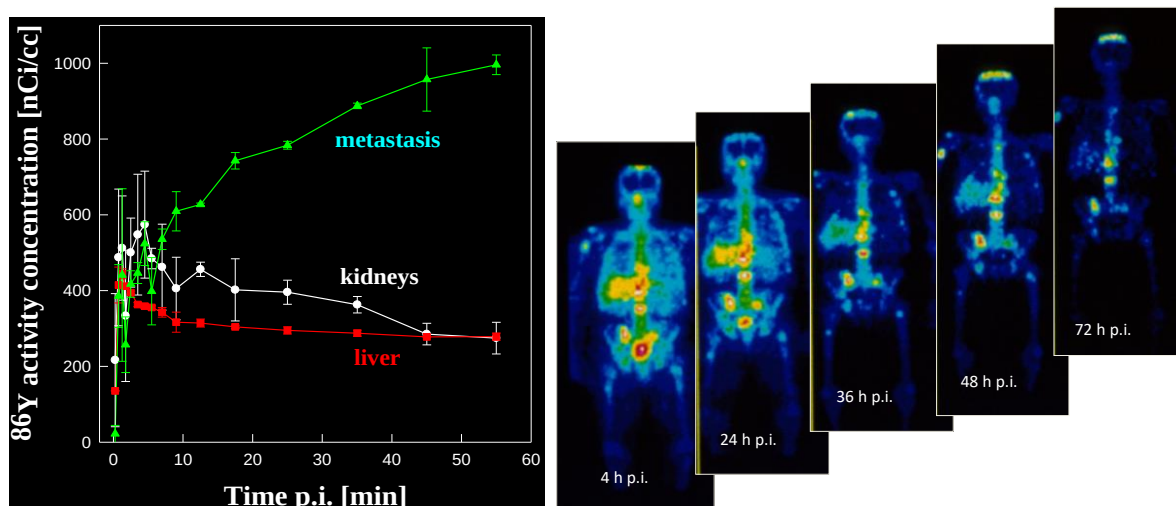


Fig. 3 ^{86}Y -Citrate PET imaging in patient suffering from bone metastasis [26, 28]

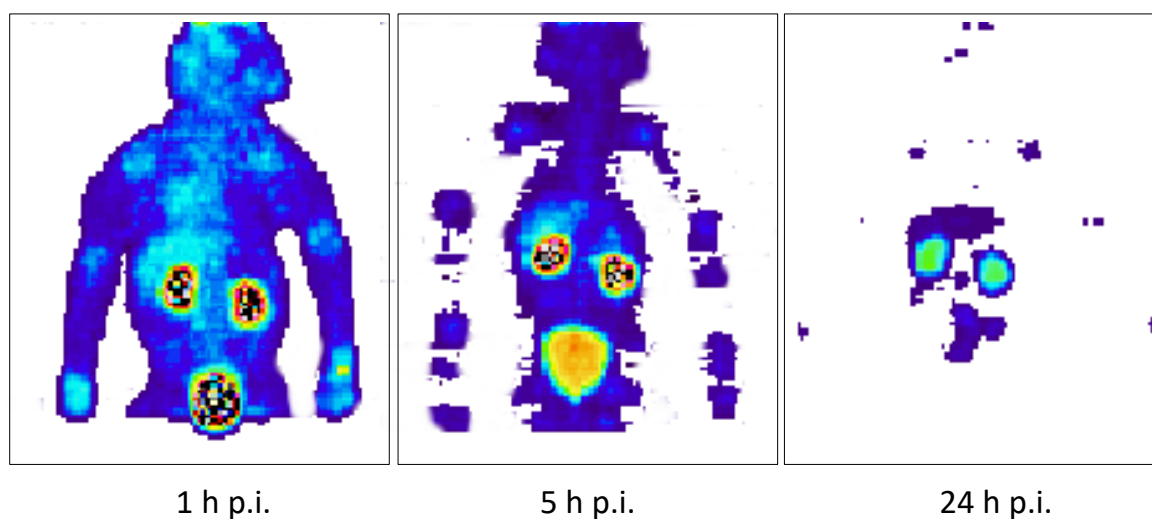


Fig. 4 ^{86}Y -DOTA-TOC PET imaging in baboons [29, 30]

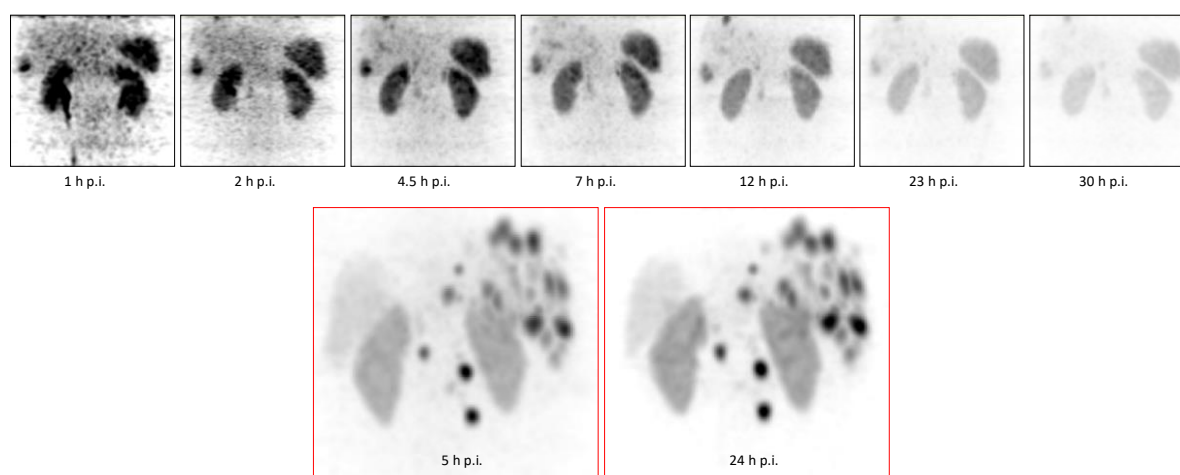


Fig. 5 ^{86}Y -DOTA-TOC PET imaging in patients suffering from neuroendocrine cancer [31, 34]

3. The 2000s: ^{177}Lu / ^{68}Ga

3.1 Biological targets

The most important radiotracer for nuclear medicine oncology imaging is 2-deoxy-2- ^{18}F fluoro-D-glucose (^{18}F FDG). Cancer cells obtain their energy mainly through aerobic glycolysis which is very inefficient. To compensate for this, the glycolysis rate is upregulated as well as the oxidative phosphorylation (Warburg effect). ^{18}F FDG is a glucose analogue which is likewise phosphorylated by the enzyme hexokinase. The ^{18}F FDG-6-phosphate on the other hand cannot be metabolized further which results in an accumulation in the cancer cell (metabolic trapping) that can be visualized via PET. Consequently, this makes ^{18}F FDG an almost universal oncological imaging tool. However, radiopharmaceutical chemistry does not have a therapeutic counterpart of glycolysis metabolism. Therefore, for tumor therapy, alternative oncological targets need to be identified and the appropriate radiopharmaceuticals developed, ideally for both PET/CT imaging and therapeutics. Fig. 6 provides a schematic overview of the relevant targets and classes of target vectors for this purpose.

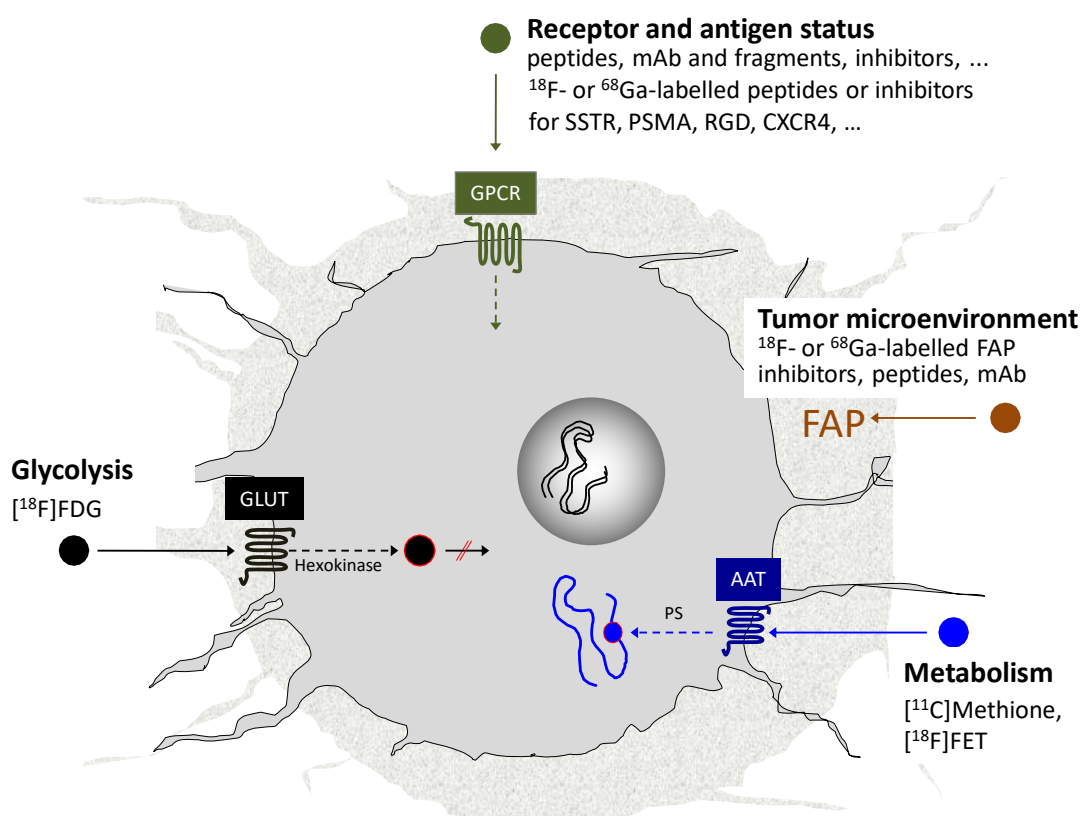


Fig. 6 Selected targets for PET imaging in oncology and corresponding radiotracers: 2-deoxy-2- ^{18}F fluoro-D-glucose (^{18}F FDG), O-(2- ^{18}F fluoroethyl)-L-tyrosine (^{18}F FET). GLUT = glucose transporter, AAT = amino acid transporter, GPCR = G protein-coupled receptors, SST = somatostatin, mAb = monoclonal antibody, PSMA = prostate-specific membrane antigen, FAP = fibroblast activation protein, PS = protein synthesis [35]

Other targets are based on the increased metabolism and requirement of substances and building blocks for the high proliferation rate of tumor cells. Radiotracers as analogs of DNA and RNA building blocks are compounds such as 5-[^{18}F]fluorouracil, [^{11}C]thymidine, or 3'-deoxy-3'-[^{18}F]fluorothymidine, which allow determination of the proliferation status of tumor cells by PET imaging.

Another important mechanism for oncological targeting is enhanced amino acid transporter activity by tumor cells. *O*-(2-[^{18}F]fluoroethyl)-L-tyrosine ([^{18}F]FET) and [^{11}C -methyl]-L-methionine are the most commonly used radiolabeled amino acid analogs. In the context of theranostics, iodinated analogs are another approach.

However, it appeared that the transmembrane receptors represent the most attractive target in radiotheranostics. Typically, the corresponding targeting vectors are radiometal-labeled tracers, which is in particular relevant for their therapeutic application. For diagnostic analogs, also ^{18}F -labeled modifications are being applied.

3.2 Radionuclides

The turn of the millennium saw a dramatic change in the theranostic isotopes utilized in the 1990s: The approach to produce carrier free ^{177}Lu and the appearance of the $^{68}\text{Ge}/^{68}\text{Ga}$ -radionuclide generator.

Although ^{90}Y was and still is an extremely important therapeutic isotope in theranostics and nuclear medicine in general, ^{177}Lu ($t_{1/2} = 6.65\text{ d}$) became relevant because of the lower energy of the emitted β^- particles. The $^{176}\text{Lu}(\text{n},\gamma)$ production pathway was easily established and gives good yields due to the high cross section, yet the content of ^{176}Lu carrier and the contamination with the long-lived isomer $^{177\text{m}}\text{Lu}$ appeared to be critical for theranostics targeting e.g. saturable oncological targets such as GPC receptors. The indirect $^{176}\text{Yb}(\text{n},\gamma)^{177}\text{Yb} \xrightarrow{\beta^-} ^{177}\text{Lu}$ production pathway suggested an alternative option [36]. It is now commercially realized at TBq scale and GMP levels [37].

With the start of the new millennium, the Obninsk team provided a $^{68}\text{Ge}/^{68}\text{Ga}$ -radionuclide generator providing the positron-emitting trivalent radiometal ^{68}Ga ($t_{1/2} = 68\text{ min}$) in a chemical environment easily to label radiopharmaceuticals by its diluted hydrochloric acid eluent system and its low contamination levels of long-lived parent isotope ^{68}Ge ($t_{1/2} = 271\text{ d}$) [38]. The post-processing chemistry introduced by ZHERNOSEKOV et al. [39] and ASTI et al. [40] translated this generator to nuclear medicine. This was crucial to the increasing success of ^{68}Ga and its routine applications in PET diagnosis. This has been discussed in several reviews in detail, e.g. by Rösch and Riss [41] as well as Rösch and Baum [42], or different reports and book chapters [43, 44].

4. The 2000s: ^{177}Lu / ^{68}Ga and theranostic octreotides for NET

A look at the surface proteins expressed by tumor cells reveals an astonishing number and variety of potential targets. The metabolism and other cellular functions of tumor cells appear to be completely unregulated. As a result, the expression of surface proteins is dramatically increased on tumor cells, which can easily reach a million-fold overexpression compared to normal cells. Moreover, several tumor entities (over)express (specific) transmembrane proteins that are hardly or only very restrictedly expressed in healthy tissues. Transmembrane receptors are large protein structures located in the cell membrane. These receptors have three main domains: the transmembrane, extracellular, and intracellular domains. The extracellular domain is the part of the cell surface that is recognized by specific ligands. In contrast, the intracellular domain is the part that generates a signal inside the cell by activating (effector) proteins or enzymes.

One prominent family is the G protein-coupled receptors (GPCRs), in which the intracellular domain is coupled to the so-called G protein, which is responsible for further signal transduction in the cell. Some GPCRs undergo endocytosis after ligand binding, which is a major advantage because it effectively traps the radiotracer inside the tumor cell.

Clearly, these surface structures are generally potential targets for tumor diagnosis and therapy. The most important transmembrane receptor class of the last decade in nuclear medicine is the human somatostatin receptor (hSSTR) family. Out of the five subtypes, hSSTR2 is the most important subtype, and to a much lesser extent hSSTR1, 3, and 5. hSSTRs are highly overexpressed in neuroendocrine tumors and some other human tumor types. Expression of hSSTR2 on tumor cells can reach 2.5-million-fold overexpression. Physiological expression of hSSTR2 in healthy tissues is very limited and is mainly found in the spleen. Therefore, hSSTR2 offers ideal properties as a target for tumor imaging (and therapy). In addition, the hSSTR family undergoes endocytosis after ligand binding, resulting in very efficient trapping of the ligand in the addressed cell.

The native ligand, somatostatin, is a peptide hormone of 14 or 28 amino acids (two isoforms). Somatostatin has a plasma half-life of only 2 minutes and is therefore unsuitable for molecular imaging. The truncated derivative octreotide, a much smaller cyclic peptide, has been developed as a somatostatin analog for the treatment of acromegaly and for the treatment of neuroendocrine tumors. In an initial approach, octreotide was conjugated to DTPA as a chelating system for radiolabeling with indium-111. ^{111}In -labeled DTPA-octreotide is available as Octreoscan® for SPECT imaging [45, 46]. Further developments led to the corresponding derivative, [DOTA^o-D-Phe¹-Tyr³]-octreotide (DOTA-TOC), with the chelating agent DOTA for radiolabeling with various radiometals and a strongly enhanced affinity for hSSTR2 [47–50]. The theranostic pair $^{86}\text{Y}/^{90}\text{Y}$ was used for research and development as described in the previous chapter. [29–31, 34] Later

on, for clinical routine application the theranostic pair was changed to $^{68}\text{Ga}/^{177}\text{Lu}$ due to the better availability of these radionuclides. Together with another variant, DOTA-TATE, DOTA-TOC is the major precursor for radiolabeled somatostatin analogs [51]. ^{68}Ga -DOTA-TOC/TATE are the most commonly used tracers in routine clinical practice for PET imaging of hSSTR2-positive tumors and metastases (cf. Fig. 7).

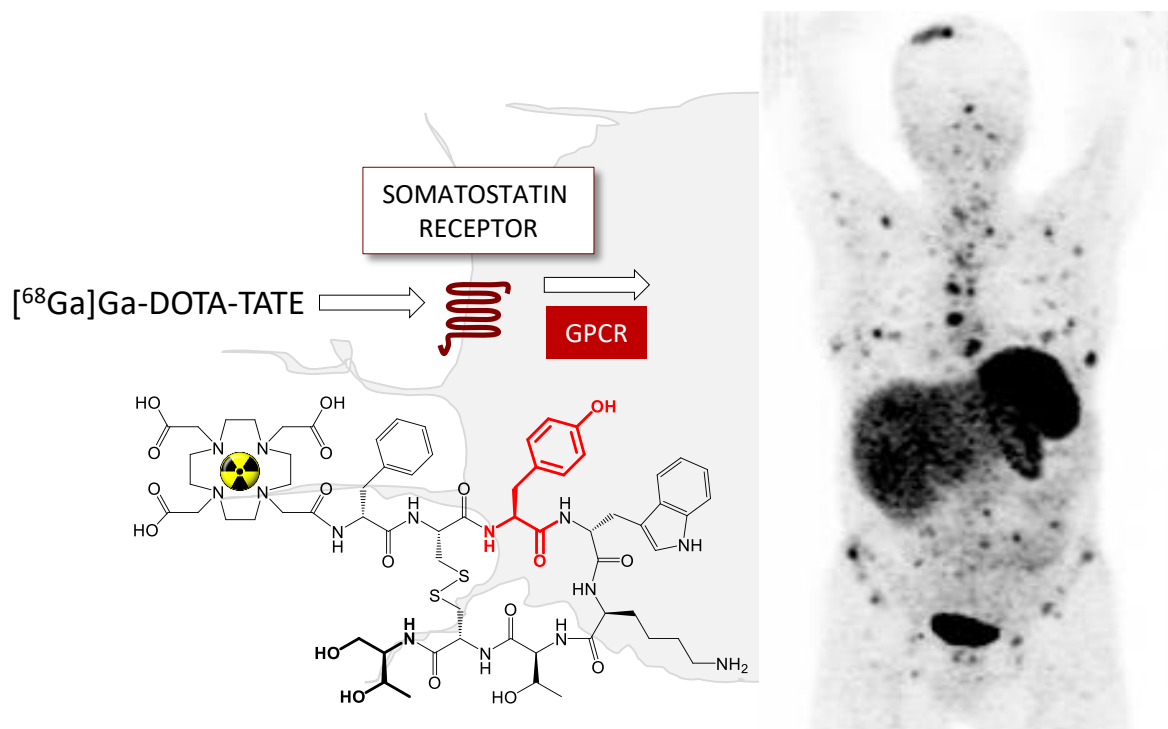


Fig. 7 PET imaging with ^{68}Ga -DOTA-TATE in patients suffering from neuroendocrine cancer [35]

Lessons to learn for the design of theranostic monomers:

The key component is the system oncological target / specific targeting vector. The chelator is covalently attached either directly to the targeting vector (amid coupling is the preferred method usually utilizing a -COOH group at the chelator and an amin at the targeting vector).

A first lesson to learn was that for therapeutic application macrocyclic chelators are much better suited because of the thermodynamic and kinetic stability of the chelates, cf. ^{111}In -labeled DTPA-octreotide for SPECT imaging vs. ^{90}Y -, ^{177}Lu or ^{225}Ac -labeled DOTA-TOC/-TATE therapeutics [51]. Pioneering work by H. Mäcke and others revealed, that the chelate is influencing the pharmacology of the theranostic compound [52, 53]. Accordingly, careful tailoring of the chelator and the radiometal belong to the challenges in radiopharmaceutical chemistry. Fig. 8 depicts some of the non-macrocyclic and cyclic chelators and some bifunctional derivatives introduced around the turn of the millennium.

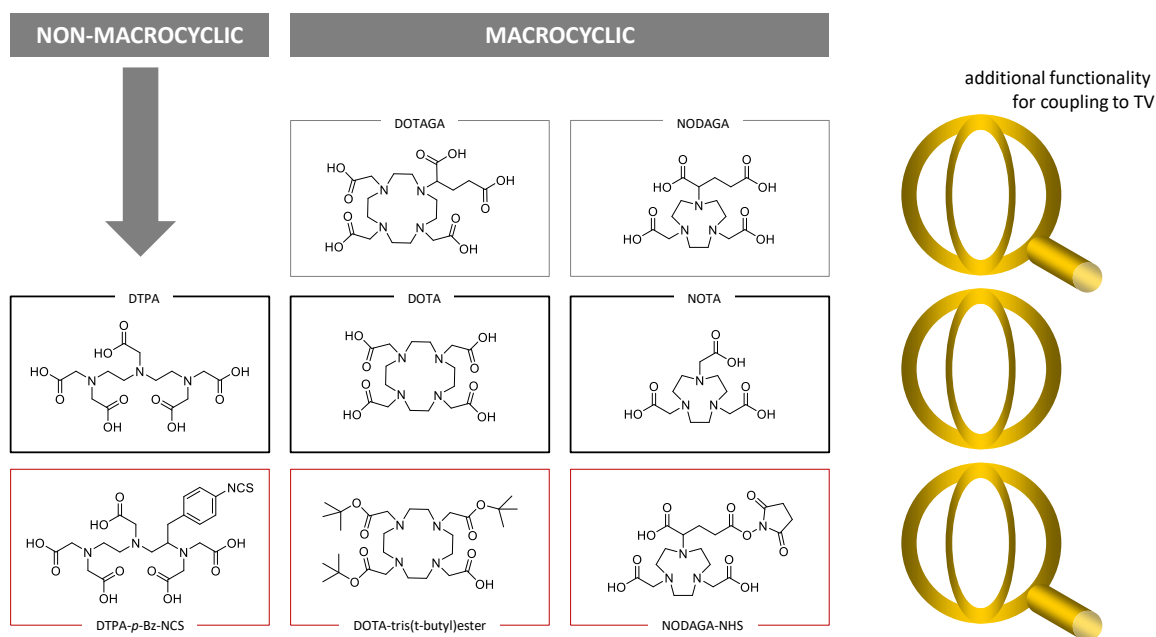


Fig. 8 Some of the non-macrocytic and cyclic chelators and some bifunctional derivatives introduced around the turn of the millennium.

The proof-of-principle peptidic radiometal theranostics: ^{90}Y -, ^{177}Lu -, ^{225}Ac -labeled DOTA-TOC/-TATE to treat neuroendocrine tumors: ^{90}Y -DOTA-TOC therapies of neuroendocrine started in the last years of the last millennium, and systematic experiences were obtained in the 2000s [47, 48, 54–56]. Fig. 9 gives a representative illustration of such applications. Obviously, the theranostic concept became a great success, and many patients benefited from this treatment. However, only decades later, this was validated by a systematic and successful clinical phase III study [57].

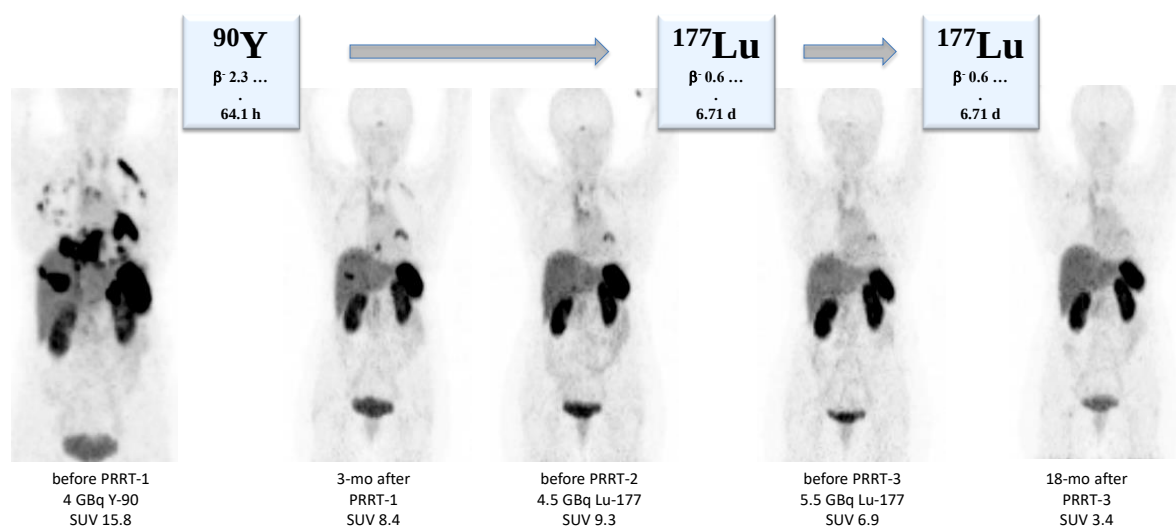


Fig. 9 ^{90}Y -DOTA-TOC therapies of neuroendocrine cancer; PRRT = peptide receptor radiation therapy. ^{68}Ga -DOTA-TOC PET images prior (left) treatment and after / between successive applications of first ^{90}Y -DOTA-TOC followed by two cycles of ^{177}Lu -DOTA-TOC (Courtesy by R. P. Baum) [58]

5. The 2010s: more oncological vectors

In addition to the key/lock systems SSTR/octreotide derivatives for neuroendocrine tumors and PSMA/PSMA inhibitors for prostate cancer (PCa), there are other examples that have been validated for the diagnosis of other cancers in nuclear medicine. The chemical design is exactly the one of option D in Fig. 1. The key component is the system oncological target / specific targeting vector, while the chelator remains a common chelate.

5.1 PSMA / PSMA inhibitor

The prostate-specific membrane antigen (PSMA) has been identified as a suitable target for prostate cancer diagnosis and therapy. The corresponding binding motif is a PSMA inhibitor. Over time the very small peptidomimetic structure of Glu-urea-Lys (KuE) has established itself as the by far most commonly used PSMA-targeting vector. Several radiolabeled derivatives have been developed for molecular imaging with ^{18}F , ^{68}Ga and other radionuclides. The most successful and clinically used diagnostic ligand is the ^{68}Ga -labeled ligand from DKFZ Heidelberg, which uses an HBED chelate: PSMA-11 ((EuK)-Ahx-HBED-CC, see Fig. 11A) [59, 60]. A comparative study with [^{18}F]fluoromethylcholine showed a much higher contrast which enabled the visualization of small metastases despite low PSA levels [61]. Fig. 10 shows PET imaging of prostate cancer with [^{68}Ga]Ga-PSMA-11.

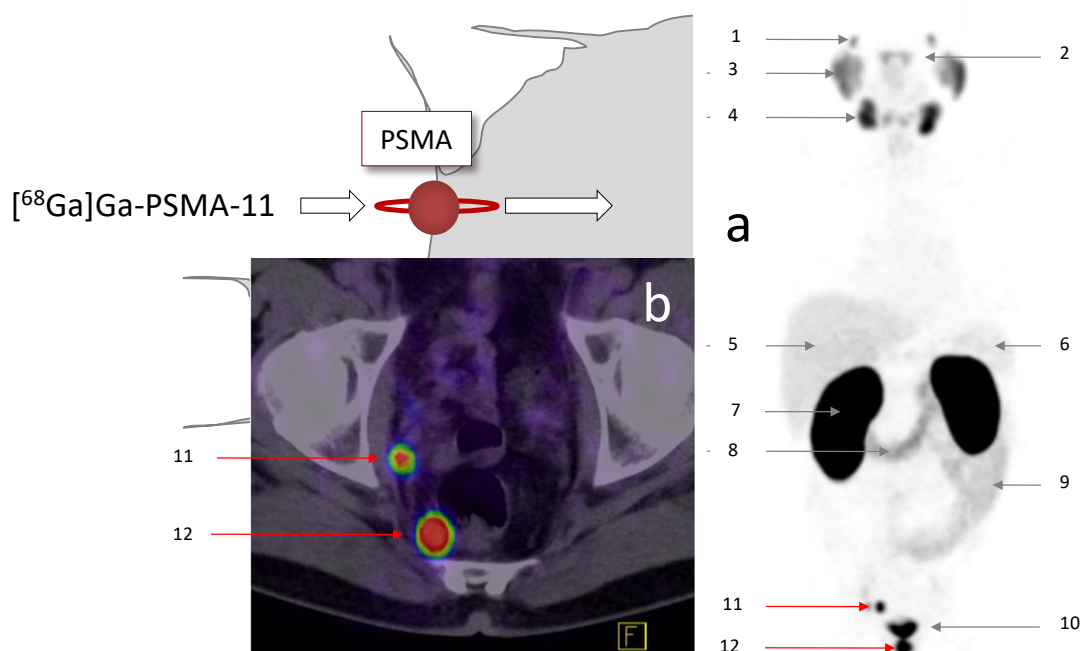


Fig. 10 [^{68}Ga]Ga-PSMA-11 PET imaging in patients suffering from prostate cancer [58]. (a) Maximum Intensity Projection (MIP) of a PET/CT at 1 h p.i. (b) Fusion PET/CT image. The primary prostate cancer tumor (12) as well as the lymph node metastases (11) can be visualized with a very high contrast (Courtesy of Afshar-Oromieh, DKFZ, Heidelberg, Germany) [35].

Fig. 11 shows three of the most common KuE-based radiopharmaceuticals routinely used for the diagnosis of prostate cancer by PET/CT.

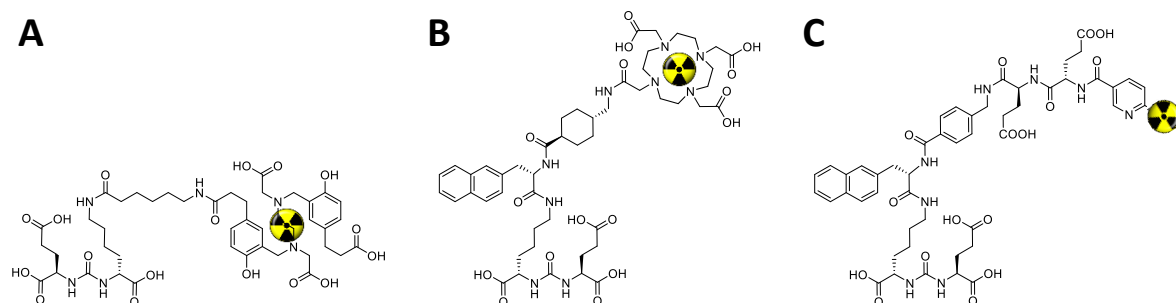


Fig. 11 Representative theranostic PSMA inhibitors: PSMA-11 labeled with ^{68}Ga (A), PSMA-617 labeled with ^{68}Ga , ^{177}Lu , ^{225}Ac and others (B), PSMA-1007 labeled with ^{18}F (C).

PSMA-617 (Fig. 11 B) has a DOTA chelator which enables theranostic application in contrast to PSMA-11 which can only be labeled with ^{68}Ga for diagnostic PET. Unlike with ^{177}Lu]Lu-DOTA-TATE, where over 15 years have passed between the first application and the approval [51], the approval process ^{177}Lu]Lu-PSMA-617 is going a lot faster. Between the original unleashing in 2015 by BENESOVA et al. [62] and the start of the phase III clinical VISION trial in 2018 only 3 years and only 7 years until its recent approval passed [63]. Several big studies were published quickly in the early years which helped to accelerate the process [64, 65].

Besides that, efforts were made to establish ^{18}F -labeled PSMA-inhibitors where ^{18}F]F-DCFPyL was investigated heavily which lead to two phase III studies that resulted in a recent FDA approval [66, 67]. ^{18}F]F-PSMA-1007 (Fig. 11 C) has also increasingly found its way into clinical routine over the last few years [68–70].

5.2 Hydroxyapatite in bone metastases / Bisphosphonates

Malignant tumors of the prostate, breast and other organs often lead to the formation of painful bone metastases in advanced stages. The disease is related to the activity of osteoblasts and osteoclasts, which are the active components of bone growth. The inorganic bone matrix itself consists mainly of calcium hydroxyapatite. Conventional drugs and diagnostic and therapeutic radiopharmaceuticals all share a high binding affinity to the inorganic matrix and/or the enzymes that regulate bone growth. Drugs for the treatment of osteoporosis are often used as bisphosphonate components. Bisphosphonates resemble pyrophosphate but are metabolically stable due to the replacement of the central oxygen atom with a carbon atom. In addition, this

carbon atom allows the attachment of two functional groups. By using a hydroxy group as a substituent, the affinity for hydroxyapatite can be increased.

Utilizing bisphosphonates as a targeting vector, a series of theranostic tracers were developed with high binding potency to bone metastases. The first generation of bisphosphonates were the DOTA-conjugated precursors BPAMD, BPAPD and BPPED that were investigated as ^{68}Ga -labeled derivatives in PET diagnosis [7–9]. Further the precursor were labeled with the therapeutic nuclides ^{177}Lu - and ^{225}Ac and have been successfully applied in patient studies [11, 71–73]. Fig. 12 shows the structure of BPAMD and its PET- and ERT-application *in vivo*.

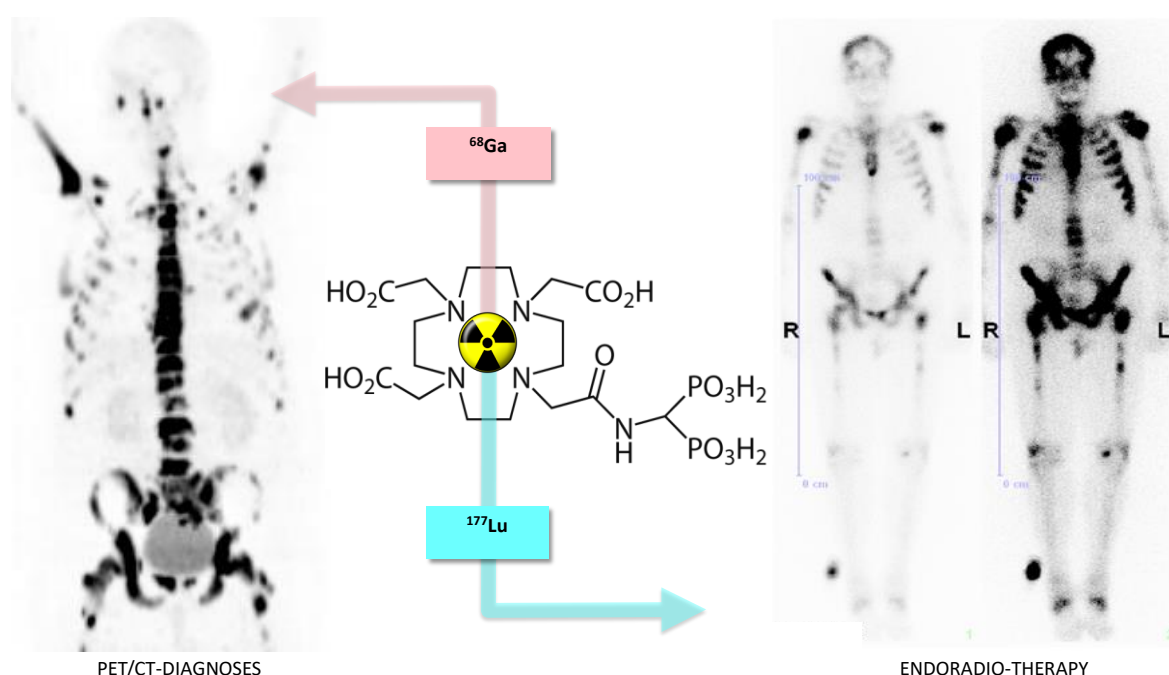


Fig. 12 BPAMD, a DOTA-conjugated first-generation bisphosphonate labeled with ^{68}Ga or ^{177}Lu and its use in theranostics of disseminated bone metastases [35]

5.3 CAFs / FAP inhibitors

In contrast to specific molecular tumor targets such as the somatostatin receptor in neuroendocrine tumors and the prostate-specific membrane antigen in prostate cancer, the tumor microenvironment has recently been identified as a suitable target for cancer diagnosis and therapy. Cancer is a heterogeneous disease that arises in an extremely complex microenvironment. Malignant tumors are composed not only of cancer cells but also of a large majority of endogenous host stromal cells (e.g., fibroblasts, vascular and immune cells) and extracellular matrix (ECM) components, collectively referred to as the tumor microenvironment (TME) [74]. The stromal cells within the tumor (tumor stroma) comprise the majority of the total tumor mass (up to 90%) and are connected by a desmoplastic reaction [75]. Among all cells within

the TME matrix, fibroblasts are considered dominant cells whose biological functions play a strong role in all stages of cancer progression and metastasis. Cancer-associated fibroblasts (CAFs) are thought to have potent tumor-modulating effects and are found in most solid tumors. In general, CAFs account for up to 80% of all fibroblasts in TME. CAFs are identified by the expression of several specific biomarkers on their surface, such as SMA (Smooth Muscle Actin), S100A4 or FSP-1 (Fibroblast-Specific Protein 1), PDGFR/ (Platelet-Derived Growth Factor Receptors), and FAP (Fibroblast Activation Protein). Fibroblast Activation Protein alpha (FAP α or FAP), also known as Seprase, is a type II membrane-bound serine protease associated with fibrosis, tissue repair, inflammation, and ECM degradation. FAP contains two types of enzymatic activity: dipeptidyl peptidase and endopeptidase. Endopeptidase enables FAP to mediate proteolytic processing of collagen I cleaved by matrix metalloproteinase, leading to prevention of morphogenesis, tissue remodeling and repair [76–78].

FAP appears to be a promising target in oncology because it is not expressed in normal fibroblasts and in the stroma of benign epithelial tumors, whereas it is significantly increased in the stromal compartments of various malignant tumors. The challenge for radiopharmaceutical chemistry is to develop molecular targeting vectors with high affinity for FAP and high selectivity toward related proteases. Recently, molecular antibodies, small peptides, and inhibitors have been transformed into molecular imaging probes, with inhibitors utilizing the (4-quinolinoyl)-glycyl-2-cyanopyrrolidine scaffold representing the most exciting class [79, 80]. The inhibitor structure UAMC1110 showed nanomolar affinity and the best selectivity towards the concurring enzymes prolyl endopeptidase (PREP) and dipeptidyl peptidases DPPIV, DPPVIII and DPPIX [80].

Radiopharmaceutical chemists at the German Cancer Research Center in Heidelberg pioneered the translation of this inhibitor into effective diagnostic radiopharmaceuticals by coupling chelators to the amine of the quinoline moiety of the inhibitor and introducing a series of linker and spacer motifs [81, 82]. At the University in Mainz (Germany) a linker based on squaric acid (SA), which links several bifunctional chelators (DATA^{5m}, DOTA, etc.) to the inhibitor was used [83, 84]. Currently, ⁶⁸Ga-labeled derivatives such as FAPI-04, FAPI-46, DATA^{5m}.SA.FAPi and DOTA.SA.FAPi (cf. Fig. 13) are being investigated *in vivo*.

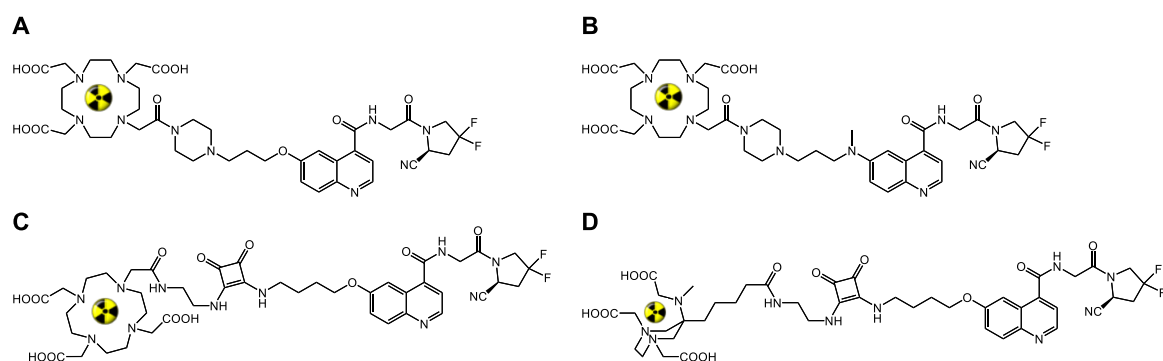


Fig. 13 Representative FAP inhibitor-based precursor for potential theranostic application: FAPI-04 (A), FAPI-46 (B), DOTA.SA.FAPi (C), DATA^{5m}.SA.FAPi (D).

These first generation of FAPi-radiotracers show promising value in the diagnosis of many different tumor types and provide new information, also in comparison with established oncologic tracers such as [¹⁸F]FDG [85, 86]. Fig. 14 shows an exemplary PET image of a thyroid cancer patient visualized with [⁶⁸Ga]Ga-DOTA.SA.FAPi.

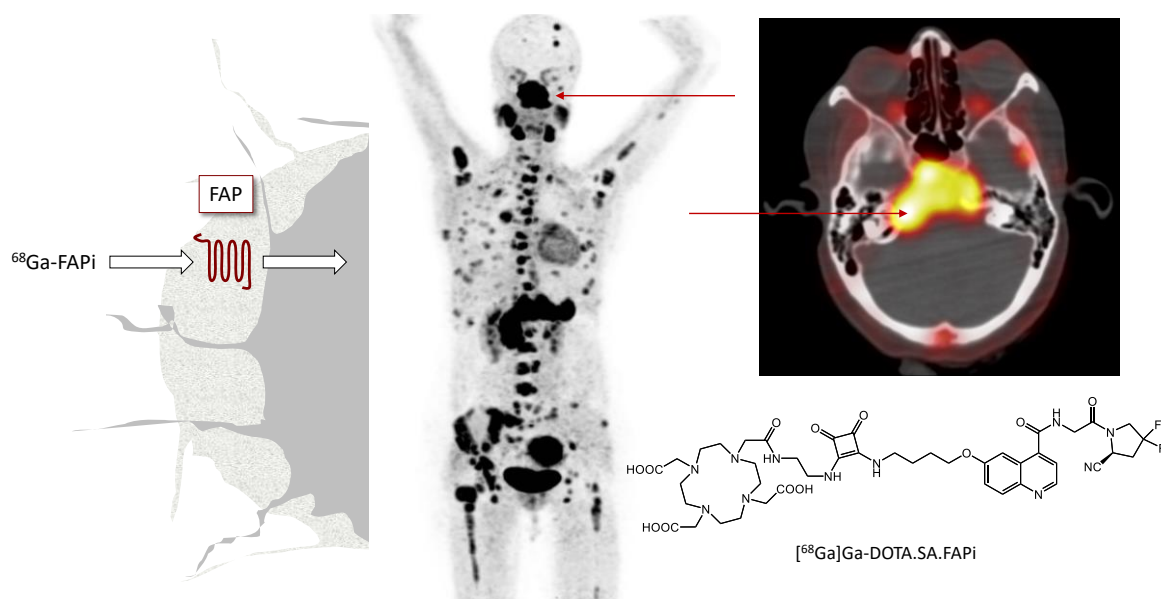


Fig. 14 [⁶⁸Ga]Ga-DOTA.SA.FAPi PET imaging in patients suffering from thyroid cancer (Courtesy by C. S. Bal)

6. Outlook: More oncological vectors and new radioisotopes

6.1 Radioisotopes

In radiotherapy, it is the desired action of particle-induced ionization of α or β^- emitters on tumor DNA. It requires the identification of the most successful radionuclide candidates which offer adequate nuclear parameters, specific activities and effective production routes including yield, purity, and cost. Routine clinical availability is the key issue. As of today, the number of such therapeutic isotopes is relatively low, yet important carrier-free β^- emitters such as ^{90}Y and ^{177}Lu are available even at a GMP level. Availability of ^{225}Ac , in contrast, is limited.

However, there is a substantial risk that the supply chain of those radionuclides cannot satisfy the demand of e.g. radiolabeled PSMA inhibitors. The growing number of registered theranostic radiopharmaceuticals and their dramatically growing demand even for the well-established radionuclides ask for upscaling production capacities. Interestingly, the use of nuclear reactors dedicated to nuclear energy also for isotope production is a fascinating avenue. In parallel, alternative production routes such as the potential of photonuclear reactions are being investigated – asking for dedicated facilities. Finally, research and development towards new radionuclides must continue – with ^{161}Tb representing an important example (cf. Fig. 15). Other candidates are still pending routine commercial availability on larger activities such as ^{211}At or ^{212}Pb .

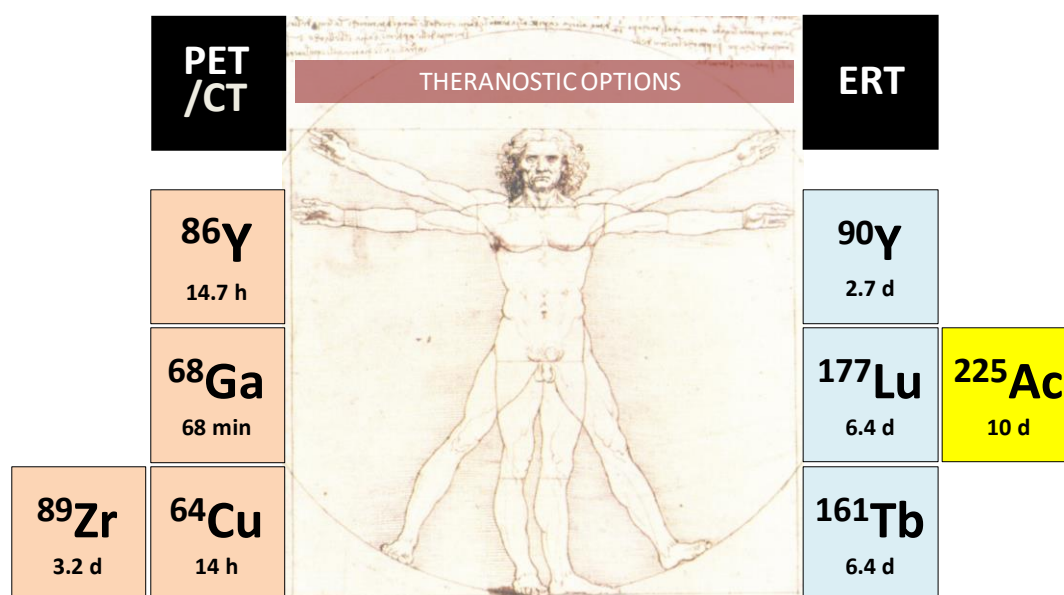


Fig. 15 Selected positron emitters (left) and α or β^- emitters (right) with established theranostic application with ^{161}Tb being about to enter the clinics.

6.2 New oncological targets and new design of targeting vectors

The dominant systems of target / targeting vectors are Somatostatin receptors (NET patients) / octreotides and PSMA (prostate cancer patients) / PSMA inhibitors – both quantified in successful clinical phase III trials and being registered drugs. In addition, in the last decade new systems have been translated from bench to bedside such as the TME / FAP inhibitors or peptides as mentioned, as well as the inorganic vectors for the system Hydroxyapatite (bone metastases) / bisphosphonates. Yet there are many more examples, just to mention bombesin-based targeting vectors targeting Gastrin-releasing protein receptor (GRPR) also known as bombesin type-2 receptor for use in prostate cancer [87, 88], the chemokine receptor CXCR4 ligands [89] and RGD-containing cyclic peptides targeting $\alpha_v\beta_3$ and other integrins [90–92] for different types of cancer, new SSTR antagonists for NET [93–95] etc. This is an ongoing progress.

However, there are also some new developments in radiopharmaceutical chemistry. One example to mention is the creation of homo- and hetero-multimers. The principal designs are illustrated in Fig. 16 with a bifunctional chelator as the central unit which has at least two functional groups for the coupling with the linker and the targeting vector. Furthermore, additional spacer units may be required.

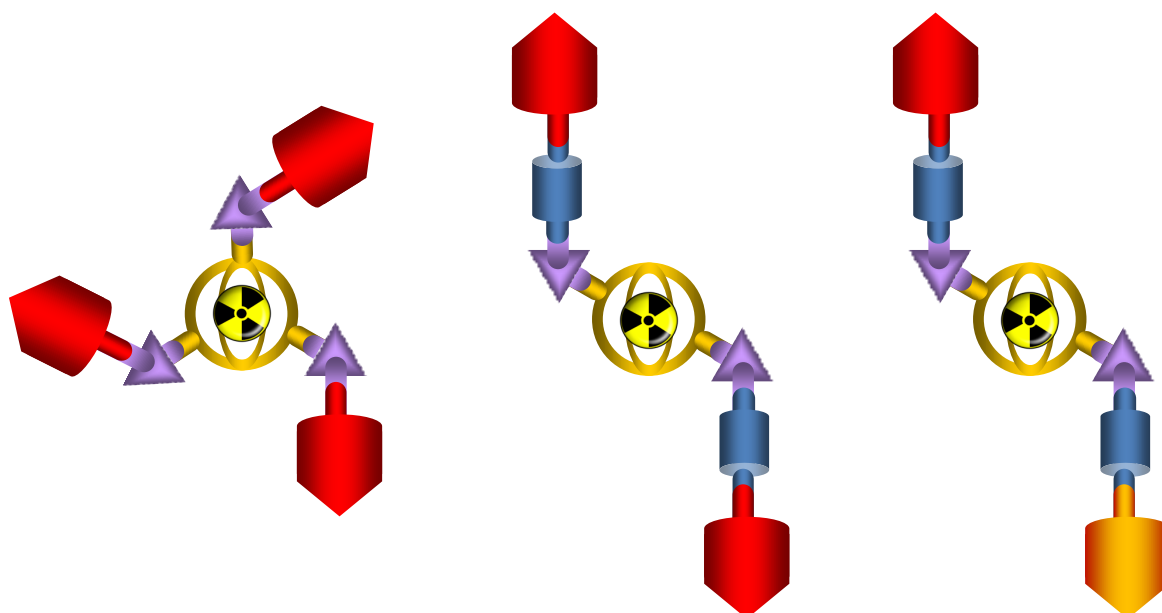


Fig. 16 Schematic designs of chelator-based multimeric radiopharmaceuticals

The effect of multivalency on affinity, avidity and selectivity of receptor-ligand interactions is known for several decades [96, 97]. Based on this, multimeric designs found its way into radiopharmaceuticals in the 2000s mainly targeting the integrin $\alpha_v\beta_3$ with RGD-containing cyclic

peptides such as c[RGDfK] as targeting vectors [98–100] but also with aptamers targeting MUC₁ [101]. A ⁶⁴Cu-labeled RGD-tetramer (⁶⁴Cu-DOTA-E{E[c(RDGfK)]₂}) showed first promising results indicating a prolonged tumor retention time while tumor uptake and blood clearance were rapid [100].

NOTNI et al. developed a new bifunctional chelator TRAP (triazacyclononane-triphosphinate) which showed superior ⁶⁸Ga-labeling properties compared to DOTA and NOTA (milder reaction conditions, broader pH range during labeling, higher specific activities while retaining high complex stability) and simultaneously allowed the conjugation of three targeting vectors [102]. A following study with ⁶⁸Ga-TRAP(RGD)₃ ("⁶⁸Ga-Avebtrin", Fig. 17) could demonstrate the superiority of the RGD trimer compared to RGD monomers [103].

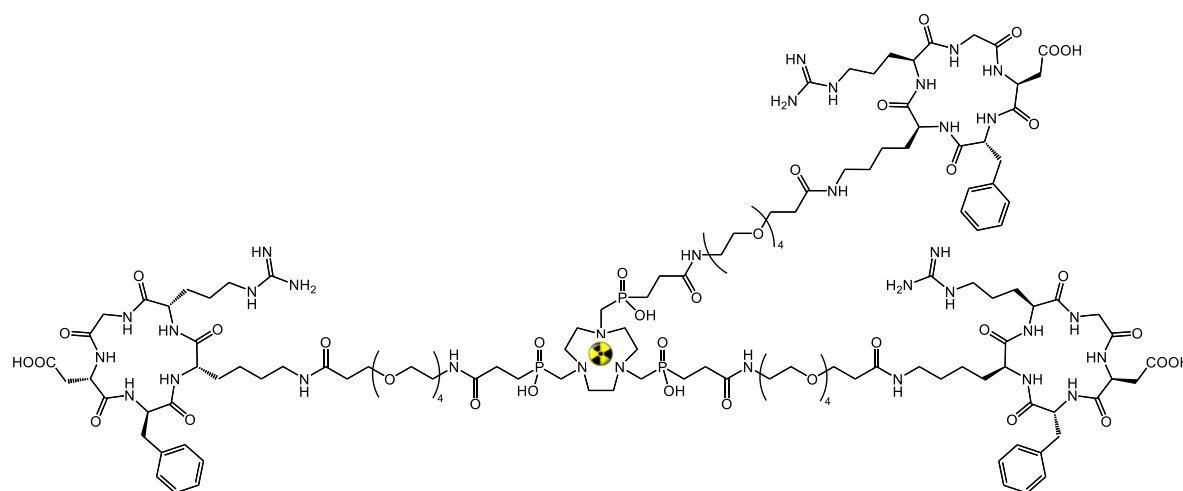


Fig. 17 Structure of TRAP(RGD)₃ which can be labeled with ⁶⁸Ga [103]

The concept was expanded to tetraazacyclododecane scaffold which resulted in the chelator DOTPI [104]. This allowed for the synthesis of tetramers and the radiolabeling with a broader range of radiometals such as ¹⁷⁷Lu which enables therapeutic applications. As a proof-of-concept DOTPI was conjugated with the PSMA-targeting vector KuE via Cu^I-catalyzed alkyne-azide cycloaddition (CuAAC, Huisgen-reaction) and copper-free strain-promoted alkyne-azide cycloaddition (SPAAC) with DBCO (dibenzoylcyclooctyne) and investigated [105].

The chelator sarcophagine (Sar) is especially suited to form stable and kinetically inert complexes with Cu^{II}. Fig. 18A shows the PSMA dimer SarbisPSMA which was labeled with ⁶⁴Cu. *In vivo*-investigation in comparison to the monomer SarPSMA showed far superior tumor uptake, tumor-to-background ratio and longer tumor retention of the dimer [106].

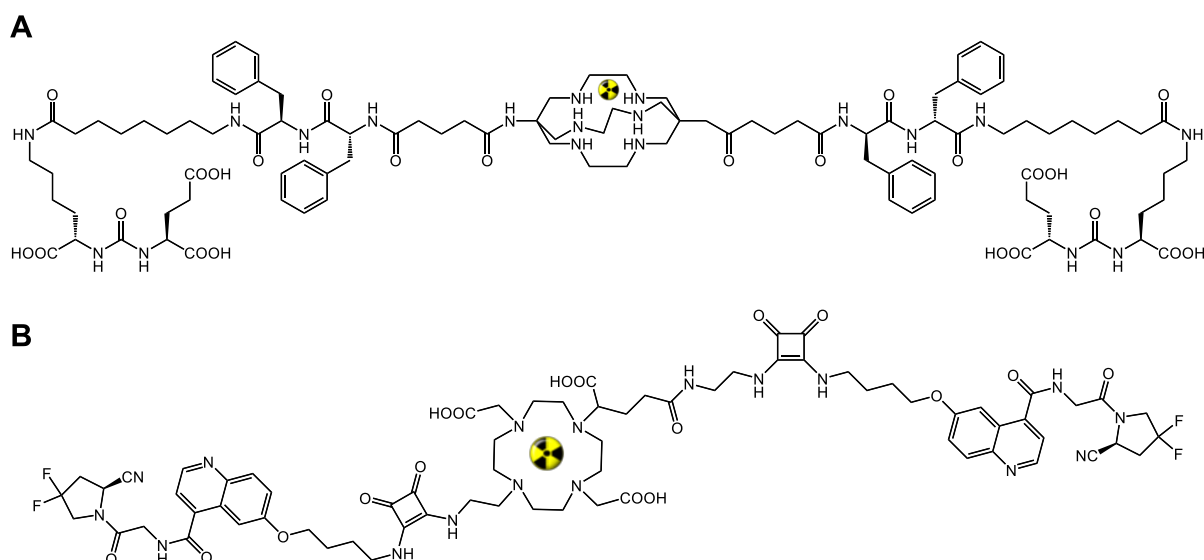


Fig. 18 Structure of SarbisPSMA (A) and DOTAGA.(SA.FAPI)₂ (B) [106, 107]

Besides PSMA also Gastrin-releasing peptide receptors (GRPRs) are interesting targets and strongly expressed in several types of cancer such as prostate cancer which can be targeted with bombesin (BBN)-derived peptides. Here, also several attempts were made to investigate homodimeric bombesin radiotracers [88, 108, 109].

Recently the dimeric concept was translated to FAP inhibitor-based radiopharmaceuticals since the first-generation of FAPIs like FAPI-04 and FAPI-46 and other monomers (cf. Fig. 13) suffer from short tumor retention time limiting its translation towards therapy using radionuclides with longer half-lives such as ¹⁷⁷Lu or ²²⁵Ac. Fig. 18B shows the structure of DOTAGA.(SA.FAPI)₂ which was labeled with ⁶⁸Ga and ¹⁷⁷Lu [107]. In comparison with [¹⁷⁷Lu]Lu-DOTA.SA.FAPI, [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPI)₂ showed higher tumor uptake, faster clearance and longer tumor retention time. Even after 7 days p.i. the tumor lesions could still be visualized via whole-body scintigraphy [110]. The slightly higher uptakes in colon and kidneys were tolerated well by the patients. Other groups also published FAPI dimers such as [¹⁷⁷Lu]Lu-BiOncoFAP [111] and [⁶⁸Ga]Ga-DOTA-2P(FAPI)₂ [112] recently and showed similar results in murine *in vivo* studies regarding the biodistribution and pharmacokinetics.

Heterodimers also appeared in recent years having a similar molecular design than the homodimers but with two different targeting vectors. Most of the combinations try to simultaneously target GRPRs and integrins with a bombesin derivative and a cyclic RGD peptide [113–115]. PSMA-BBN [116, 117] and recently the first PSMA-FAP heterodimer [118] have also been published. Some PCa patients are PSMA-negative so they do not overexpress PSMA. This means that cannot be visualized via a monomeric PSMA radiotracer. A heterodimer could

therefore be interesting to improve the rate of successful diagnosis or therapy. It has also been reported that the enzymes are both overexpressed in different malignant tumors at the same time [119, 120].

Besides that, a combination of one of an established oncological targeting vector and a human serum albumin (HSA) binding moiety emerged in radiopharmaceuticals. In most cases 4-(*p*-iodophenyl)butyric acid or Evans Blue (EB) are used as a HSA binding moiety [121]. These moieties bind reversibly to albumin which results in a prolonged circulation in the blood and therefore a delayed renal clearance. This results in a prolonged tumor retention also which is why a higher accumulated radiation dose can be deposited in the tumor region. This approach has been investigated for SSTR and PSMA in humans. This could be confirmed e.g. with ^{177}Lu -EB-TATE and ^{177}Lu -EB-PSMA-617 with a 7.9- and 5.7-fold higher dose to the tumor compared to the monomeric derivatives, respectively [121–123]. But the drawbacks were also observed since the radiation dose is increased in healthy tissue as well which can result in overall lower tumor-to-background ratios. Much higher uptake was also observed in the kidneys (3.2- and 6-fold) and especially the bone marrow (18.2- and 6-fold) which can be critical. Even when lower initial activities are needed to treat a patient (particularly interesting for ^{225}Ac) the uptake in healthy tissue increases roughly in the same order of magnitude than in the tumor [121–123].

Conclusion

Since the 1990s huge progress was made in radiometal-based theranostics in every field concerning radiopharmaceuticals. This includes new methods to produce radionuclides (improved production routes of established radionuclides as well as making new radionuclides available for routine use), improved labeling chemistry (acyclic vs. macrocyclic chelators), new oncologic targets (SSTR, PSMA, FAP, ...) combined with new targeting vectors (TOC/TATE, PSMA and FAP inhibitors, ...), sophisticated linker and spacer chemistry. The development in this field still is ongoing with new molecular designs (dimers and multimers with multiple targeting vectors) and most importantly the ever-increasing interaction of diagnosis and therapy.

Conflicts of interest: The authors declare no conflict of interest.

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3.2. Novel Generation of FAP Inhibitor-Based Homodimers for Improved Application in Radiotheranostics

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The following **Summary** and **Author Contributions** sections do not belong to the publication. The actual publication begins after that and was not altered in any way.

Summary

Fibroblast activation protein alpha (FAP) is highly expressed in the tumor microenvironment by cancer-associated fibroblasts (CAFs) in over 90% of epithelial carcinomas. Therefore, it is an ideal biological target for theranostic applications. Studies with radiopharmaceuticals based on the highly potent FAP inhibitor (FAPi) UAMC-1110 as a targeting vector have shown great potential in molecular imaging but the short tumor retention time of the monomers (one targeting vector) do not match the half-lives of therapeutic radionuclides lutetium-177, actinium-225 (and yttrium-90). This could be improved by a dimer (two targeting vectors), however pharmacokinetic and radiolabeling properties still need further optimization.

This study aimed to optimize the previously reported FAPi homodimer DOTAGA.(SA.FAPi)₂ from our group by synthesizing the novel homodimeric FAP inhibitor structures DO3A.Glu.(FAPi)₂ and DOTAGA.Glu.(FAPi)₂. Particular focus was directed to ²²⁵Ac-labeling to enable FAP-targeted alpha-particle therapy (FAP-TAT).

DO3A.Glu.(FAPi)₂ and DOTAGA.Glu.(FAPi)₂ were both synthesized and radiolabeled with gallium-68 and lutetium-177 at 95 °C in 1 M HEPES buffer (pH = 4.5 or 5.5). Both precursors were labeled successfully with gallium-68 (radiochemical conversion RCC > 95% after 5 min) and also with lutetium-177 (10 – 20 nmol/GBq) in quantitative RCC > 99%. ⁹⁰Y-labeling results were similar to ¹⁷⁷Lu-labeling. The log*D*_{7.4} values were determined via shake-flask method for the ⁶⁸Ga- and ¹⁷⁷Lu-complexes. Complex stabilities of the radiolabeled compounds in human serum (HS) and phosphate-buffered saline (PBS) were investigated at 37 °C over two half-lives. All radiolabeled products were stable over 2 half-lives in HS and PBS and the hydrophilicity was high (–log*D*_{7.4} = 1.77 – 2.08) for [⁶⁸Ga]Ga- and [¹⁷⁷Lu]Lu-DO3A.Glu.(FAPi)₂ and even higher for [⁶⁸Ga]Ga- and [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ (–log*D*_{7.4} = 2.48 – 2.77). The DO3A precursor was further labeled with yttrium-90 under the same conditions in 1 M HEPES buffer (pH = 4.5) and gave similar results than ¹⁷⁷Lu-labeling. The DOTAGA precursor was additionally radiolabeled with actinium-225 in 0.1 M sodium ascorbate (pH = 7.0) at 95 °C. For [²²⁵Ac]Ac-DOTAGA.Glu.(FAPi)₂ the labeling yield reached 90 – 95% after 15 min with 30 – 40 nmol/MBq. The final formulation was stable. The stability was also high in HS over 20 days and in PBS for at least 2 days.

In vitro affinities were evaluated by determining the *IC*₅₀ values of the precursor and the Lu-complex [^{nat}Lu]Lu-DOTAGA.Glu.(FAPi)₂ for FAP, prolyl endopeptidase (PREP) and the dipeptidyl peptidases (DPPs) DPP4, DPP8 and DPP9. *In vitro* studies showed very high subnanomolar affinity for FAP (<1 nM) and high selectivity over the related proteases (PREP and DPPs) for both compounds. Both compounds showed improved affinity and selectivity compared to first-generation homodimer DOTAGA.(SA.FAPi)₂ and especially DOTAGA.Glu.(FAPi)₂ showed very promising *in vitro* properties on top of the satisfactory radiolabeling properties and was further considered for subsequent application *in vivo*.

The first proof-of-concept study in a patient with medullary thyroid cancer was carried out. The patient was previously treated with [^{177}Lu]Lu-DOTAGA.(SA.FAPi)₂, where besides high uptake and long tumor retention, a high uptake in the liver (at 24 h p.i.) and colon (48 h p.i.) was observed. In contrast, the new compound [^{177}Lu]Lu-DOTAGA.Glu.(FAPi)₂ showed negligible liver and colon uptake at 24 h p.i. besides high tumor uptake.

To conclude, the novel FAPi dimer DOTAGA.Glu.(FAPi)₂ showed improved radiolabeling properties compared to the DO3A derivative and first-generation FAPi dimer DOTAGA.(SA.FAPi)₂. For both new compounds the *in vitro* affinity and selectivity were superior to those of DOTAGA.(SA.FAPi)₂ which made them promising candidates for improved theranostic application. A first proof-of-concept patient study indicates that [^{177}Lu]Lu-DOTAGA.Glu.(FAPi)₂ is an ideal candidate for *in vivo* application with superior pharmacological properties compared to DOTAGA.(SA.FAPi)₂ with a significantly reduced radiotracer uptake to the non-target and critical organs (liver, colon) while maintaining the long tumor retention. The improved ^{225}Ac -radiolabeling properties of DOTAGA.Glu.(FAPi)₂ compared to DOTAGA.(SA.FAPi)₂ also are promising results to translate [^{225}Ac]Ac-DOTAGA.Glu.(FAPi)₂ to the clinic for FAP-TAT in the near future.

Author Contributions

Conceptualization, planning, supervision and project administration was done by Frank Roesch, Chandrasekhar Bal, Ingrid De Meester and Pieter Van Der Veken and were supported by MARCEL MARTIN. MARCEL MARTIN carried out the organic synthesis and did all the radiochemical investigation, evaluation and validation including all synthesis, analysis and interpretation of the results. He was additionally responsible for translation of optimized radiolabeling protocol into the clinical routine together with Sanjana Ballal and Madhav Prasad Yadav. *In vitro* investigations were carried out and analyzed by Yentl Van Rymenant, Emile Verhulst and Joni De Loose who also did the interpretation of the *in vitro* results together with MARCEL MARTIN.

Clinical studies were carried out and the clinical data were analyzed by Sanjana Ballal and Madhav Prasad Yadav under the supervision of Chandrasekhar Bal. They were supported by MARCEL MARTIN in the interpretation of the results. MARCEL MARTIN wrote the original draft of the manuscript. Reviewing and revisions of the manuscript was done MARCEL MARTIN together with all co-authors.

Novel Generation of FAP Inhibitor-Based Homodimers for Improved Application in Radiotheranostics

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Simple Summary

Radiopharmaceuticals targeting the fibroblast activation protein alpha (FAP) can be used in many different cancer types since FAP is highly expressed in the tumor microenvironment of almost all epithelial cancers. Monomeric radiotracers have shown great potential in molecular imaging (diagnosis), but the tumor retention time is relatively short (few hours). For effective radioligand therapy (RLT), the biological half-life of the radiotracer should ideally match the physical half-life of the important therapeutic radionuclides ¹⁷⁷Lu and ²²⁵Ac (6.7 and 9.9 days). The tumor retention was improved with the FAPi homodimer DOTAGA.(SA.FAPi)₂. In terms of optimizing, the new FAPi homodimers DO3A.Glu.(FAPi)₂ and DOTAGA.Glu.(FAPi)₂ were synthesized. DOTAGA.Glu.(FAPi)₂ showed superior radiolabeling properties (including successful ²²⁵Ac-labeling, higher hydrophilicity), in vitro affinity and selectivity compared to DOTAGA.(SA.FAPi)₂. In addition, significantly reduced uptake in the critical organs (liver, colon) compared to [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂ was observed with [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ in a first patient study (medullary thyroid cancer) while maintaining high and prolonged tumor uptake.

Abstract

Radiopharmaceuticals based on the highly potent FAP inhibitor (FAPi) UAMC-1110 have shown great potential in molecular imaging, but the short tumor retention time of the monomers do not match the physical half-lives of the important therapeutic radionuclides ^{177}Lu and ^{225}Ac . This was improved with the dimer DOTAGA.(SA.FAPi)₂, but pharmacological and radiolabeling properties still need optimization. Therefore, the novel FAPi homodimers DO3A.Glu.(FAPi)₂ and DOTAGA.Glu.(FAPi)₂ were synthesized and quantitatively radiolabeled with ^{68}Ga , ^{90}Y , ^{177}Lu and ^{225}Ac . The radiolabeled complexes showed high hydrophilicity and were generally stable in human serum (HS) and phosphate-buffered saline (PBS) at 37 °C over two half-lives, except for [^{225}Ac]Ac-DOTAGA.Glu.(FAPi)₂ in PBS. In vitro affinity studies resulted in subnanomolar IC₅₀ values for FAP and high selectivity for FAP over the related proteases PREP and DPP4 for both compounds as well as for [^{nat}Lu]Lu-DOTAGA.Glu.(FAPi)₂. In a first proof-of-principle patient study (medullary thyroid cancer), [^{177}Lu]Lu-DOTAGA.Glu.(FAPi)₂ was compared to [^{177}Lu]Lu-DOTAGA.(SA.FAPi)₂. High uptake and long tumor retention was observed in both cases, but [^{177}Lu]Lu-DOTAGA.Glu.(FAPi)₂ significantly reduces uptake in non-target and critical organs (liver, colon). Overall, the novel FAPi homodimer DOTAGA.Glu.(FAPi)₂ showed improved radiolabeling in vitro and pharmacological properties in vivo compared to DOTAGA.(SA.FAPi)₂. [^{177}Lu]Lu-DOTAGA.Glu.(FAPi)₂ and [^{225}Ac]Ac-DOTAGA.Glu.(FAPi)₂ appear promising for translational application in patients.

Keywords

theranostics; radiometals; positron emission tomography (PET); radionuclide therapy (RNT); radioligand therapy (RLT); targeted alpha-particle therapy (TAT); fibroblast activation protein alpha (FAP); FAPi dimers; homodimeric FAPi-based radiopharmaceuticals; medullary thyroid cancer

1. Introduction

Fibroblast activation protein alpha (FAP), also called Seprase, is a S9B family serine protease and an integral type-II transmembrane glycoprotein [1]. It is mainly expressed on activated fibroblasts such as cancer-associated fibroblasts (CAFs), fibroblasts present during wound healing [2], at sites of inflammation (e.g., arthritis) [3] and fibrosis (e.g., liver cirrhosis) [4]. However, FAP is absent in resting fibroblasts of normal healthy tissue. CAFs behave as activated stromal fibroblasts and occupy a major part of the tumor microenvironment (TME) that further contains of vascular, inflammatory and immune cells as well as extracellular matrix components (ECM) [5]. The TME plays an essential role in tumorigenesis, tumor growth, metastasis and angiogenesis and can account for up to 90% of the total tumor mass [5–8]. Each epithelial tumor more than 1 mm in diameter is surrounded by an extensive TME. More than 90% of these epithelial tumors strongly express FAP, including prostate, breast, lung, ovarian and colorectal cancer, among others [6].

FAP is only enzymatically active as a dimer and has unique dual enzymatic activity, both dipeptidyl peptidase and prolyl endopeptidase, whereas the similar serine proteases prolyl endopeptidase (PREP) and other post-proline dipeptidyl peptidases (DPP4, DPP8 and DPP9) only have one of these activities [8–12]. Natural substrates have been identified including several neuropeptides (neuropeptide Y, peptide YY etc.) [13], α_2 -antiplasmin [4,14] and denatured type I collagen [15,16]. The actual *in vivo* role of FAP is not fully understood yet but seems to be non-essential in healthy tissue [8]. In contrast, FAP is considered to play an important role in fibroblast growth, tissue remodeling of the ECM, epithelial carcinogenesis, tumor growth (proliferation) and angiogenesis in malignant tissue [8,17–22].

Compared to other tumor targets that are vastly used in nuclear medicine such as prostate-specific membrane antigen (PSMA) or somatostatin receptor (SSTR), radiopharmaceuticals targeting FAP do not address the cancer cells directly but indirectly via its TME. PSMA inhibitor-based tracers can basically only be applied in prostate cancer and octreotide-based SSTR-targeting tracers for neuroendocrine tumors (NETs), while targeting FAP allows for a broader application in many different types of cancers, which makes it a more universal pan-tumor target. Jeremie Calais discussed FAP as potentially being “the next billion dollar nuclear theranostics target” following [^{177}Lu]Lu-PSMA-617 and [^{177}Lu]Lu-DOTA-TATE that has had a great impact in nuclear medicine in the last years [23].

The FAP inhibitor (FAPi) UAMC-1110 (Figure 1a) was the first small molecule that showed high affinity for FAP and high selectivity over PREP and the DPPs [24]. Since then, several FAPi-based radiopharmaceuticals have been synthesized [25–34]. Some of them have already been systematically investigated in human positron emission tomography (PET) studies such as FAPI-04, FAPI-46, and DOTA.SA.FAPi (Figure 1b) [35–40].

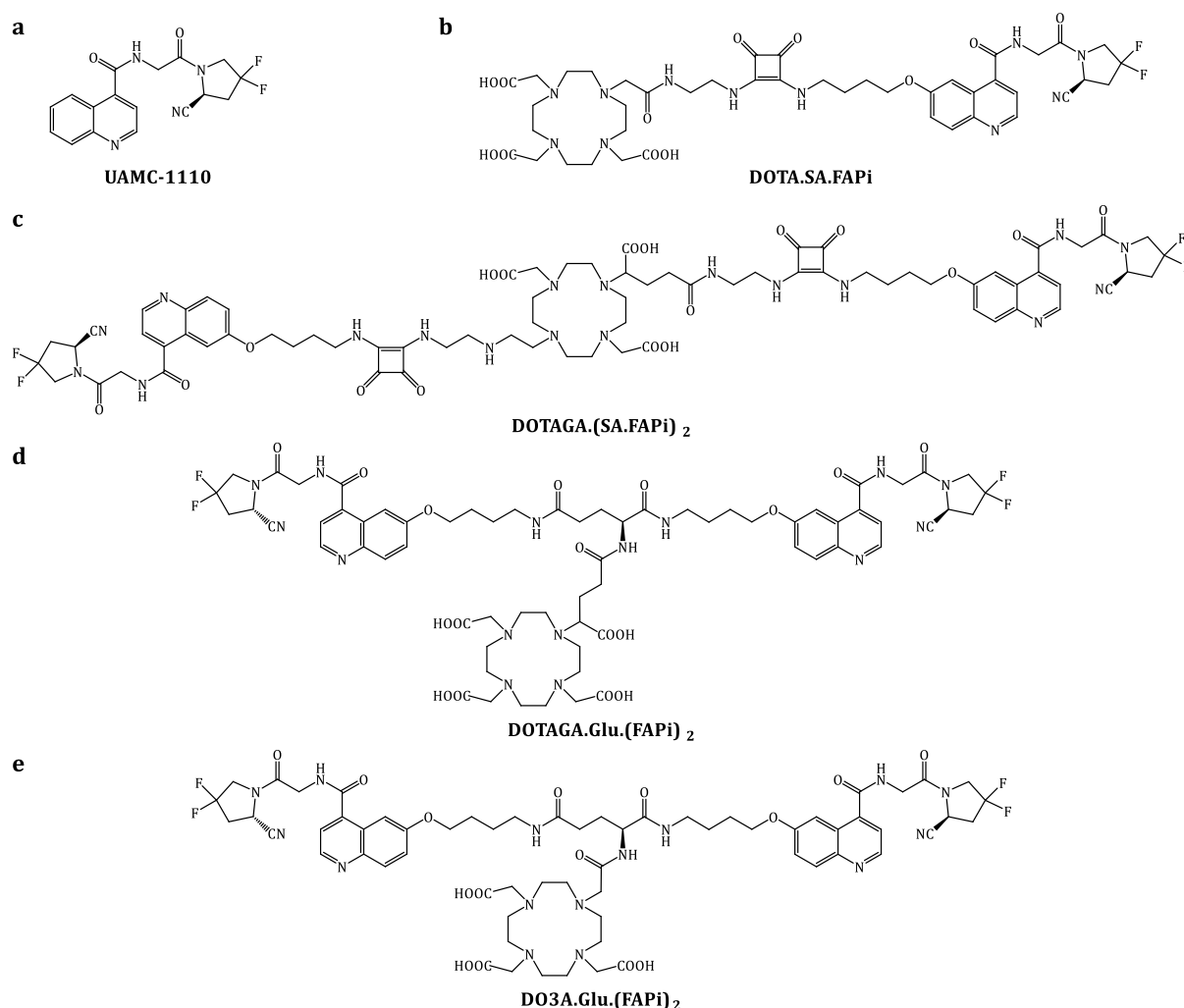


Figure 1. Structures of the original FAP inhibitor UAMC-1110 (**a**), the FAPi-based monomer DOTA.SA.FAPi (**b**), the first-generation linear FAPi dimer DOTAGA.(SA.FAPi)₂ (**c**) and the second-generation FAPi dimers DOTAGA.Glu.(FAPi)₂ (**d**) and DO3A.Glu.(FAPi)₂ (**e**).

Most of them are conjugated with bifunctional chelators such as DOTA to enable PET imaging with ⁶⁸Ga. The rapid renal clearance of monomers such as the squaramide (SA)-based [⁶⁸Ga]Ga-DOTA.SA.FAPi or [⁶⁸Ga]Ga-FAPi-04 resulted in high-contrast PET images in various cancers even at early time points (10 min p.i.) [25,40]. However, the short tumor retention time and as such the biological half-life does not match the physical half-life of therapeutic radionuclides such as ¹⁷⁷Lu ($t_{1/2}$ = 6.7 d) or ²²⁵Ac ($t_{1/2}$ = 9.9 d), which limits the use of these compounds for radioligand therapy (RLT) with the beta minus particle emitter ¹⁷⁷Lu or targeted alpha-particle therapy (TAT) with the alpha emitter ²²⁵Ac.

Recently, peptides were introduced as FAP-targeting vectors in compounds such as FAP-2286, which showed prolonged retention in CAFs-expressing tissue and which represent a new approach regarding FAP radiotherapeutics with a similar approach to TOC or TATE used for peptide receptor radionuclide therapy by targeting SSTRs of NETs [41,42].

Another approach is to apply the dimeric concept, which has already shown, to improve accumulation and to prolong tumor retention time in the context of different targets (e.g., PSMA) beforehand [43–47]. This has led to the development of the first homodimeric structure DOTAGA.(SA.FAPi)₂ (see Figure 1c). Other groups have adapted this approach, and several other dimeric FAPi-based radiopharmaceuticals were published recently [48–51]. ZHAO et al. developed the dimer DOTA-2P(FAPi)₂ based on the FAPi-46 structure, and the ⁶⁸Ga-labeled derivative was investigated in vivo [48,49]. GALBIATI et al. developed the dimeric BiOncoFAP-DOTAGA [50]. The publication mainly focused on the ¹⁷⁷Lu-labeled therapeutic agent that showed promising results in preclinical studies. Both studies confirmed that the homodimeric concept is a successful strategy to target FAP and to prolong tumor retention as first demonstrated with DOTAGA.(SA.FAPi)₂.

The ¹⁷⁷Lu-labeled derivative [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂ has already been investigated extensively in RNT of radioiodine-refractory differentiated thyroid cancer (RR-DTC) patients, among others (e.g., breast cancer) [39,52]. Much longer tumor residence times of up to one week could be measured via planar scintigraphy and single-photon emission computed tomography (SPECT) imaging [39]. The colon was identified as the critical organ regarding the radiation dose due to slow biliary excretion [39,52]. Furthermore, the linear molecular design with the central chelator may have resulted in steric hindrance during complexation, which led to slower reaction kinetics and/or lower radiochemical yields, in particular with ²²⁵Ac.

On the one hand, the new compounds DO3A.Glu.(FAPi)₂ and DOTAGA.Glu.(FAPi)₂ (Figure 1d,e) were designed, synthesized and investigated to improve radiolabeling, especially regarding ²²⁵Ac and therefore allowing for potential application in TAT. On the other hand, the pharmacokinetic and pharmacodynamic properties should be optimized while maintaining or even further improving the desired long tumor retention time. In contrast to the original linear dimer DOTAGA.(SA.FAPi)₂, these new FAPi dimers are designed in a branched structure where the two FAPi targeting vectors and the terminal chelator are coupled via a central glutamic acid (Glu) linker. The further use of squaramide as a linker or spacer was renounced with the intention to synthesize more hydrophilic compounds instead. This could accelerate clearance from non-target tissues as well as excretion to reduce the radiation dose for healthy organs such as colon and liver. Following the organic synthesis of the precursors, the radiolabeling properties with different radiometals (⁶⁸Ga, ¹⁷⁷Lu, ²²⁵Ac and ⁹⁰Y) as well as the complex stabilities were investigated. In addition, the lipophilicity of the ⁶⁸Ga- and ¹⁷⁷Lu-labeled derivatives was evaluated. The new FAPi dimers were tested for their in vitro affinity for FAP as well as PREP, DPP4, DPP8, and DPP9. Beyond that, first proof-of-concept patient investigations were carried out with [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂, which demonstrated faster excretion kinetics and therefore reduced the radiation dose for healthy organs such as the colon and liver. All these studies were performed in comparison to DOTAGA.(SA.FAPi)₂.

2. Materials and Methods

2.1. Chemicals and Equipment

All basic chemicals were purchased from Sigma-Aldrich and Merck (Darmstadt, Germany), TCI (Eschborn, Germany), ABCR (Karlsruhe, Germany), Alfa Aesar by Thermo Fisher Scientific (Kandel, Germany), Acros Organics (Schwerte, Germany) and VWR (Bruchsal, Germany). Dry solvents were bought from Acros Organics, Alfa Aesar by Thermo Fischer Scientific and Merck and deuterated solvents from Deutero (Kastellaun, Germany). The chelators DOTA-tri(^tBu)ester **8** (2-(4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)acetic acid) and DOTA-GA(^tBu)₄ **5** (5-(*tert*-butoxy)-5-oxo-4-(4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetra-azacyclododecan-1-yl)pentanoic acid) were obtained from CheMatech (Dijon, France). (*S*)-6-(4-aminobutoxy)-*N*-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide (FAPi-NH₂ **1**) was purchased from KE Biochem Co. (Shanghai, China) or synthesized according to Figure S1.

Thin-layer chromatography (TLC) plates coated with silica gel 60 F₂₅₄ from Merck were used. For analysis, they were investigated via UV lamp (λ = 254 and 366 nm) as well as staining with potassium permanganate. Silica 60 (0.040–0.063 mm particle size) was used for column chromatography.

Nuclear magnetic resonance (NMR) spectra were recorded in deuterated solvents on an Avance II 400 spectrometer (400 MHz, 5 mm BBFO sample head with z-gradient and ATM, SampleXPress 60 sample changer) or an Avance III 300 spectrometer (300 MHz, 5 mm BBFO sample head with z-gradient and ATM, BACS 60 sample changer) from Bruker (Rheinstätten, Germany). MestReNova 14.2.0 software from Mestrelab Research (Santiago de Compostela, Spain) was used to analyze the spectra.

Liquid chromatography coupled with electrospray ionization mass spectrometry (ESI-LC/MS) was measured using Agilent Technologies 1220 Infinity LC coupled to an Agilent Technologies 6130B Single Quadrupole LC/MS system with an Agilent Zorbax SB-C18 column (21 × 50 mm, 1.8 μ m) with linear gradients of acetonitrile (MeCN)/Milli-Q® water (H₂O) + 0.05% formic acid (HFO) and a flow rate of 0.5 mL/min.

High-pressure liquid chromatography coupled with electrospray ionization mass spectrometry (ESI-HPLC-MS) measurements were performed using an Agilent Technologies G6545A Q-ToF with electron spray ionization coupled to a 1260 Infinity II HPLC system (Agilent Technologies) with G7111B 1260 Quaternary Pump, G7129A 1260 Vial Sampler, and G7116A Multicolumn Thermostat. Separation was performed using an Agilent Poroshell 120 EC-C8 column (2.1 × 100 mm, 2.7 μ m) with H₂O + 2% MeCN/MeCN + 2% H₂O + 0.05% HFO and a flow rate of 0.1 mL/min.

Semi-preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) was performed using LaChrom HPLC (7000 series) from Merck Hitachi with L-7100 pump, L-7400 UV detector ($\lambda = 254$ nm), a D-7000 interface and autosampler. Separation was performed using a Phenomenex Synergi Max-RP C18 column (250 $\times$ 10 mm, 4 μ m) and linear gradient of MeCN/H₂O + 0.1% trifluoacetic acid (TFA) and a flow rate of 5 mL/min.

For radiolabeling, only chemicals of the highest purity (TraceSelect if available) were used. Radio-TLCs were developed using 0.1 M citrate buffer (pH = 4.0). Additional TLCs were developed using 1 M ammonium acetate (pH = 4.0)/methanol (AmOAc/MeOH, 1:1). All radio-TLCs were evaluated using a CR-35 Bio Test-Imager from Duerr-ndt (Bietigheim-Bissingen, Germany) and AIDA software from Elysia-Raytest (Straubenhardt, Germany). Some radio-TLCs of ²²⁵Ac-labelings were additionally analyzed via high-resolution gamma spectroscopy with a HPGe detector. Analytical radio-HPLC was performed also using a Merck Hitachi LaChrom HPLC (7000 series). Separation was performed using Phenomenex Luna C18 column (250 $\times$ 4.6 mm, 5 μ m) and linear gradient of MeCN/H₂O + 0.1% TFA and flow rate of 1 mL/min. The radio-HPLC is equipped with a Ramona analog radio detector from Elysia Raytest with its energy window set to 100–1200 keV for ⁶⁸Ga and 100–250 keV for ¹⁷⁷Lu.

2.2. Organic Synthesis

Figure 2 shows the synthesis scheme of the precursors DOTAGA.Glu.(FAPi)₂ **7** and DO3A.Glu.(FAPi)₂ **11**.

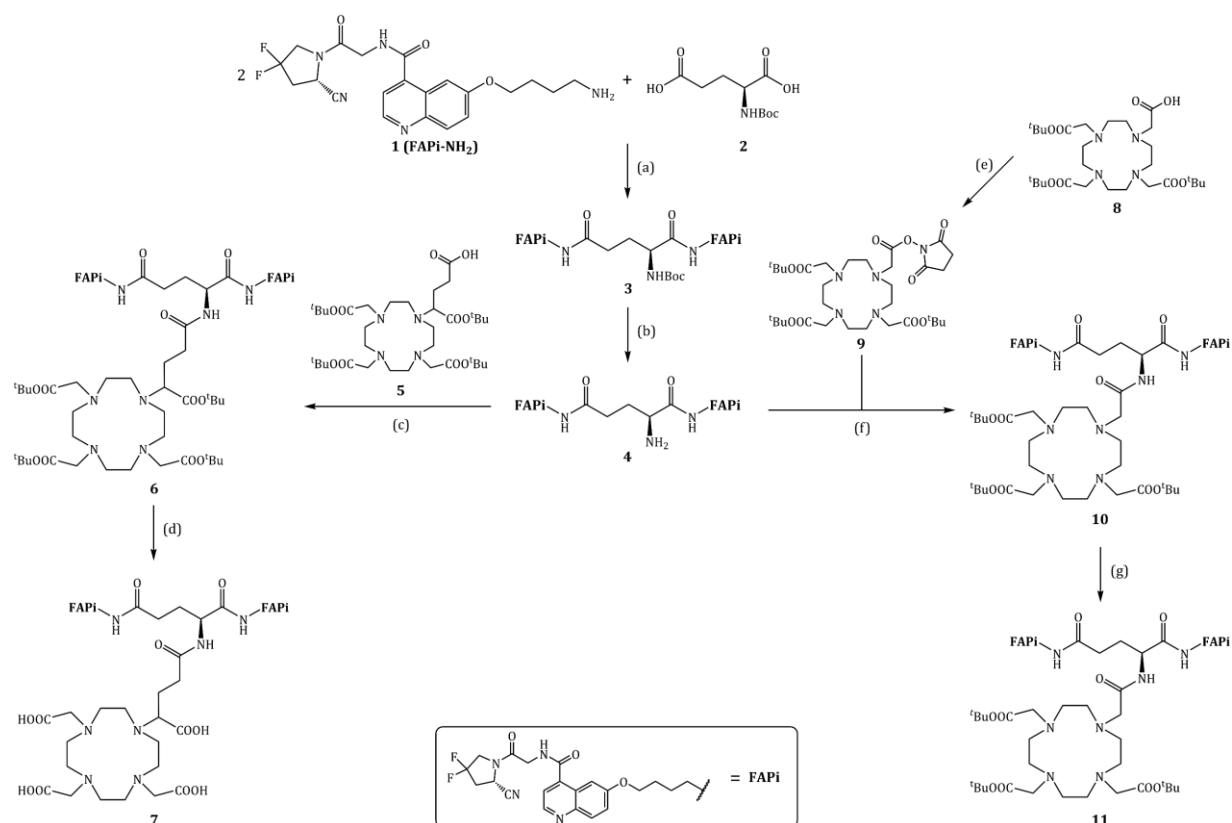


Figure 2. Organic synthesis of DOTAGA.Glu.(FAPi)₂ **7** and DO3A.Glu.(FAPi)₂ **11**: (a) HOBt, EDC*HCl, DIPEA, DMF, RT, 1 d, 74%; (b) 4 M HCl in 1,4-dioxane, MeCN, 0 °C–RT, 6 h, 95%; (c) HATU, DIPEA, DMF, 30 °C, 3 d; (d) TFA:MeCN:TIPS:H₂O (85:10:5:2.5), RT, 5 h, 28% (over 2 steps); (e) NHS, HBTU, MeCN, DMF, 30 °C, 1 d, 96%; (f) DIPEA, DMF, 40 °C, 1 d; (g) TFA:TIPS:H₂O (95:2.5:2.5), RT, 8 h, 29% (over 2 steps).

2.2.1. Synthesis of Glu.(FAPi)₂

tert-Butyl ((*S*)-1,5-bis((4-((2-((*S*)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)-quinolin-6-yl)oxy)butyl)amino)-1,5-dioxopentane-2-yl)carbamate (**3**, Boc-Glu.(FAPi)₂)

N-*tert*-Butoxycarbonyl-L-glutamic acid **2** (Boc-Glu-OH, 49.5 mg, 200 μmol, 1.00 eq), 1-hydroxybenzotriazole (HOBt, 70 mg, 518 μmol, 2.59 eq) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC*HCl, 100 mg, 522 μmol, 2.61 eq) were dissolved in dry *N,N*-dimethylformamide (DMF, 4 mL), and *N,N*-diisopropylethylamine (DIPEA, 17.0 μL, 100 μmol, 0.50 eq) was added under argon atmosphere. The solution was stirred at room temperature (RT) for one hour. Then, a solution of FAPi-NH₂ **1** (173 mg, 400 μmol, 2.00 eq) and DIPEA (102 μL, 600 μmol, 3.00 eq) in DMF (2 mL) was added. After another 4 h at RT, further HOBt (13.5 mg, 100 μmol, 0.50 eq) and EDC*HCl (19.2 mg, 100 μmol, 0.50 eq) were added, and after another 30 min, a solution of FAPi-NH₂ **1** (43.4 mg, 100 μmol, 0.50 eq) and DIPEA (17.0 μL, 100 μmol, 0.50 eq) in DMF (1 mL) was added again. This addition of HOBt, EDC*HCl (0.50 eq each) followed by FAPi-NH₂ (28.5 mg, 66 μmol, 0.33 eq) and DIPEA (0.50 eq) was repeated one more time, and after 4 more hours, the solvent was removed in vacuo. After column chromatography (CHCl₃/MeOH, 100:10–15) **3** was obtained as a yellowish solid (158 mg, 147 μmol, 74%). ¹H-

NMR (400 MHz, DMF- d_7): δ [ppm] = 8.98 (t, J = 6.0 Hz, 1H), 8.83 (d, J = 4.3 Hz, 1H), 8.29 – 8.17 (m, 1H), 8.03 – 7.94 (m, 3H), 7.93 – 7.87 (m, 1H), 7.80 – 7.71 (m, 1H), 7.58 (d, J = 4.3 Hz, 1H), 7.52 – 7.43 (m, 2H), 7.34 (dd, J = 8.5, 2.0 Hz, 1H), 7.25 (dd, J = 8.4, 2.5 Hz, 1H), 6.83 (d, J = 7.8 Hz, 1H), 5.31 (dd, J = 9.6, 2.5 Hz, 1H), 4.53 – 3.70 (m, 11H), 3.37 – 3.01 (m, 8H), 2.33 – 2.25 (m, 2H), 2.16 – 1.83 (m, 4H), 1.78 – 1.66 (m, 2H), 1.62 – 1.44 (m, 2H), 1.42 (s, 9H), 1.40 – 1.37 (m, 6H). ^{19}F -NMR (300 MHz, DMF- d_7): δ [ppm] = -74.48 (s). MS (ESI $^+$): m/z (%) = 487.8 (100, [M-Boc+H] $^{2+}$), 537.8 (73, [M+H] $^{2+}$), 1074.4 (9, [M+H] $^+$), 1075.4 (6, [M+H] $^+$), calculated for $\text{C}_{52}\text{H}_{59}\text{F}_4\text{N}_{11}\text{O}_{10}$: 1073.44 [M].

(S)-2-Amino- N^1,N^5 -bis(4-((4-((2-((*S*)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)-quinolin-6-yl)oxy)butyl)pentanediamide (**4**, Glu.(FAPi) $_2$)

Boc-Glu.(FAPi) $_2$ **3** (158 mg, 147 μmol , 1.00 eq) was dissolved in dry MeCN (3 mL) and under argon atmosphere and at 0 $^\circ\text{C}$, 4 M hydrochloric acid (HCl) in 1,4-dioxane (300 μL , 1.20 mmol, 8.57 eq) was added. The reaction was allowed to warm to RT, and after 6 h, it was diluted with MeOH (20 mL), and the solvent was completely removed in vacuo. Glu.(FAPi) $_2$ **4** (136 mg, 140 μmol , 95%) was obtained as a yellowish solid. ^1H -NMR (300 MHz, MeOD): δ [ppm] = 8.84 (dd, J = 8.4, 4.9 Hz, 2H), 8.01 (dd, J = 8.8, 2.6 Hz, 4H), 7.72 (dd, J = 6.6, 4.8 Hz, 2H), 7.55 (ddd, J = 9.3, 4.6, 2.7 Hz, 2H), 5.16 (dt, J = 9.3, 3.4 Hz, 2H), 4.35 (d, J = 2.1 Hz, 4H), 4.31 – 4.07 (m, 8H), 3.91 (t, J = 6.2 Hz, 1H), 3.58 – 3.36 (m, 2H), 3.11 – 2.71 (m, 6H), 2.47 – 2.37 (m, 2H), 2.17 – 2.00 (m, 2H), 2.00 – 1.87 (m, 4H), 1.86 – 1.70 (m, 4H). ^{19}F -NMR (300 MHz, MeOD): δ [ppm] = -77.29 (s). MS (ESI $^+$): m/z (%) = 325.6 (100, [M-Boc+H] $^{3+}$), 487.8 (28, [M+H] $^{2+}$), 974.3 (5, [M+H] $^+$), calculated for $\text{C}_{47}\text{H}_{51}\text{F}_4\text{N}_{11}\text{O}_8$: 973.39 [M].

2.2.2. Synthesis of DOTAGA.Glu.(FAPi) $_2$

*tri-tert-Butyl 2,2',2''-(10-(5-(((*S*)-1,5-bis((4-((4-((2-((*S*)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-1,5-dioxopentan-2-yl)amino)-1-(tert-butoxy)-1,5-dioxopentan-2-yl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate* (**6**, DOTAGA($t\text{Bu}$) $_4$.Glu.(FAPi) $_2$)

DOTAGA($t\text{Bu}$) $_4$ **5** (56.1 mg, 80.0 μmol , 1.15 eq), 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo [4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU, 30.3 mg, 80.0 μmol , 1.15 eq) and DIPEA (15.0 μL , 88 μmol , 1.25 eq) were dissolved in dry DMF (1 mL) under argon atmosphere and stirred at 30 $^\circ\text{C}$ for one hour. A solution of Glu.(FAPi) $_2$ (68.0 mg, 70.0 μmol , 1.00 eq) and DIPEA (30.0 μL , 175 μmol , 2.50 eq) in dry DMF (1 mL) was added. It was stirred for two days at 30 $^\circ\text{C}$, and then, more HATU (15.2 mg, 40.0 μmol , 0.50 eq) was added. After another day of stirring at 30 $^\circ\text{C}$, the solvent was removed in vacuo. A yellow oil was obtained, which was directly used in the next step without further purification. MS (ESI $^+$): m/z (%) = 414.97 (13, [M+H] $^{4+}$), 415.22 (12, [M+H] $^{4+}$), 552.95 (100, [M+H] $^{3+}$), 553.29 (97, [M+H] $^{3+}$), 553.62 (51, [M+H] $^{3+}$), 553.96 (18,

[M+H]³⁺), 828.93 (82, [M+H]²⁺), 829.43 (78, [M+H]²⁺), 829.93 (40, [M+H]²⁺), 830.43 (15, [M+H]²⁺), 1656.85 (87, [M+H]⁺), 1657.85 (85, [M+H]⁺), 1658.85 (43, [M+H]⁺), 1659.86 (15, [M+H]⁺), calculated for C₈₂H₁₁₃F₄N₁₅O₁₇: 1655.84 [M].

2,2',2''-(10-(4-(((S)-1,5-bis((4-((2-((S)-2-Cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)-carbamoyl)quinolin-6-yl)oxy)butyl)amino)-1,5-dioxopentan-2-yl)amino)-1-carboxy-4-oxobutyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (7, DOTAGA.Glu.(FAPi)₂)

DOTAGA(^tBu)₄.Glu.(FAPi)₂ **6** was dissolved in TFA (850 µL), MeCN (100 µL), triisopropylsilane (TIPS, 50 µL) and H₂O (25 µL) and stirred at RT for 5 h. After co-distillation with MeOH (5 × 10 mL) and evaporation to dryness, the crude product was purified by semipreparative RP-HPLC (22–24% MeCN in 20 min, *t_R* = 17–18 min), and **7** was obtained as a yellow solid (28.0 mg, 19.5 µmol, 28%). MS (ESI⁺): *m/z* (%) = 358.85 (65, [M+H]⁴⁺), 369.05 (24, [M+MeCN+H]⁴⁺), 478.30 (100, [M+H]³⁺), 717.30 (6, [M+H]²⁺), 1432.40 (1, [M+H]⁺), 1454.70 (1, [M+Na]⁺), calculated for C₆₆H₈₁F₄N₁₅O₁₇: 1431.59 [M].

2.2.3. Synthesis of DO3A.Glu.(FAPi)₂

tri-tert-Butyl 2,2',2''-(10-(2-(((S)-1,5-bis((4-((2-((S)-2-dioxopyrrolidin-1-yl)oxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (9, DOTA(^tBu)₃-NHS)

DOTA-tris(*tert*-butyl ester) **8** (573 mg, 1.00 mmol, 1.00 eq) and HBTU (417 mg, 1.10 mmol, 1.10 eq) were dissolved in dry MeCN (12 mL) and dry DMF (5 mL), and then, *N*-hydroxysuccinimide (NHS, 127 mg, 1.10 mmol, 1.10 eq) was added. After stirring at 30 °C for 6 h, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 190 mg, 501 µmol, 0.50 eq) and NHS (57.5 mg, 500 µmol, 0.50 eq) were added, and stirring was continued overnight. After all solvents were removed in vacuo, purification by column chromatography (dichloromethane/MeOH (100:10)) gave **9** as a colorless solid (643 mg, 961 µmol, 96%). ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 2.97 – 2.77 (m, 12H), 2.66 – 2.02 (m, 16H), 1.44 (s, 27H). MS (ESI⁺): *m/z* (%) = 335.7 (100, [M+H]²⁺), 670.4 (50, [M+H]⁺), 671.4 (18, [M+H]⁺), calculated for C₃₂H₅₅N₅O₁₀: 669.39 [M].

tri-tert-Butyl 2,2',2''-(10-(2-(((S)-1,5-bis((4-((4-((2-((S)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-1,5-dioxopentan-2-yl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (10, DOTA(^tBu)₃.Glu.(FAPi)₂)

DOTA(^tBu)₃-NHS **9** (40.2 mg, 60.0 µmol, 1.20 eq) and Glu.(FAPi)₂ **4** (48.7 mg, 50.0 µmol, 1.00 eq) were dissolved in dry DMF (2 mL), and DIPEA (200 µL) was added. After stirring at 40 °C under argon atmosphere for one day, the solvents were completely removed in vacuo. A yellow oil was obtained and directly used in the next step without further purification. MS (ESI⁺): *m/z* (%) =

382.95 (22, [M+H]⁴⁺), 383.20 (19, [M+H]⁴⁺), 491.57 (34, [M-^tBu+H]³⁺), 491.90 (28, [M-^tBu+H]³⁺), 492.24 (13, [M-^tBu+H]³⁺), 510.26 (100, [M+H]³⁺), 510.59 (90, [M+H]³⁺), 510.93 (44, [M+H]³⁺), 511.26 (14, [M+H]³⁺), 764.88 (42, [M+H]²⁺), 765.38 (37, [M+H]²⁺), 765.89 (17, [M+H]²⁺), 1528.76 (25, [M+H]⁺), 1529.76 (22, [M+H]⁺), 1530.77 (10, [M+H]⁺), calculated for C₇₅H₁₀₁F₄N₁₅O₁₅: 1527.75 [M].

*2,2',2''-(10-(2-(((S)-1,5-bis((4-((2-((S)-2-Cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)-carbamoyl)quinolin-6-yl)oxy)butyl)amino)-1,5-dioxopentan-2-yl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (**11**, DO3A.Glu.(FAPi)₂)*

DOTA(^tBu)₃.Glu.(FAPi)₂ **10** was dissolved in TFA (1.9 mL), TIPS (50 µL) and H₂O (50 µL) and stirred at RT for 8 h. After co-distillation with MeOH (4 × 10 mL), the solvents were completely removed under reduced pressure again. After semipreparative RP-HPLC (22–23% MeCN in 16 min, *t_R* = 14–15 min), **11** was obtained as a yellow solid (19.9 mg, 14.6 µmol, 29%). MS (ESI⁺): *m/z* (%) = 340.85 (42, [M+H]⁴⁺), 351.00 (57, [M+MeCN+H]⁴⁺), 361.35 (13, [M+2MeCN+H]⁴⁺), 454.15 (100, [M+H]³⁺), 468.00 (20, [M+MeCN+H]³⁺), 680.85 (9, [M+H]²⁺), calculated for C₆₃H₇₇F₄N₁₅O₁₅: 1359.57 [M].

2.2.4. Synthesis of ^{nat}Lu-Complexes

*[^{nat}Lu]Lu-DOTAGA.Glu.(FAPi)₂ (^{nat}Lu-**7**)*

DOTAGA.Glu.(FAPi)₂ **7** (8.6 mg, 6.0 µmol, 1.00 eq) was dissolved in 500 µL 1 M HEPES (pH = 5.5), 100 µL 1 M ammonium acetate (pH = 5.5) and 200 µL EtOH. Then, 0.1 M LuCl₃ (150 µL, 15.0 µmol, 2.50 eq) solution in 1 M ammonium acetate (pH = 5.5) was added and shaken at 90 °C for 6 h. Subsequent semi-preparative RP-HPLC (22–24% MeCN in 12 min, *t_R* = 11.5 min) yielded [^{nat}Lu]Lu.DOTAGA.Glu.(FAPi)₂ as a yellow solid (5.3 mg, 3.3 µmol, 55%). MS (ESI⁺): *m/z* (%) = *m/z* (%) = 535.50 (100, [M+H]³⁺), 802.95 (36, [M+H]²⁺), calculated for C₆₆H₇₈F₄LuN₁₅O₁₇: 1603.50 [M].

*[^{nat}Lu]Lu-DO3A.Glu.(FAPi)₂ (^{nat}Lu-**11**)*

DO3A.Glu.(FAPi)₂ **11** (2.8 mg, 2.0 µmol, 1.00 eq) was dissolved in 500 µL of 1 M HEPES buffer (pH = 5.5). Then, 0.1 M LuCl₃ solution (40 µL, 4.0 µmol, 2.00 eq) was added and shaken at 90 °C for 4 h. Subsequent semi-preparative RP-HPLC (20–25% MeCN in 20 min, *t_R* = 14–15 min) yielded [^{nat}Lu]Lu-DO3A.Glu.(FAPi)₂ as a yellow solid (0.7 mg, 0.46 µmol, 23%). MS (ESI⁺): *m/z* (%) = 511.55 (100, [M+H]³⁺), 766.75 (14, [M+H]²⁺), calculated for C₆₃H₇₄F₄LuN₁₅O₁₅: 1531.48 [M].

2.3. Radiosynthesis

2.3.1. ⁶⁸Ga-Radiolabeling

Gallium-68 was eluted from a ITM (Isotope Technologies Munich AG, Germany) ⁶⁸Ge/⁶⁸Ga-generator with 5 mL of 0.05 M HCl (TraceSelect). For the labeling of DOTAGA.Glu.(FAPi)₂ **7**, the eluate (100–400 MBq in 0.5–2.0 mL) was directly used. The equivalent volume of 1 M 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES, pH = 4.5) was added, followed by precursor **7** (10–40 nmol, 10–40 µL 1 mM stock solution).

For DO3A.Glu.(FAPi)₂ **11**, cationic post processing of the ⁶⁸Ge/⁶⁸Ga-generator was carried out according to the procedure developed by EPPARD et al. [53]. Precursor **11** (5–20 nmol, 5–20 µL 1 mM stock solution) was added to 400 µL 1 M HEPES (pH = 4.5 or 5.5) buffer solution followed by the addition of gallium-68 (ca. 100 MBq).

The reaction solutions were heated at 95 °C, and reaction controls were taken at different time points to investigate reaction kinetics analyzed via radio-TLC. Additional AmOAc/MeOH (1:1) radio-TLCs and radio-HPLC were carried out for the last reaction control to double-check radiochemical conversion (RCC) and purity (RCP).

2.3.2. ¹⁷⁷Lu-Radiolabeling

[¹⁷⁷Lu]LuCl₃ (in 0.04 M HCl) was purchased from ITM (Isotope Technologies Munich SE, Munich, Germany). Then, 400 µL 1 M HEPES (pH = 5.5) buffer solution and the respective quantity of precursor (1 mM stock solution) were added to lutetium-177 (50–100 MBq) and heated at 95 °C. Reaction controls were taken at different time points to investigate reaction kinetics analyzed via radio-TLC. Additional AmOAc/MeOH (1:1) radio-TLCs and radio-HPLC were carried out for the last reaction control to double-check radiochemical conversion (RCC) and purity (RCP).

2.3.3. ⁹⁰Y-Radiolabeling

[⁹⁰Y]YCl₃ (in 0.04 M HCl) was purchased from EZAG (Eckert & Ziegler Strahlen- und Medizintechnik AG, Berlin, Germany). Then, 400 µL 1 M HEPES (pH = 4.5) buffer solution and DO3A.Glu.(FAPi)₂ (5 or 10 nmol, 5 or 10 µL of 1 mM stock solution) were added to yttrium-90 (30–60 MBq) and heated at 95 °C. Reaction controls were taken at different time points to investigate reaction kinetics analyzed via radio-TLC.

2.3.4. ²²⁵Ac-Radiolabeling

[²²⁵Ac]AcCl₃ (in 0.04 M HCl) was purchased from ITM (Isotope Technologies Munich SE, Munich, Germany).

Small scale labeling was carried out as follows: ca. 500 kBq [^{225}Ac]AcCl₃ (in 50 μL 0.04 M HCl) was added to 500 μL 0.1 M sodium ascorbate (pH = 7.0) buffer solution, and then, the respective quantity of precursor **11** (10 or 20 nmol, 10 or 20 μL of 1 mM stock solution) was added and heated at 95 °C for 60 min.

Bigger scale labeling was carried out similarly: 1.6–3.2 MBq [^{225}Ac]AcCl₃ (in 100 μL 0.04 M HCl) was added to 1000 μL 0.1 M sodium ascorbate (pH = 7.0) buffer solution, and then, the respective quantity of precursor **11** (30–40 nmol/MBq from 1 mM stock solution) was added and heated at 95 °C for 60 min.

Reaction controls were taken at different time points to investigate reaction kinetics. The radio-TLCs were dried with a heating gun and imaged at different time points (1 h, 1 d) after the TLC had been developed. Additional AmOAc/MeOH (1:1) radio-TLCs were developed for the last reaction control.

Subsequent purification using SepPak® Light C18 cartridge yielded the product. The cartridge was conditioned with 2 mL EtOH and 5 mL H₂O. The reaction solution was pushed through the cartridge. The reaction vial was rinsed with 0.1 M sodium ascorbate, and the step was repeated once. The product was washed with 1 mL H₂O and eluted with 2 mL EtOH/saline (1:1). It was diluted with 8 mL of saline containing 100 mg sodium ascorbate to give the final formulation. The radiochemical yield (>98%) was determined via radio-TLC and high-resolution gamma spectroscopy with HPGe detector. For the gamma spectroscopy, a TLC was developed in standard 0.1 M citrate buffer, cut into two pieces at $R_f = 0.2$ – 0.3 and measured separately after one hour for 15 min each. The integral of the ^{221}Fr line ($E_\gamma = 218$ keV) of each of the two pieces was considered for the RCC determination.

2.3.5. Complex Stability Measurements

After radiolabeling was carried out (RCP > 95%), the stability in human serum (HS) and phosphate-buffered saline (PBS) was investigated ($n = 3$) by incubating ca. 10 MBq of the labeled tracer solution in 0.5 mL HS and PBS at 37 °C for 120 min (^{68}Ga), for 6 d (^{90}Y) and 14 d (^{177}Lu). The complex stability was determined via radio-TLC with 0.1 M citrate buffer (pH = 4.0) as the mobile phase.

For ^{225}Ac , 350–400 kBq was incubated in 0.5 mL HS and PBS as well as 200 kBq in 1 mL final formulation solution (“pure”). The complex stability was investigated over 20 days, and the citrate radio TLCs were imaged 1 d after they were developed.

2.3.6. Determination of $\log D_{7.4}$ (Lipophilicity Measurement)

After radiolabeling was carried out (RCP > 95%), the $\log D_{7.4}$ value was determined by diluting 10 MBq of the labeled tracer solution to 700 μL with PBS ($n = 4$). To each, 700 μL of 1-octanol was

added and shaken vigorously for 1–2 min (1500 rpm) followed by centrifugation for 1–2 min. Then, 400 μL of the organic and aqueous phase were separated into new Eppendorf tubes. Samples of 3 μL (PBS) or 6 μL (1-octanol) were pipetted onto a TLC plate. Since most of the activity was in the aqueous phase, this was diluted to 700 μL with PBS, and again, 700 μL of 1-octanol was added. The procedure was repeated twice. The TLC plate was imaged (exposure time: 5–10 min), and the integral of each spot (octanol phase: I_o , aqueous PBS phase: I_{PBS}) was determined. The $\log D_{7.4}$ value was calculated using Equation (1) where the different volumes of $V_o = 6 \mu\text{L}$ und $V_{PBS} = 3 \mu\text{L}$ were taken into account.

$$\log D_{7.4} = \log \left(\frac{I_o}{2 \cdot I_{PBS}} \right) \quad (1)$$

Only the values of the second and third extraction were considered in the calculation.

2.4. In Vitro Inhibition Assays (IC_{50} Measurements)

rhFAP (recombinant human fibroblast activation protein), DPP8 (dipeptidyl peptidase VIII) and DPP9 (dipeptidyl peptidase IX) were expressed and purified as described previously [54,55]. DPP4 (dipeptidyl peptidase IV) was purified from human seminal plasma as described previously [56]. Human recombinant PREP (prolylendopeptidase) was expressed and purified as described before [54].

IC_{50} measurements for FAP and PREP were carried out as described before [30,55]. Briefly, Z-Gly-Pro-7-amino-4-methylcoumarine (AMC, Bachem) at a final concentration of 50 μM at pH 8.0 in 0.05 M Tris-HCl buffer, 1 mg/mL BSA and 140 mM NaCl was used as the substrate for FAP IC_{50} measurements. For PREP, N-succinyl-Gly-Pro-AMC (Bachem) at a final concentration of 250 μM at pH 7.4 in 0.1 M K-phosphate, 1 mM EDTA, 1 mM DTT and 1 mg/mL BSA buffer was used.

IC_{50} measurements for DPP4, DPP8 and DPP9 were performed as published before, using Ala-Pro-pNA as the substrate at the respective concentrations of 25 μM (DPP4), 300 μM (DPP8) and 150 μM (DPP9) at pH 7.4 in 0.05 M HEPES-NaOH buffer with 0.1% Tween-20, 0.1 mg/mL BSA and 150 mM NaCl [30,57].

All enzymatic activities were determined kinetically for 15 min at 37 °C by measuring initial velocities of AMC release ($\lambda_{\text{ex}} = 380 \text{ nm}$ and $\lambda_{\text{em}} = 465 \text{ nm}$) or pNA release (405 nm) from the substrates mentioned above with an Infinite 200 plate reader from Tecan Group Ltd. (Männedorf, Switzerland). The Magellan software was used to process the data, and data fitting was performed using a non-linear fitting model in Grafit 7.4. All IC_{50} measurements were carried out in triplicate with at least eight different inhibitor concentrations tested.

2.5. Patient Study

2.5.1. Radiosynthesis

[⁶⁸Ga]Ga-DOTA.SA.FAPi was synthesized as described by BALLAL et al. [58]. [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂ was synthesized as described by BALLAL et al. [39].

[¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ was radiolabeled by adding 6.1 GBq [¹⁷⁷Lu]LuCl₃ (obtained from BRIT (India) in 0.01 M supra pure HCl) in 0.1 M sodium acetate buffer (pH = 5.0) followed by 150 nmol DOTAGA.Glu.(FAPi)₂ **7** (≈ 25 nmol/GBq). The solution was heated at 95 °C for 30 min followed by purification through Sep-Pak C18 light cartridge and eluted with 1 mL EtOH/H₂O (1:1). Radiochemical quality control was carried out using the instant thin-layer chromatography method with 0.1 M trisodiumcitrate buffer (pH = 4.0) as solvent. The product was administered with a radiochemical purity of 95%. The radiochemical yield was 90%.

2.5.2. Clinical Image Acquisition and Analysis

The patient underwent PET scans with [¹⁸F]F-FDG and [⁶⁸Ga]Ga-DOTA.SA.FAPi mean injected activities of 185 and 222 MBq, respectively. The scans were acquired within a month time interval with a GE Discovery 710* 128 Slice PET/CT Scanner (40 mm detector at 0.35 s rotation speed). The whole-body PET/CT scans were acquired one hour post-injection. An initial scout image was acquired first, followed by a CT scan, and PET acquired at 2 min per bed. The whole-body CT scan parameters included 300–350 mAs, 120 kVp, slice thickness of 5 mm, and pitch 1 was acquired. [¹⁸F]F-FDG and [⁶⁸Ga]Ga-DOTA.SA.FAPi PET/CT scans were loaded simultaneously and co-registered using carina as an anatomical landmark registration technique. The scan interpretations were conducted by two experienced nuclear medicine physicians and reviewed by a third. ROIs were drawn according to the PET Response Criteria in Solid Tumors (PERCIST 1.0). For quantitative assessment of standardized uptake values (SUV), corrected for lean body mass, 3D auto-contour ROI at a 40% threshold of SULpeak technique was used.

Planar whole-body scans after administration of [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂ or [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ were performed on a Dual Head Gamma Camera (GE Discovery NM/CT 670) using a high-energy general purpose (HEGP) collimator. Scans were acquired in the lutetium-177 window at a 20% energy window with peaks at 208 and 113 keV. The matrix size for the whole-body scan was 256 × 1024. Anterior and posterior views were acquired by using both detectors. The clinical study was approved by the Institute Ethics committee at All India Institute of Medical Sciences (IECPG-22/27.02.2020). All patients gave their written informed consent.

3. Results and Discussion

3.1. Organic Synthesis

FAPi-NH₂ **1** was synthesized according to the procedure in the supporting information (cf. Figure S1).

The synthesis of DOTAGA.Glu.(FAPi)₂ **7** and DO3A.Glu.(FAPi)₂ **11** is shown in Figure 2 and was started from FAPi-NH₂ **1** and Boc-Glu-OH **2**.

First, **1** was coupled twice to obtain Boc-Glu.(FAPi)₂ **3**. Here, HOBT and EDC·HCl with DIPEA in DMF were found to be the best coupling conditions, and **1** needs to be used in slight excess (2.5–3.0 eq). The cleavage of the Boc-protective group can be carried out under very mild conditions with 4 M HCl in 1,4-dioxane (8.6 eq) and acetonitrile as solvent, alternatively using trifluoroacetic acid (TFA). The following coupling of the FAPi dimer Glu.(FAPi)₂ **4** with DOTA-tris(tert-butyl ester) **8** and DOTAGA(^tBu)₄ **5** were both carried out with HATU and DIPEA in DMF. For DO3A, only small amounts of product could be found in LC-MS measurements, and no product could be isolated after column chromatography. Therefore, an alternative route was tried out. Compound **8** was first converted into DOTA(^tBu)₃-NHS **9**. After coupling with **4** with DIPEA in DMF, the *tert*-butyl ester groups of the chelator were cleaved and following RP-HPLC purification yielded DO3A.Glu.(FAPi)₂ **11** with 20% over four steps. DOTAGA.Glu.(FAPi)₂ **7** could be obtained with similar yields.

The ^{nat}Lu-complexes were obtained by adding 0.1 M LuCl₃ solution (2.0 eq) to 1 M HEPES buffer (pH = 5.5) and the corresponding precursors **7** and **11**, respectively. Subsequent RP-HPLC purification gave the Lu-complexed derivatives ^{nat}Lu-**7** and ^{nat}Lu-**11**.

3.2. Radiosynthesis

3.2.1. [⁶⁸Ga]Ga-DOTAGA.Glu.(FAPi)₂ (⁶⁸Ga-**7**)

MOON et al. [31] observed that the ⁶⁸Ga-radiolabeling of the dimers DOTA.(SA.FAPi)₂ and DOTAGA.(SA.FAPi)₂ reached better radiochemical conversion (RCC) rates with HEPES buffer compared to ammonium acetate (AmOAc) and sodium acetate (NaOAc). This could also be observed with the new dimers. Labeling of ca. 100 MBq ⁶⁸Ga in 500 μL 1 M AmOAc (pH = 4.5) only gave 57% RCC with 10 nmol DOTAGA.Glu.(FAPi)₂ **7** (cf. Figure S4). Furthermore, 20 nmol was needed to give good yields of > 90%. In comparison, 10 nmol **7** and 100 MBq ⁶⁸Ga in 500 μL 1 M HEPES (pH = 4.5) gave > 90% after 1.5 min and > 95% after 15 min (Figure 3). Similar results were achieved in a fourfold upscale with 400 MBq ⁶⁸Ga. The second TLC system 1 M AmOAc/MeOH (1:1) (RCC = 98.8 ± 0.5%) and Radio-HPLC (RCP = 99.1%, cf. Figure S13) gave confirming results.

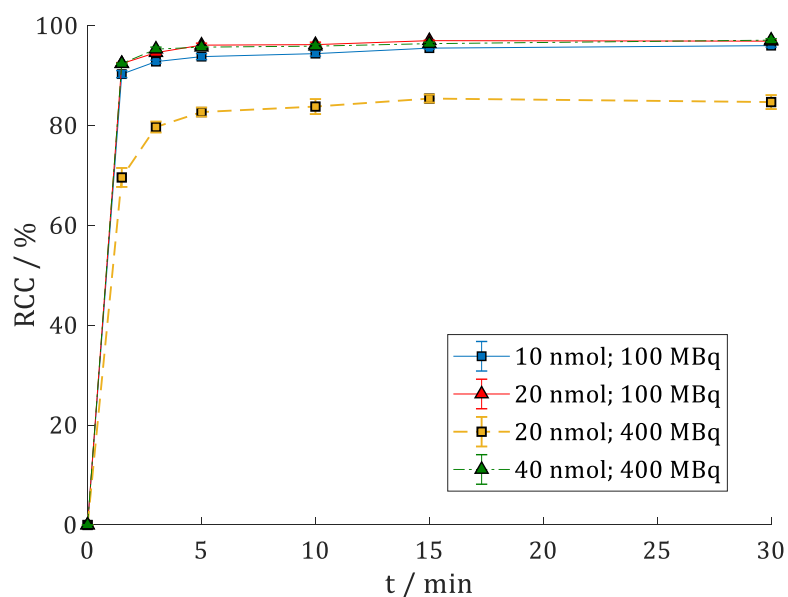


Figure 3. Reaction kinetics (radiochemical conversion (RCC) in %) of $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu.}(\text{FAPi})_2$ $^{68}\text{Ga-7}$ in 1 M HEPES (pH = 4.5) at 95 °C with 100 MBq ^{68}Ga (n = 4) or 400 MBq ^{68}Ga (n = 2).

The complex stability was determined in human serum (HS) and phosphate-buffered saline (PBS) in triplicate each (n = 3) by incubating ca. 10 MBq of $^{68}\text{Ga-7}$ in 0.5 mL of HS/PBS at 37 °C for 120 min and analyzing via radio-TLC. The complex is stable under these conditions (Figure S5).

3.2.2. $[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu.}(\text{FAPi})_2$ ($^{177}\text{Lu-7}$)

Labeling of **7** with lutetium-177 was carried out in 400 μL 1 M HEPES buffer (pH = 5.5) at 95 °C and gave excellent results with RCC > 99% (1 min) with 2 and 5 nmol **7** and > 98% (1 nmol) with just 1 nmol that was given to the buffer solution with 50–100 MBq $[^{177}\text{Lu}]\text{LuCl}_3$ (Figure 4).

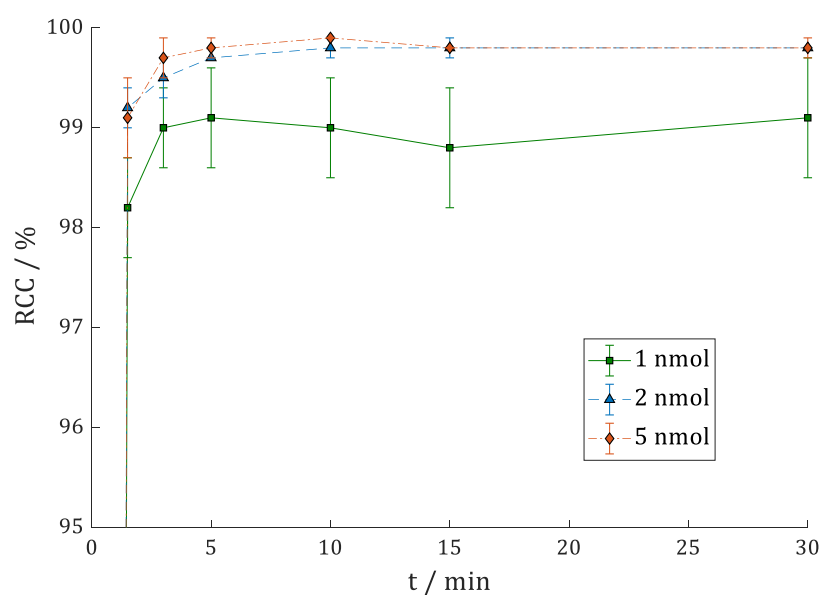


Figure 4. Reaction kinetics (radiochemical conversion (RCC) in %) of $[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu.}(\text{FAPi})_2$ $^{177}\text{Lu-7}$ in 1 M HEPES (pH = 5.5) at 95 °C with 50–100 MBq ^{177}Lu (n = 3).

In a proportional upscale, this would lead to 10 nmol per GBq lutetium-177. The second radio-TLC system (RCC > 99%) and radio-HPLC (cf. Figure S14: RCP = 99.8%) also showed quantitative labeling without any subsequent purification step to separate free noncomplexed lutetium-177. The complex stability of $^{177}\text{Lu-7}$ was determined similarly to the ^{68}Ga -complex $^{68}\text{Ga-7}$, except that the incubation at 37 °C was carried out for 14 days. $^{177}\text{Lu-7}$ showed no degradation, and the complex was stable over two half-lives (Figure S6).

3.2.3. [^{225}Ac]Ac-DOTAGA.Glu.(FAPi) $_2$ ($^{225}\text{Ac-7}$)

Actinium-225 ($t_{1/2} = 9.9$ d) is part of the neptunium cascade and has no dominant gamma-ray emission lines itself. Therefore, the imaging has to be performed via its daughter nuclides. The gamma lines of francium-221 ($t_{1/2} = 4.9$ min, $E_\gamma = 218$ keV) and bismuth-213 ($t_{1/2} = 45.6$ min, $E_\gamma = 440$ keV) are the predominant ones that can be used in TLC imaging.

The radio-TLCs were always imaged after one hour and one day. Waiting at least one hour is necessary since the equilibrium between actinium-225 and its daughters is disrupted while running the TLC, and the $^{225}\text{Ac}/^{221}\text{Fr}$ -equilibrium has to be reestablished. At this time point, bismuth-213 also contributed to the TLC imaging, which is why the ^{225}Ac -RCY/RCC is systematically underestimated at this time point. This was broadly investigated with a statistic model by KELLY et al. [59] where they found that a RCP > 90% after 2 h results in a “true” RCP > 97% after 1 day. After one day, bismuth-213 and all the other daughter nuclides decayed. Imaging at this time point gives the distribution of actinium-225. In gamma spectroscopy the gamma-ray lines of francium-221 and bismuth-213 can be distinguished. The amount of actinium-225 can be calculated via the ^{221}Fr peak; thus, waiting one hour is sufficient in this case.

At first, test labeling experiments were carried out. Therefore, 10 or 20 nmol of **7** were added to a solution of ca. 500 kBq [^{225}Ac]AcCl $_3$ and 0.1 M sodium ascorbate (pH = 7.0), which was heated at 95 °C for 60 min ($n = 2$). For 10 nmol, the RCC was $86.7 \pm 3.6\%$ (60 min, 1 d) when imaging after one day. As expected, the RCCs are slightly lower when imaging after one hour, in this case $84.3 \pm 4.0\%$ (60 min, 1 h). For 20 nmol, the RCCs were > 90% (15 min) and $91.8 \pm 4.7\%$ (60 min, 1 h) and $96.5 \pm 1.9\%$ (60 min, 1 d). Exemplary reaction kinetics are shown in Figure S5. It can be seen that at early imaging time points (e.g., 1 h), four spots can be seen, with $R_f \approx 0.5$ being free actinium-225 and $R_f = 0.0$ being the ^{225}Ac -complex [^{225}Ac]Ac-DOTAGA.Glu.(FAPi) $_2$ $^{225}\text{Ac-7}$. They can also be seen when the exact same TLC is measured again the next day. The other two spots disappear at the radio-TLC after one day. When measuring at additional time points (e.g., 2 h, 3 h etc.), gradually decreasing intensity is observed. This most likely fits the physical half-life of ^{213}Bi and the ability of Bi to form complexes with DOTA conjugates as a trivalent metal. Therefore, $R_f > 0.7$ is free ^{213}Bi , whereas the spot at $R_f = 0.1\text{--}0.2$ is considered to be [^{213}Bi]Bi-DOTAGA.Glu.(FAPi) $_2$, also due its gradual increasing intensity during the reaction.

Higher amounts were used for labeling in similar conditions: 1.6–3.2 MBq [^{225}Ac]AcCl₃ and 30–40 nmol/MBq were heated in 1 mL 0.1 M sodium ascorbate (pH = 7.0) at 95 °C for 60 min (n = 3). Using 40 instead of 30 nmol/MBq did not significantly influence the RCC; hence, the data were merged. The reaction kinetics were analyzed via radio-TLCs (Figure 5) that were imaged one hour after the TLC had been developed, and the exact same TLC was measured again the next day.

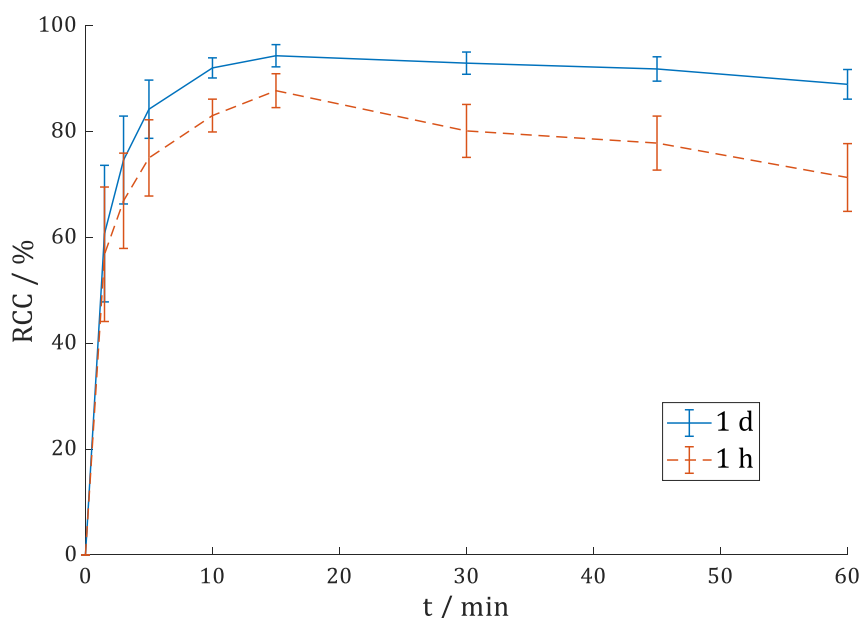


Figure 5. Reaction kinetics (radiochemical conversion (RCC) in %) of [^{225}Ac]Ac-DOTAGA.Glu.(FAPi)₂ ^{225}Ac -7 in 0.1 M sodium ascorbate (pH = 7.0) at 95 °C with 1.6–3.2 MBq ^{225}Ac (n = 3).

Figure 5 shows the underestimation of the imaging after one hour compared to imaging after one day. The maximum RCC of $87.7 \pm 3.2\%$ (1 h) and $94.3 \pm 2.1\%$ (1 d) is reached after 15 min of heating. The RCC is not quantitative, but increasing to 40 nmol/MBq did not increase RCC. Higher activity concentrations per volume (MBq/mL) might improve the yield, for example by using more activity, less volume or both.

With longer heating times, the RCC decreases again, which might indicate radiolysis. The effect is stronger in the data after one hour. KELLY et al. [59] also found out that the underestimation is not linear and varies depending on the value of RCP/RCC, which might play a role here. In conclusion, the heating should be shortened (15–30 min) in future ^{225}Ac -labelings of **7**. Lower reaction temperatures should also be investigated in future labeling experiments since the solution became slightly yellow after long heating times, which is probably due to the oxidation of ascorbate.

The RCCs were determined with AmOAc (pH = 4.0)/MeOH (1:1) radio-TLC and gamma spectroscopy measurements of a citrate TLC strip match and confirm the results (Table 1).

Table 1. Comparison of the determined radiochemical conversion rates (RCCs) with the different methods at different time points of the reaction (15 and 60 min: n = 3; after C18 cartridge purification: n = 1). The imaging/measurement was carried out 1 hour (1 h) or 1 day (1 d) after the radio TLC had been developed.

RCC/%	1 h (Citrate)	1 d (Citrate)	1 d (AmOAc/MeOH)	1 h (γ)
after 15 min	87.7 $\pm$ 3.2%	94.3 $\pm$ 2.1%	-	-
after 60 min	71.3 $\pm$ 6.4%	88.9 $\pm$ 2.8%	90.8 $\pm$ 2.3%	94.8 $\pm$ 2.5%
after C18	93.9%	98.1%	97.9%	100%

For the complex stability measurements of ^{225}Ac -**7** in HS and PBS, 350–400 kBq of the labeling solution was directly added to HS and PBS (0.5 mL, n = 3) and incubated for 20 days at 37 °C (Figure S9). The complex ^{225}Ac -DOTAGA.Glu.(FAPi)₂ remains stable in PBS for a couple of days (89.9 $\pm$ 0.7% after 2 d), but then, gradual radiolysis occurs (45.7 $\pm$ 0.2% after 20 d). In contrast, the stability in HS is much higher despite having the same activity concentration (85.5 $\pm$ 1.7% after 20 d). Higher dilution and the addition of sodium ascorbate and ethanol results in a high stability of the pure final formulation (95.4 $\pm$ 1.0% after 20 d). Both are known to be scavengers and are able to reduce radiolysis. It seems reasonable to dilute the final product and add scavengers as much as possible, especially for ^{225}Ac -radiopharmaceuticals due to the high-energy alpha-particles and the high potential for radiolysis.

3.2.4. [^{68}Ga]Ga-DO3A.Glu.(FAPi)₂ (^{68}Ga -**11**)

Labeling of DO3A.Glu.(FAPi)₂ **11** with gallium-68 was performed similarly to labeling of **7**. Since the generator had much lower activities, cationic post-processing was carried out before labeling [53]. In addition, 1 M HEPES buffer was tested at two different pH values (4.5 and 5.5). Three different precursor amounts were used to label 100 MBq ^{68}Ga at each pH value (Figure 6).

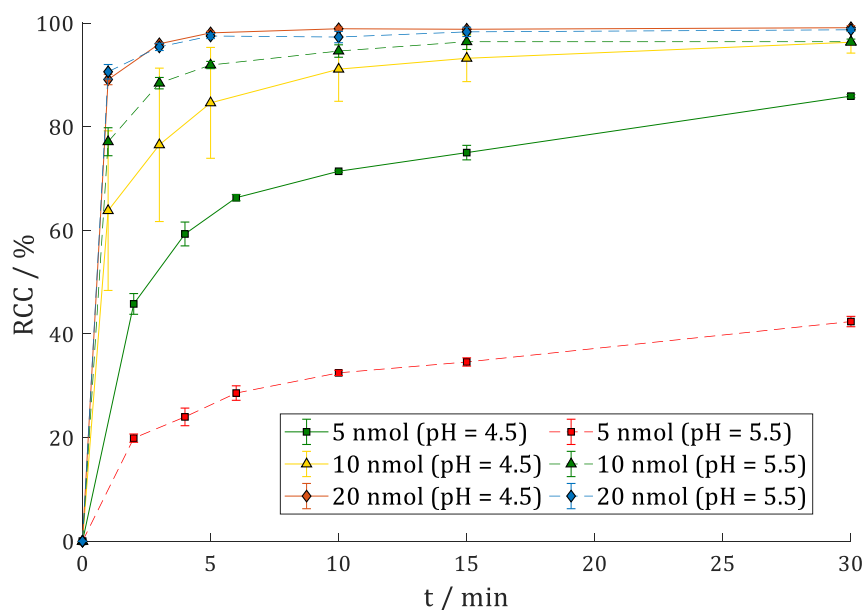


Figure 6. Reaction kinetics (radiochemical conversion (RCC) in %) of $[^{68}\text{Ga}]\text{Ga-DO3A.Glu.}(\text{FAPi})_2$ **$^{68}\text{Ga-11}$** in 1 M HEPES (pH = 4.5 or 5.5) at 95 °C with 100 MBq ^{68}Ga ($n = 3$).

Only with 5 nmol of precursor **11** the differences were significant, and pH = 4.5 seems to be better suited than pH = 5.5 ($85.9 \pm 0.3\%$ vs. $42.4 \pm 1.0\%$ after 30 min). For 10 nmol ($96.3 \pm 2.1\%$ vs. $96.4 \pm 0.5\%$) and 20 nmol ($99.1 \pm 0.4\%$ vs. $98.7 \pm 0.3\%$), the RCCs were almost identical. This suggests that the pH value of the reaction is flexible to a certain extent. Radio-HPLC (Figure S15) shows RCP = 98.7%.

The complex stability of **$^{68}\text{Ga-11}$** was determined analogously to **$^{68}\text{Ga-7}$** . Almost no radiolysis had taken place, and after 120 min, still > 98% of complexes were intact in both HS and PBS (Figure S10).

3.2.5. $[^{177}\text{Lu}]\text{Lu-DO3A.Glu.}(\text{FAPi})_2$ (**$^{177}\text{Lu-11}$**)

$[^{177}\text{Lu}]\text{Lu-DO3A.Glu.}(\text{FAPi})_2$ **$^{177}\text{Lu-11}$** was synthesized similarly to the DOTAGA derivative **$^{177}\text{Lu-7}$** . When comparing the reaction kinetics in Figure 7 with Figure 4, the labeling kinetics of **$^{177}\text{Lu-11}$** are somewhat slower than those of **$^{177}\text{Lu-7}$** .

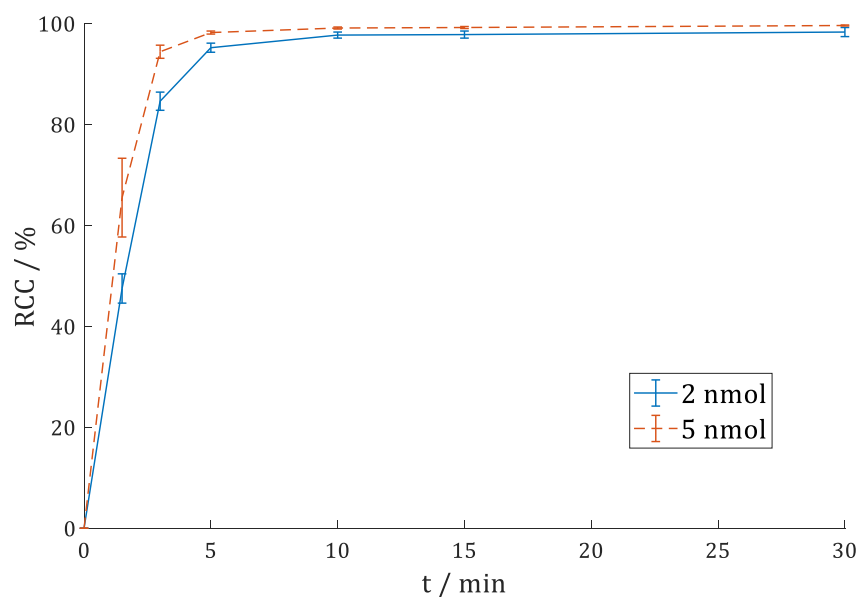


Figure 7. Reaction kinetics (radiochemical conversion (RCC) in %) of $[^{177}\text{Lu}]\text{Lu-DO3A.Glu.(FAPi)}_2$ $^{177}\text{Lu-11}$ in 1 M HEPES (pH = 5.5) at 95 °C with 50–100 MBq ^{177}Lu ($n = 5$).

Whereas the RCC is > 99% after 1.5 min for 2 nmol **13**, it takes 5 min to reach > 95% with $^{177}\text{Lu-11}$, and even 5 min to obtain RCC > 98% with 5 nmol $^{177}\text{Lu-11}$. Nevertheless, $^{177}\text{Lu-11}$ can be synthesized in practically quantitative RCCs (> 98% with 2 nmol for 30 min; > 99% with 5 nmol for 10 min). The second TLC system and radio-HPLC (cf. Figure S16) also gave almost quantitative RCC/RCP.

The complex stability measurements of $^{177}\text{Lu-11}$ were performed as described before for $^{177}\text{Lu-7}$. Slight decomposition could be observed in PBS ($94.7 \pm 2.0\%$ intact conjugates after 14 days), whereas $^{177}\text{Lu-11}$ seems to be stable in HS, with both having 20 MBq/mL in the beginning (Figure S11).

$^{177}\text{Lu-11}$ is slightly less stable than $^{177}\text{Lu-7}$ since the additional carboxyl group of DOTAGA might be able to take part in the complexation of lutetium-177, which could give it additional stability. Lutetium can form a complex with unconjugated DOTA where all eight donor ligands ($4 \times \text{N}$ in the macrocyclic + $4 \times \text{O}^-$ from the acetate arms) are part of the complex [60].

3.2.6. $[^{90}\text{Y}]\text{Y-DO3A.Glu.(FAPi)}_2$ ($^{90}\text{Y-11}$)

DO3A.Glu.(FAPi)₂ **11** was additionally labeled with yttrium-90 in 1 M HEPES buffer (pH = 4.5). For these reaction kinetic studies, 5 and 10 nmol **11** were labeled with 30–60 MBq $[^{90}\text{Y}]\text{YCl}_3$ in 400 μL buffer at 95 °C. The reaction kinetics were analyzed via radio-TLC and (see Figure 8).

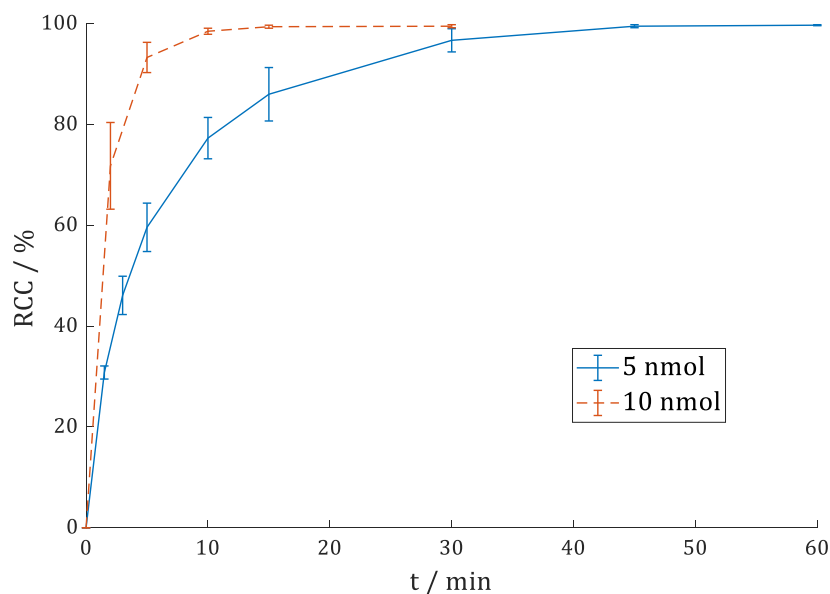


Figure 8. Reaction kinetics (radiochemical conversion (RCC) in %) of $[^{90}\text{Y}]\text{Y-DO3A.Glu.}(\text{FAPi})_2$ **$^{90}\text{Y-11}$** in 1 M HEPES (pH = 4.5) at 95 °C with 30–60 MBq ^{90}Y ($n = 3$).

Quantitative RCC > 99% could be achieved with 10 nmol in 15 min. With 5 nmol, the reaction is much slower, but after 45 min, RCC > 99% could also be reached.

Subsequent complex stability studies of **$^{90}\text{Y-11}$** were carried out the same way as with ^{68}Ga - and ^{177}Lu -complexes. The complex can be considered stable (>98%) in both HS and PBS after more than two half-lives (6 days) at 37 °C (see Figure S12).

3.2.7. Lipophilicity

The lipophilicity was investigated experimentally via the shake-flask method for the ^{68}Ga -complexes **$^{68}\text{Ga-7}$** and **$^{68}\text{Ga-11}$** as well as for the ^{177}Lu -complexes **$^{177}\text{Lu-7}$** and **$^{177}\text{Lu-11}$** with ca. 10 MBq per vial ($n = 4$) with PBS and 1-octanol. The $\log D_{7.4}$ values were obtained by determining the integral of each spot on the radio-TLC and calculation with Equation (1). Table 2 gives an overview and compares the $\log D_{7.4}$ values with those of previous works from our group [29,31].

Table 2. $\log D_{7.4}$ values of different labeled FAPi-based radiopharmaceuticals. The values for $[^{68}\text{Ga}]\text{Ga-DO3A.SA.FAPi}$ and $[^{68}\text{Ga}]\text{Ga-DOTAGA.}(\text{SA.FAPi})_2$ are taken from MOON et al. [29] and MOON et al. [31].

Complex	$\log D_{7.4}$
$[^{68}\text{Ga}]\text{Ga-DO3A.SA.FAPi}$ [29]	-2.68 ± 0.06
$[^{68}\text{Ga}]\text{Ga-DOTAGA.}(\text{SA.FAPi})_2$ [31]	-2.02 ± 0.06
$[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu.}(\text{FAPi})_2$ ($^{68}\text{Ga-7}$)	-2.48 ± 0.05
$[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu.}(\text{FAPi})_2$ ($^{177}\text{Lu-7}$)	-2.77 ± 0.10
$[^{68}\text{Ga}]\text{Ga-DO3A.Glu.}(\text{FAPi})_2$ ($^{68}\text{Ga-11}$)	-2.08 ± 0.07
$[^{177}\text{Lu}]\text{Lu-DO3A.Glu.}(\text{FAPi})_2$ ($^{177}\text{Lu-11}$)	-1.77 ± 0.10

3.2.8. Comparison with DOTAGA.(SA.FAPi)₂

The monomeric radiotracer [⁶⁸Ga]Ga-DOTA.SA.FAPi showed relatively strong hydrophilic characteristics. This may be one of the reasons that in vivo PET studies indicated a relatively fast renal clearance [58]. In contrast, the dimer [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂ predominantly showed biliary excretion over renal excretion [39]. The washout from the hepatobiliary system/liver was slower than that of the monomeric radiotracers (the same for the renal excretion). In particular, [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂ remained in the large intestines/colon region for a longer time during excretion. The colon was identified as the critical organ, and the radiation dose to the kidneys was increased compared to the monomer [39,52]. Therefore, we tried to synthesize compounds that are more hydrophilic than DOTAGA.(SA.FAPi)₂ to reduce biliary excretion and thus the radiation dose to the colon.

The DOTAGA complexes **⁶⁸Ga-7** and **¹⁷⁷Lu-7** are definitely more hydrophilic than the first-generation dimer DOTAGA.(SA.FAPi)₂ and the DOTA complexes **⁶⁸Ga-11** and **¹⁷⁷Lu-11**. The hydrophilicity of **⁶⁸Ga-7** is comparable to the monomer [⁶⁸Ga]Ga-DOTA.SA.FAPi. Radioligand therapy (RLT) with radiometals such as lutetium-177 is the main objective of these new dimers. [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ **¹⁷⁷Lu-7** shows the highest hydrophilicity of all complexes ($\log D_{7.4} = -2.77 \pm 0.10$). There is no $\log D_{7.4}$ value for [¹⁷⁷Lu]Lu-DOTA.SA.FAPi that allows for a direct comparison. Nonetheless, these results are very promising to translate this molecule from bench to bedside.

The molecular design of the FAPi homodimers DOTA.(SA.FAPi)₂ and DOTAGA.(SA.FAPi)₂ may create radiolabeling limitations due to steric hindrance as a result of the chelator being in the center of the molecule. The DOTA chelator only has six donor ligands because it is attached to the two targeting vectors on two binding sites, which only allow for labeling with gallium-68 [31]. The DOTAGA derivative has seven donor ligands for complexation and can be labeled with lutetium-177, while it was cumbersome in the case of actinium-225. The new FAPi dimer DOTAGA.Glu.(FAPi)₂ **7** has eight donor ligands and can be labeled with actinium-225 with high RCC > 90%. ²²⁵Ac-labeling of DO3A.Glu.(FAPi)₂ **11** was just as problematic as with DOTAGA.(SA.FAPi)₂. In general, DOTA-conjugated precursors have been investigated for ²²⁵Ac-labeling quite extensively and allow for efficient labeling when the chelator is in exo-position [61–66].

In summary, DOTAGA.Glu.(FAPi)₂ showed superior labeling properties compared to the other dimeric compounds.

3.3. In Vitro Inhibition Assays

High affinity for FAP and low affinity for the very similar proteases PREP and DPPs (DPP4, DPP8 and DPP9) are important requirements for FAPi-based radiopharmaceuticals to be promising candidates for subsequent in vivo studies.

The IC_{50} values were determined in in vitro studies. Table 3 summarizes the results of the IC_{50} measurements. The selectivity index (SI) is the ratio of the IC_{50} value of the respective protease (PREP, DPP4, DPP8 and DPP9) to the IC_{50} value of FAP.

Table 3. IC_{50} value and selectivity index (SI) of different FAP inhibitors: DOTAGA.Glu.(FAPi)₂ **7**, [^{nat}Lu]Lu-DOTAGA.Glu.(FAPi)₂ **natLu-7**, DO3A.Glu.(FAPi)₂ **11** as well as DOTAGA.(SA.FAPi)₂, [^{nat}Lu]Lu-DOTAGA.(SA.FAPi)₂ and the initial FAP inhibitor UAMC-1110 from previous works (JANSEN et al. [24] and MOON et al. [31]).

Compound	DOTAGA.Glu.(FAPi) ₂ (7)	[^{nat} Lu]Lu-DOTAGA.Glu.(FAPi) ₂ (natLu-7)	DO3A.Glu.(FAPi) ₂ (11)	DOTAGA.(SA.FAPi) ₂ [31]	[^{nat} Lu]Lu-DOTAGA.(SA.FAPi) ₂ [31]	UAMC-1110 [24,31]
$IC_{50}(FAP)/nM$	0.26 ± 0.04	0.33 ± 0.02	0.60 ± 0.04	0.92 ± 0.06	1.54 ± 0.15	0.43 ± 0.02
$IC_{50}(PREP)/\mu M$	0.59 ± 0.10	0.43 ± 0.16	1.00 ± 0.14	0.39 ± 0.02	0.56 ± 0.04	1.80 ± 0.01
$IC_{50}(DPP4)/\mu M$	1.19 ± 0.08	0.65 ± 0.04	0.54 ± 0.06	0.40 ± 0.07	0.63 ± 0.07	>10
$IC_{50}(DPP8)/\mu M$	0.029 ± 0.004	0.22 ± 0.02	1.03 ± 0.18	0.42 ± 0.04	0.41 ± 0.03	>10
$IC_{50}(DPP9)/\mu M$	0.083 ± 0.0015	0.19 ± 0.01	0.95 ± 0.11	0.16 ± 0.02	0.18 ± 0.02	4.70 ± 0.40
$SI(PREP/FAP)$	2269	1292	1667	424	364	4186
$SI(DPP4/FAP)$	4577	1967	900	435	409	23,256
$SI(DPP8/FAP)$	112	661	1717	456	266	23,256
$SI(DPP9/FAP)$	319	557	1583	174	117	10,930

Compounds **7**, **natLu-7** and **11** show very high affinity for FAP, as reflected in the subnanomolar IC_{50} values. The affinities of **7** and **natLu-7** are very similar, and the different selectivity indexes do not differ substantially as well. This supports previous results where the IC_{50} values of the precursor and its respective metal complex (^{nat}Ga or ^{nat}Lu) do not differ much [29–31], as can be seen with DOTAGA.(SA.FAPi)₂ and [^{nat}Lu]Lu-DOTAGA.(SA.FAPi)₂ for example (see Table 3) [31].

The affinity and selectivity for all new compounds were better than for DOTAGA.(SA.FAPi)₂. This also includes the comparison of the Lu-complex ^{nat}Lu-**7** with [^{nat}Lu]Lu-DOTAGA.(SA.FAPi)₂.

Although selectivity indexes (SIs) are lower compared to UAMC-1110, high selectivity for FAP over PREP and DPP4 was measured, which are most relevant among the related proteases. In contrast to FAP, they are ubiquitously expressed in healthy tissue. A high affinity for PREP or DPP4 would lead to worsened tumor selectivity and a diminished tumor-to-background ratio in PET/SPECT scans and/or RNT. Overall, these results strongly suggest that compounds **7** and **11** might also have superior properties in vivo compared to DOTAGA.(SA.FAPi)₂ and that especially FAPi dimer **7** is suitable for further investigations.

The IC₅₀(FAP) values reported for BiOncoFAP-DOTAGA and [^{nat}Lu]Lu-BiOncoFAP-DOTAGA were 0.17 and 0.19 nM, respectively [50]. These values seem to be equal to **7** and ^{nat}Lu-**7**, although it has to be noted that the assay conditions were different (substrate/enzyme/buffer concentration, temperature, volume etc.). For the dimer [⁶⁸Ga]Ga-DOTA-2P(FAPi)₂, the assay setup differed drastically, as a competitive assay of the radiolabeled agent against FAPi-46 was carried out and resulted in an IC₅₀ = 3.68 ± 1.82 nM [48]. The different assay conditions, especially of ZHANG et al. [48], limit the comparability of the different IC₅₀ values. On top, no IC₅₀ values for the related proteases and therefore no selectivity indexes were reported by ZHAO et al. [48] or by GALBIATI et al. [50].

3.4. Patient Study (Medullary Thyroid Cancer)

A 40-year-old male was diagnosed with medullary thyroid cancer in 2016. The patient underwent total thyroidectomy and bilateral radical neck dissection in prior treatments.

[¹⁸F]F-FDG and [⁶⁸Ga]Ga-DOTA.SA.FAPi PET/CT scans were carried out. [¹⁸F]F-FDG PET/CT (see Figure 9a) showed an FDG-avid soft tissue lesion noted measuring 3 × 3.5 cm in the thyroid bed region (SULpeak: 3.4), avid bilateral level VI, bilateral supraclavicular lymph nodes noted, sub-centimetric tracer avid level V lymph nodes, superior mediastinal/bilateral upper paratracheal, and bilateral paraoesophageal lymph nodes. Few hypodense lesions were noted in the right lobe of the liver involving segment V with low [¹⁸F]F-FDG uptake of 1.75. Diffuse physiological FDG distribution was seen in the axial and appendicular skeleton. Unlike [¹⁸F]F-FDG, [⁶⁸Ga]-DOTA.SA.FAPi showed superior uptake in all the lesions (soft tissue neck lesion: SULpeak 17.98) corresponding to [¹⁸F]F-FDG PET/CT scan and better delineation in the liver lesions (SULpeak:10.5) on MIP itself (see Figure 9b). The patient was administered two cycles of [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂. Figure 9c shows whole-body scans at 24 and 48 h post-injection. Despite uptake in the lesions (neck nodes and mass), huge amounts of radiotracer uptake were observed in the liver (24 h p.i.) and colon (48 h p.i.), causing high radiation burden to the colon

and liver. For the third cycle of treatment, the new FAPi dimer [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ ^{177}Lu -7 was considered.

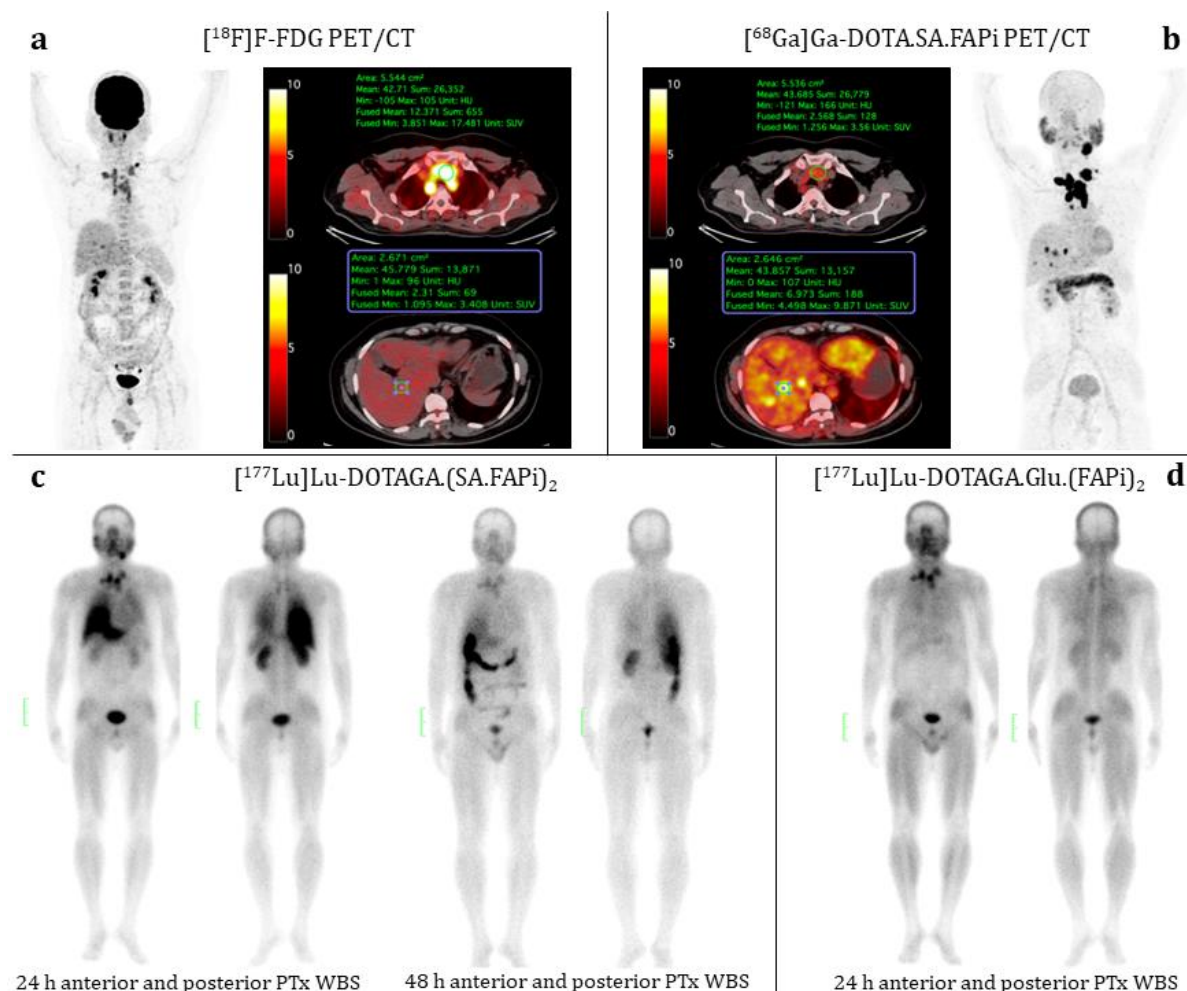


Figure 9. A 40-year-old male diagnosed with medullary thyroid carcinoma underwent [^{18}F]F-FDG PET/CT scan (a) followed by [^{68}Ga]Ga-DOTA.SA.FAPi PET/CT scan (b) with the scale being SUV in %ID/g. The patient underwent two cycles of 5.5 GBq of [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$ therapy (c). The post-treatment whole-body scan at 24 h p.i. (c, left) reveals intense uptake in the neck and mediastinal metastases but a simultaneous intense radiotracer accumulation in the liver. In addition, 48 h p.i. shows the hepatobiliary excretion (c, right). Subsequent treatment in the same patient with 5.5 GBq of the new dimer [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ ^{177}Lu -7 showed only uptake in the neck lesions on whole-body scan at 24 h p.i. (d) and negligible uptake in the liver.

The patient was administered 5.5 GBq of [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ ^{177}Lu -7, which was previously synthesized according to the procedure described in Methods and Materials (Section 2.5.1). Furthermore, 24 h p.i. high uptake in the neck and mediastinal metastases was comparable to the first-generation dimer. In contrast, only negligible radiotracer uptake in the liver and colon was observed. This suggests a different excretion pattern, possibly a higher proportion of renal instead of hepatobiliary excretion (improved pharmacodynamic profile) or a faster washout

(improved pharmacokinetic profile), or both, which results in a significantly reduced radiation dose to the critical healthy organs liver and colon.

While this is a first proof-of-concept investigation and not of quantitative dimension, the results support the assumption that the new ^{177}Lu -labeled FAPi homodimer ^{177}Lu -**7** has favorable pharmacologic properties compared to ^{177}Lu -DOTAGA.(SA.FAPi)₂. More detailed studies regarding biodistribution as well as safety and efficacy are currently ongoing, which are needed to better understand the pharmacokinetic and pharmacodynamic behavior of ^{177}Lu -DOTAGA.Glu.(FAPi)₂ ^{177}Lu -**7** in systematic studies.

4. Conclusions

DOTAGA.Glu.(FAPi)₂ and DO3A.Glu.(FAPi)₂ represent the second generation of our homodimeric FAPi compounds. They were synthesized with glutamic acid as a central linker unit and successfully labeled with different trivalent radiometals. Both precursors show excellent labeling properties with ^{68}Ga and ^{177}Lu . In contrast to DO3A.Glu.(FAPi)₂ and the first-generation dimer DOTAGA.(SA.FAPi)₂, DOTAGA.Glu.(FAPi)₂ could also be labeled successfully with ^{225}Ac in high yields. All complexes showed high stability and hydrophilicity. Beyond that, IC₅₀ values were determined to confirm the very high affinity for fibroblast activation protein alpha (FAP) and high selectivity for FAP over prolyl endopeptidase (PREP) and the dipeptidyl peptidase (DPP4). The in vitro studies showed the best results for DOTAGA.Glu.(FAPi)₂, but the results for DO3A.Glu.(FAPi)₂ were also better than for DOTAGA.(SA.FAPi)₂. It is also noteworthy that the respective FAP affinity of the new homodimeric compounds is higher than for the initial FAP inhibitor UAMC-1110 itself, which was measured in the same assay. Similar FAP affinities were reported for BiOncoFAP-DOTAGA and its ^{nat}Lu -complex by GALBIATI et al. [50], but no selectivity data were reported. The comparison of the IC₅₀ values is difficult and should be cautiously interpreted, as the inhibition assays are different from each other (especially regarding ZHAO et al. [48]).

DOTAGA.Glu.(FAPi)₂ proved to be the most promising candidate for further in vivo studies that are currently ongoing. The compound seems especially suited for therapeutic applications with ^{177}Lu , ^{90}Y and ^{225}Ac . Especially, FAP-targeted alpha-particle therapy (FAP-TAT) with ^{225}Ac -radiopharmaceuticals is a new treatment option that could potentially be more efficacious than with beta-emitting therapeutics since stromal cells, similar to how CAFs are reported to be more resistant to radiation [67–69]. Therefore, particles with higher energy and linear energy transfer such as alpha particles might be needed to address the specifics of the tumor stroma. The stroma can build a physical barrier around the tumor that is hard to penetrate for some agents (e.g., chemotherapeutics), which results in poor efficacy [5,70,71]. The destruction of tumor stroma with alpha-emitters in combination with other non-radioactive agents (e.g., chemotherapeutics,

targeted drug delivery systems, CAR T-cell therapy etc.) might also be a promising therapy approach, and [^{225}Ac]Ac-DOTAGA.Glu.(FAPi) $_2$ might be an interesting candidate for FAP-TAT in the future.

A first proof-of-concept patient study with [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ showed promising results. The pharmacological properties are superior to those of [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$ with a high radiotracer uptake and long tumor retention in lesions. At the same time, the excretion kinetics are faster, and minimized uptake in non-target tissues, especially the colon as the critical organ for [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$, was observed. Improved pharmacologic properties are essential to ensure a safe therapy without adverse side effects. Further studies are currently ongoing and will investigate biodistribution, efficacy and safety in more detail.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Organic synthesis of FAPi-NH $_2$ **1**: (a) HBr (47%), 126 °C, 4 h, 96%; (b) Boc $_2$ O, TEA, THF, RT, 19 h, 66%; (c) HBr (47%), 126 °C, 1 d, 100%; (d) SOCl $_2$, MeOH, 0 °C–RT, 2 d, 100%; (e) Cs $_2$ CO $_3$, DMF, 70 °C, 1 d, 59%; (f) 1 M LiOH, 1,4-dioxane, RT, 4 h, 57%; (g) HATU, DIPEA, DCM/DMF (1:1), RT, 19 h, 86%; (h) TFAA, pyridine, THF, DCM, RT, 3 h, 81%; (i) TFA, MeCN, RT, 5 h, 100%; (j) HBTU, HOBT, DIPEA, DMF, RT, 1 d, 72%; (k) 4 M HCl in 1,4-dioxane, MeCN, 0 °C–RT, 7 h, 100%; Figure S2: Analytical RP-HPLC of DOTAGA.Glu.(FAPi) $_2$ **7**, 20–30% MeCN + 0.1% TFA (linear gradient) in 20 min, 5 mL/min: purity = 99.0%; Figure S3: Analytical RP-HPLC of DO3A.Glu.(FAPi) $_2$ **11**, 20–30% MeCN + 0.1% TFA (linear gradient) in 20 min, 5 mL/min: purity = 98.5%; Figure S4: Reaction kinetics (radiochemical conversion (RCC) in %) of [^{68}Ga]Ga-DOTAGA.Glu.(FAPi) $_2$ **^{68}Ga -7** in 1 M AmOAc (pH = 4.5) at 95 °C with 100 MBq ^{68}Ga (n = 1); Figure S5: Complex stability of [^{68}Ga]Ga-DOTAGA.Glu.(FAPi) $_2$ **^{68}Ga -7** in human serum (HS) and phosphate-buffered saline (PBS) over 120 min (n = 3); Figure S6: Complex stability of [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ **^{177}Lu -7** in human serum (HS) and phosphate-buffered saline (PBS) over 14 days (n = 3); Figure S7: Exemplary reaction kinetic of [^{225}Ac]Ac-DOTAGA.Glu.(FAPi) $_2$ **^{225}Ac -7** at 95 °C analyzed with 0.1 M citrate buffer (pH = 4.0) radio-TLC from 0–15 min (left to right). Exemplary profiles of citrate radio-TLC after 60 minutes at 95 °C are shown in the middle. The corresponding 1 M AmOAc (pH = 4.0)/MeOH (1:1) radio-TLCs are shown on the right. All TLCs were imaged at different time points after 1 h (top) and 1 day (bottom). These measurements together make clear that: $R_f(\text{free } ^{225}\text{Ac}) \approx 0.5$ and $R_f(\text{product } ^{225}\text{Ac-7}) = 0.0$. The other two spots appearing in a) must be from one of the short-lived daughter nuclides, most likely ^{213}Bi since the spots are slowly decaying when measuring the TLC over several hours, which fits the physical half-life and Bi is a trivalent metal that can be complexed by DOTA conjugates. Therefore, $R_f(\text{free } ^{213}\text{Bi}) > 0.7$ can be assigned to be free ^{213}Bi and $R_f(^{213}\text{Bi-complex}) = 0.1\text{--}0.2$ is considered to be the $^{213}\text{Bi-complex}$; Figure S8: Reaction kinetics (radiochemical conversion (RCC) in %) of [^{225}Ac]Ac-

DOTAGA.Glu.(FAPi)₂ ²²⁵Ac-**7** in 0.1 M sodium ascorbate (pH = 7.0) at 95 °C with 500 kBq ²²⁵Ac (n = 2); Figure S9: Complex stability of [²²⁵Ac]Ac-DOTAGA.Glu.(FAPi)₂ ²²⁵Ac-**7** in human serum (HS) and phosphate-buffered saline (PBS) (0.7–0.8 MBq/mL) and final formulation (“pure”, 0.2 MBq/mL) after C18 cartridge purification over 20 days (n = 3); Figure S10: Complex stability of [⁶⁸Ga]Ga-DO3A.Glu.(FAPi)₂ ⁶⁸Ga-**11** in human serum (HS) and phosphate-buffered saline (PBS) over 120 min (n = 3); Figure S11: Complex stability of [¹⁷⁷Lu]Lu-DO3A.Glu.(FAPi)₂ ¹⁷⁷Lu-**11** in human serum (HS) and phosphate-buffered saline (PBS) over 14 days (n = 3); Figure S12: Complex stability of [⁹⁰Y]Y-DO3A.Glu.(FAPi)₂ ⁹⁰Y-**11** in human serum (HS) and phosphate-buffered saline (PBS) over 6 days (n = 3); Figure S13: Analytical Radio-HPLC of [⁶⁸Ga]Ga-DOTAGA.Glu.(FAPi)₂ ⁶⁸Ga-**7** (*t_R* = 10.5 min, 40 nmol **7**, 400 MBq ⁶⁸Ga), 20–70% MeCN + 0.1% TFA (linear gradient) in 20 min: RCP = 99.1%; Figure S14: Analytical Radio-HPLC of [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ ¹⁷⁷Lu-**7** (*t_R* = 10.3 min, 1 nmol **7**, 100 MBq ¹⁷⁷Lu), 20–50% MeCN + 0.1% TFA (linear gradient) in 20 min: RCP = 99.8%; Figure S15: Analytical Radio-HPLC of [⁶⁸Ga]Ga-DO3A.Glu.(FAPi)₂ ⁶⁸Ga-**11** (*t_R* = 9.5 min, 20 nmol **11**, 100 MBq ⁶⁸Ga), 10–90% MeCN + 0.1% TFA (linear gradient) in 20 min: RCP = 98.7%; Figure S16: Analytical Radio-HPLC of [¹⁷⁷Lu]Lu-DO3A.Glu.(FAPi)₂ ¹⁷⁷Lu-**11** (*t_R* = 10.1 min, 2 nmol, 100 MBq ¹⁷⁷Lu), 20–50% MeCN + 0.1% TFA (linear gradient) in 10 min: RCP = 99.9%.

Author Contributions: Conceptualization, F.R., C.B., I.D.M. and P.V.D.V.; methodology, M.M., S.B., M.P.Y., Y.V.R., J.D.L. and E.V.; validation, M.M., S.B., M.P.Y., Y.V.R., J.D.L. and E.V.; formal analysis, M.M., S.B., M.P.Y., Y.V.R., J.D.L. and E.V.; investigation, M.M., S.B., M.P.Y., Y.V.R., J.D.L. and E.V.; resources, F.R., C.B., I.D.M. and P.V.D.V.; data curation, M.M., S.B. and Y.V.R.; writing—original draft preparation, M.M.; writing—review and editing, M.M., S.B., Y.V.R., E.V., I.D.M., P.V.D.V. and F.R.; supervision and project administration, F.R., C.B., I.D.M. and P.V.D.V. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and was approved by the Institutional Ethics Committee of All India Institute of Medical Sciences (Ref. no. IECPG-22/27.02.2020 for the clinical use of ⁶⁸Ga-labeled FAPi radiotracers and Ref. no. IEC-483/05.06.2020 for the clinical use of ¹⁷⁷Lu-labeled FAPi radiotracers).

Informed Consent Statement: All patients gave their written informed consent to participate in this study.

Data Availability Statement: Data are contained within the article and Supplementary Material.

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Conflicts of Interest: Marcel Martin and Frank Roesch have filed a patent (WO/2022/258637) on DOTAGA.Glu.(FAPi) $_2$, DO3A.Glu.(FAPi) $_2$ and their radiolabeled derivatives.

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Supporting Information

Organic synthesis of FAPi-NH₂

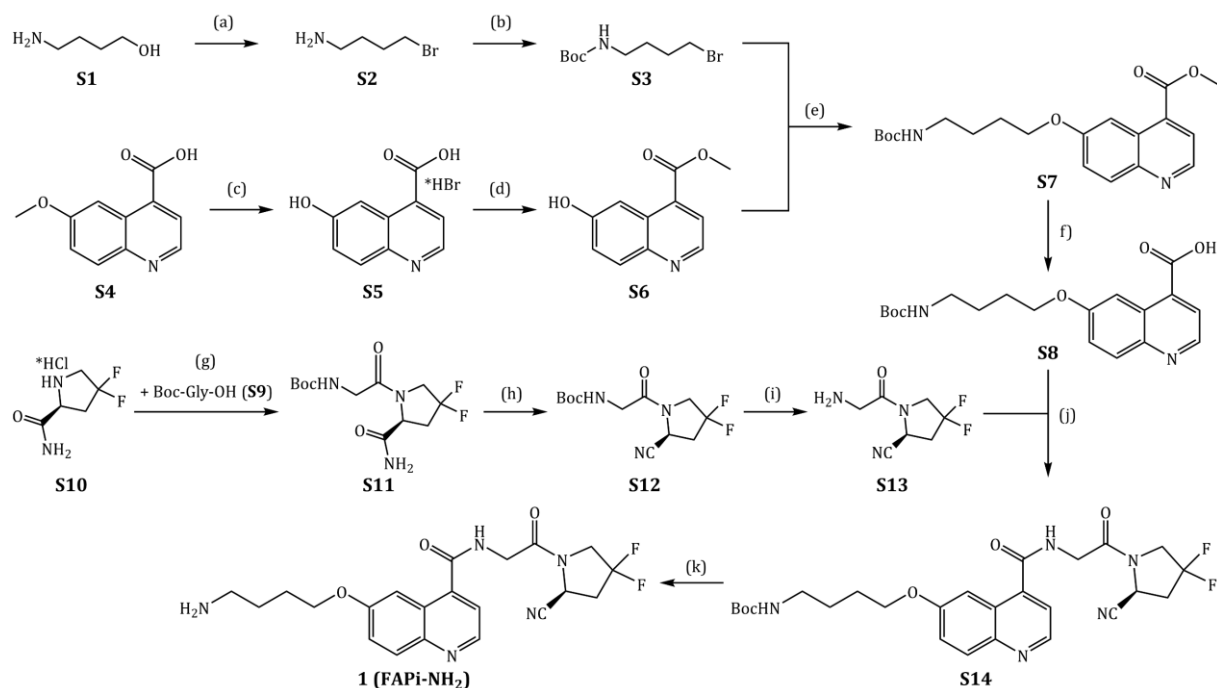


Figure S1. Organic synthesis of FAPi-NH₂ **1**: (a) HBr (47%), 126 °C, 4 h, 96%; (b) Boc₂O, TEA, THF, RT, 19 h, 66%; (c) HBr (47%), 126 °C, 1 d, 100%; (d) SOCl₂, MeOH, 0 °C–RT, 2 d, 100%; (e) Cs₂CO₃, DMF, 70 °C, 1 d, 59%; (f) 1 M LiOH, 1,4-dioxane, RT, 4 h, 57%; (g) HATU, DIPEA, DCM/DMF (1:1), RT, 19 h, 86%; (h) TFAA, pyridine, THF, DCM, RT, 3 h, 81%; (i) TFA, MeCN, RT, 5 h, 100%; (j) HBTU, HOBT, DIPEA, DMF, RT, 1 d, 72%; (k) 4 M HCl in 1,4-dioxane, MeCN, 0 °C–RT, 7 h, 100%.

4-Bromobutylamine (**S2**)

47% hydrobromic acid (HBr, 70 mL) was slowly added to 4-aminobutanol (**S1**, 5.39 g, 60.5 mmol, 1.00 eq) and then heated for 4 hours under reflux. Then, the reaction mixture was concentrated *in vacuo*. **S2** was obtained as a colorless solid (13.5 g, 58.0 mmol, 96%) and used directly in the next step without any further purification. ¹H-NMR (400 MHz, MeOD): δ [ppm] = 3.51 (t, *J* = 6.4 Hz, 2H), 2.98 (t, *J* = 7.6 Hz, 2H), 2.02 – 1.73 (m, 4H). MS (ESI⁺): *m/z* (%) = 152.0 (100, [M+H]⁺), 154.0 (98, [M+H]⁺), calculated for C₄H₁₀BrN: 151.00 [M].

tert-Butyl (4-bromobutyl)carbamate (**S3**)

4-Bromobutylamine (**S2**, 7.01 g, 30.1 mmol, 1.00 eq) and di-*tert*-butyl dicarbonate (Boc₂O, 7.34 g, 33.6 mmol, 1.12 eq) were dissolved in dry tetrahydrofuran (THF, 34 mL) under argon atmosphere. Triethylamine (TEA, 4.6 mL, 36.1 mmol, 1.20 eq) was then added, followed by MeOH (36 mL) to turn the suspension into a clear solution again. The solution was stirred overnight at RT. The solvent was removed *in vacuo* and diluted HBr was added to the residue until pH = 2.5.

The aqueous solution was extracted with diethyl ether (Et₂O, 5×80 mL) and the combined organic phases were washed once with NaHCO₃ (10 mL) and Brine (10 mL) and then dried over Na₂SO₄. The solvent was removed under reduced pressure and after column chromatography (cyclohexane/ethyl acetate (CH/EA, 5:1)) **S3** was obtained as a colorless solid (5.08 g, 20.2 mmol, 66%). ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 3.36 – 3.21 (m, 4H), 1.86 - 1.76 (m, 4H), 1.43 (s, 9H). MS (ESI⁺): m/z (%) = 196.0 (100, [M-^tBu+H]⁺), 198.0 (100, [M-^tBu+H]⁺), calculated for C₉H₁₈BrNO₂: 251.05 [M].

6-Hydroxyquinoline-4-carboxylic acid hydrobromide (S5)

6-Methoxyquinoline-4-carboxylic acid (**S4**, 2.46 g, 12.1 mmol, 1.00 eq) was dissolved in 47% HBr (28.2 mL, 242.4 mmol, 20 eq) and heated for one day under reflux. After cooling to RT, the hydrobromic acid was partially removed *in vacuo* and the precipitate was then filtered off and washed first with cold EA (20 mL) and then with cold EA/MeOH (9:1, 10 mL). **S5** was obtained as a yellow solid (3.25 g, 12.1 mmol, 100%). ¹H-NMR (400 MHz, MeOD): δ [ppm] = 9.05 (d, *J* = 5.6 Hz, 1H), 8.41 (d, *J* = 5.6 Hz, 1H), 8.33 (d, *J* = 2.6 Hz, 1H), 8.19 (d, *J* = 9.3 Hz, 1H), 7.77 (dd, *J* = 9.3, 2.6 Hz, 1H). MS (ESI⁺): m/z (%) = 190.0 (100, [M+H]⁺), 191.0 (12, [M+H]⁺), calculated for C₁₀H₈BrNO₃: 189.04 [M].

6-Hydroxyquinoline-4-carboxylic acid methyl ester (S6)

Dry MeOH (20 mL) was cooled to 0°C under argon atmosphere and SOCl₂ (4.43 mL, 61.1 mmol, 5.05 eq) was added dropwise. 6-Hydroxyquinoline-4-carboxylic acid hydrobromide (**S5**, 3.25 g, 12.1 mmol, 1.00 eq) was dissolved in dry MeOH (20 mL) and also cooled to 0°C under argon atmosphere. Then, the SOCl₂-MeOH solution was added dropwise to **S5**. At first, the reaction solution was allowed to warm to RT and then heated under reflux for one day. SOCl₂ (2.91 g, 24.4 mmol, 2.02 eq) and MeOH (20 mL) were again combined at 0 °C and added to the reaction mixture at RT. The solution was heated at reflux for an additional day. The step was repeated one more time and after another 4 hours of heating under reflux, the solvent was removed under reduced pressure. **S6** was obtained as a yellow solid (2.46 g, 12.1 mmol, 100%) which was used in the next step without any further purification. ¹H-NMR (400 MHz, MeOD): δ [ppm] = 9.02 (d, *J* = 5.5 Hz, 1H), 8.38 (d, *J* = 5.5 Hz, 1H), 8.24 (d, *J* = 2.6 Hz, 1H), 8.17 (d, *J* = 9.3 Hz, 1H), 7.75 (dd, *J* = 9.3, 2.6 Hz, 1H), 4.09 (s, 3H). MS (ESI⁺): m/z (%) = 204.0 (100, [M+H]⁺), 205.1 (12, [M+H]⁺), calculated for C₁₁H₉NO₃: 203.06 [M].

Methyl 6-(4-((tert-butoxycarbonyl)amino)butoxy)quinoline-4-carboxylate (S7, Boc-Chino-COOMe)

6-Hydroxyquinoline-4-carboxylic acid methyl ester (**S6**, 2.46 g, 12.1 mmol, 1.00 eq) and Cs₂CO₃ (4.37 g, 13.4 mmol, 1.25 eq) were suspended in dry DMF (55 mL). The reaction solution was heated to 70°C. Then, *tert*-butyl (4-bromobutyl)carbamate (**S3**, 3.76 g, 14.9 mmol, 1.22 eq),

dissolved in dry DMF (80 mL), was dropped into the reaction mixture. The solution was stirred for 3 hours at 70°C before some more **S3** (1.23 g, 4.88 mmol, 0.4 eq., again dissolved in dry DMF (20 mL), was added. It was stirred overnight at 70°C. After another addition of **S3** (308 mg, 1.22 mmol, 0.1 eq) and another 3 hours at 70°C, the solvent was removed *in vacuo* and the residue was taken up in diluted HBr (150 mL, pH = 2.6). It was extracted with EA (5×80 mL), and the organic phase was washed with Brine (20 mL) and dried over Na₂SO₄. The solvent was removed under reduced pressure and the product **S7** was purified via column chromatography (CHCl₃/MeOH, 100:1) to give a yellowish solid (2.68 g, 7.17 mmol, 59%). ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 8.84 (d, *J* = 4.5 Hz, 1H), 8.21 (d, *J* = 2.8 Hz, 1H), 8.07 (d, *J* = 9.2 Hz, 1H), 7.92 (d, *J* = 4.5 Hz, 1H), 7.41 (dd, *J* = 9.2, 2.8 Hz, 1H), 4.66 (s, 1H), 4.15 (t, *J* = 6.2 Hz, 2H), 4.02 (s, 3H), 3.23 (q, *J* = 6.7 Hz, 2H), 1.90 (tt, *J* = 8.6, 6.0 Hz, 2H), 1.77 – 1.68 (m, 2H), 1.44 (s, 9H). MS (ESI⁺): *m/z* (%) = 375.2 (100, [M+H]⁺), 376.2 (23, [M+H]⁺), calculated for C₂₀H₂₆N₂O₅: 374.18 [M].

6-(4-((tert-Butoxycarbonyl)amino)butoxy)quinoline-4-carboxylic acid (S8, Boc-Chino-COOH)

Boc-Chino-COOMe (**S7**, 3.34 g, 8.92 mmol, 1.00 eq) was dissolved in 1,4-dioxane (40 mL) and 1 M LiOH (17.8 mL, 17.8 mmol, 2.00 eq) was added and stirred for 4 hours at RT. The organic solvent was removed *in vacuo* and the solution was adjusted to pH = 3.5 with 1 M HCl. The aqueous solution was extracted with EA (8×80 mL), the organic phase was dried over Na₂SO₄ and the solvent was removed under reduced pressure. **S8** was obtained as a yellowish solid (1.82 g, 5.05 mmol, 57%). ¹H-NMR (400 MHz, DMSO-d₆): δ [ppm] = 8.86 (d, *J* = 4.5 Hz, 1H), 8.15 (d, *J* = 2.8 Hz, 1H), 8.02 (d, *J* = 9.3 Hz, 1H), 7.92 (d, *J* = 4.4 Hz, 1H), 7.49 (dd, *J* = 9.2 Hz, 2.8 Hz, 1H), 6.87 (t, *J* = 5.8 Hz, 1H), 4.10 (t, *J* = 6.3 Hz, 2H), 3.00 (q, *J* = 6.6 Hz, 2H), 1.78 (q, *J* = 11.8, 6.5 Hz, 2H), 1.62 – 1.51 (m, 2H), 1.37 (s, 9H). MS (ESI⁺): *m/z* (%) = 261.1 (20, [M-Boc+H]⁺), 361.2 (100, [M+H]⁺), 362.2 (22, [M+H]⁺), calculated for C₁₉H₂₄N₂O₅: 360.17 [M].

tert-Butyl (S)-(2-(2-carbamoyl-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamate (S11, Boc-Gly-Pro-CONH₂)

Boc-Gly-OH (**S9**, 1.38 g, 7.88 mmol, 1.05 eq) and HBTU (3.12 g, 8.20 mmol, 1.1 eq) were dissolved in dry dichloromethane (DCM, 8 mL) and DMF (8 mL) under argon atmosphere. DIPEA (1.53 mL, 8.97 mmol, 1.20 eq) was added and the solution was stirred for one hour at RT. In another reaction vessel, 4,4-difluoro-L-prolinamide hydrochloride (**S10**, 1.40 g, 7.50 mmol, 1.00 eq) and DIPEA (2.54 mL, 14.90 mmol, 2.00 eq) were dissolved in dry DCM (5 mL) and DMF (5 mL). The solutions were combined and stirred overnight at RT. The precipitate was filtered off and the filtrate was cooled overnight to complete the precipitation. The two precipitates were combined and **S6** was obtained as a colorless solid (1.97 g, 6.41 mmol, 86%). ¹H-NMR (400 MHz, DMSO-d₆): δ [ppm] = 7.40 (s, 1H), 7.16 (s, 1H), 6.87 (dt, *J* = 10.4, 5.8 Hz, 1H), 4.45 (dd, *J* = 9.0 Hz, 1H), 4.15 – 3.85 (m, 2H), 3.86 – 3.63 (m, 2H), 2.81 – 2.27 (m, 2H), 1.37z (s, 9H). MS (ESI⁺): *m/z* (%) = 207.8 (62,

[M–Boc+H]⁺), 251.8 (100, [M–^tBu+H]⁺), 307.9 (39, [M+H]⁺), 329.9 (24, [M+Na]⁺), calculated for C₁₂H₁₉F₂N₃O₄: 307.13 [M].

tert-Butyl (S)-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamate (**S12**, Boc-Gly-Pro-CN)

Boc-Gly-Pro-CONH₂ (**S11**, 1.97 g, 6.41 mmol, 1.00 eq) was dissolved in dry THF (50 mL) under argon atmosphere and cooled to 0°C. Pyridine (4.1 mL, 51.3 mmol, 8.00 eq) was added. In another reaction vessel, Trifluoroacetic anhydride (TFAA, 2.7 mL, 19.2 mmol, 3.00 eq) was dissolved in dry DCM (35 mL) and slowly dropped to the reaction solution. The reaction solution was allowed to warm to RT while stirring for another 3 hours. Then, 1 M HCl (80 mL) was added and the aqueous solution was extracted with DCM (5×80 mL). The combined organic phases were washed once with Na₂CO₃ (10 mL) and Brine (10 mL) and then dried over Na₂SO₄. The solvent was removed *in vacuo* and the product **S12** was purified via column chromatography (CH/EA 3:2) to give a colorless solid (1.49 g, 4.81 mmol, 81%). ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 5.35 (s, 1H), 4.97 (t, *J* = 6.5 Hz, 1H), 4.04 – 3.78 (m, 4H), 2.81 – 2.69 (m, 2H), 1.45 (s, 9H). MS (ESI⁺): *m/z* (%) = 190.0 (31, [M–Boc+H]⁺), 233.9 (100, [M–^tBu+H]⁺), calculated for C₁₂H₁₇F₂N₂O₃: 289.12 [M].

(S)-4,4-Difluoro-1-glycylpyrrolidine-2-carbonitrile trifluoroacetic acid (**S13**, Gly-Pro-CN)

Boc-Gly-Pro-CN (**S12**, 1.15 g, 3.97 mmol, 1.00 eq) was dissolved in dry MeCN (2 mL) and TFA (2 mL) was slowly added under argon atmosphere. After stirring for 5 hours at RT the solvent was removed under reduced pressure and codistilled with MeOH (5×25 mL). **S13** was obtained as a yellowish oil (1.20 g, 3.97 mmol, 100%) which was used in the next step without any further purification. ¹H-NMR (400 MHz, MeOD): δ [ppm] = 8.25 (s, 2H), 5.22 – 5.06 (m, 1H), 4.33 – 3.75 (m, 4H), 3.02 – 2.73 (m, 2H). MS (ESI⁺): *m/z* (%) = 189.9 (100, [M+H]⁺), 231.0 (20, [M+MeCN+H]⁺), calculated for C₇H₉F₂N₃O: 189.07 [M].

tert-Butyl (S)-(4-((4-((2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)carbamate (**S14**, FAPi-NHBoc)

Boc-Chino-COOH (**S8**, 1.64 g, 4.55 mmol, 1.15 eq) and DIPEA (0.93 mL, 5.46 mmol, 1.37 eq) were dissolved in dry DMF (16 mL) under argon atmosphere. HOBt (0.68 g, 5.01 mmol, 1.26 eq) and HBTU (1.90 g, 5.01 mmol, 1.26 eq) were then added and the reaction mixture was stirred for one hour at RT. Gly-Pro-CN (**S13**, 1.20 g, 3.97 mmol, 1.00 eq), also dissolved in dry DMF (10 mL) and mixed with DIPEA (1.93 mL, 11.38 mmol, 2.86 eq), was then added and the whole reaction mixture was stirred for one day at RT. Then the solvent was removed *in vacuo* and the residue was taken up in EA (100 mL). The organic phase was washed with 1 M citric acid, saturated Na₂CO₃ and Brine (10 mL each). The aqueous phase was extracted with EA (3×100 mL) and the combined organic extracts were dried over Na₂SO₄. The solvent was removed under reduced pressure and the

product **S14** purified via column chromatography (CHCl₃/MeOH, 100:3) to give a colorless solid (1.74 g, 3.27 mmol, 72%). ¹H-NMR (400 MHz, MeOD): δ [ppm] = 8.74 (d, *J* = 4.4 Hz, 1H), 7.96 (d, *J* = 9.3 Hz, 1H), 7.93 – 7.88 (m, 1H), 7.56 (d, *J* = 4.4 Hz, 1H), 7.46 (dd, *J* = 9.3, 2.7 Hz, 1H), 5.15 (dd, *J* = 9.4, 3.1 Hz, 1H), 4.39 – 3.98 (m, 8H), 3.19 – 3.09 (m, 2H), 3.02 – 2.70 (m, 2H), 1.94 – 1.83 (m, 2H), 1.76 – 1.65 (m, 2H), 1.43 (s, 9H). MS (ESI⁺): *m/z* (%) = 432.0 (33, [M–Boc+H]⁺), 476.1 (46, [M–^{*t*}Bu+H]⁺), 532.4 (100, [M+H]⁺), calculated for C₂₆H₃₁F₂N₅O₅: 531.23 [M].

(S)-6-(4-Aminobutoxy)-*N*-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide (**1**, FAPi-NH₂)

FAPi-NHBoc (**S14**, 531.6 mg, 1.0 mmol, 1.0 eq) was dissolved in dry MeCN (10 mL) at 0 °C under argon atmosphere. 4 M HCl in 1,4-dioxane (5.0 mL, 5.0 mmol, 5.00 eq) was added and the reaction solution was slowly allowed to warm to RT. After 3 hours more 4 M HCl in 1,4-dioxane (2.5 mL, 2.5 mmol, 2.5 eq) was added. After another 4 hours it was diluted with MeCN (30 mL) and then completely concentrated *in vacuo*. FAPi-NH₂ **1** was obtained as a colorless solid (467 mg, 1.0 mmol, 100%). ¹H-NMR (400 MHz, MeOD): δ [ppm] = 9.10 (d, *J* = 5.5 Hz, 1H), 8.32 (d, *J* = 2.7 Hz, 1H), 8.24 (d, *J* = 9.3 Hz, 1H), 8.08 (d, *J* = 5.5 Hz, 1H), 7.86 (dd, *J* = 9.4, 2.6 Hz, 1H), 5.15 (dd, *J* = 9.4, 3.1 Hz, 1H), 4.48 – 4.33 (m, 4H), 4.32 – 4.07 (m, 2H), 3.06 (t, *J* = 6.5 Hz, 2H), 3.02 – 2.74 (m, 2H), 2.09 – 1.87 (m, 4H). MS (ESI⁺): *m/z* (%) = 216.7 (100, [M+H]²⁺), 237.2 (27, [M+MeCN+H]²⁺), 432.1 (22, [M+H]⁺), calculated for C₂₁H₂₃O₅F₂N₅O₃: 431.18 [M].

Analytical RP-HPLC

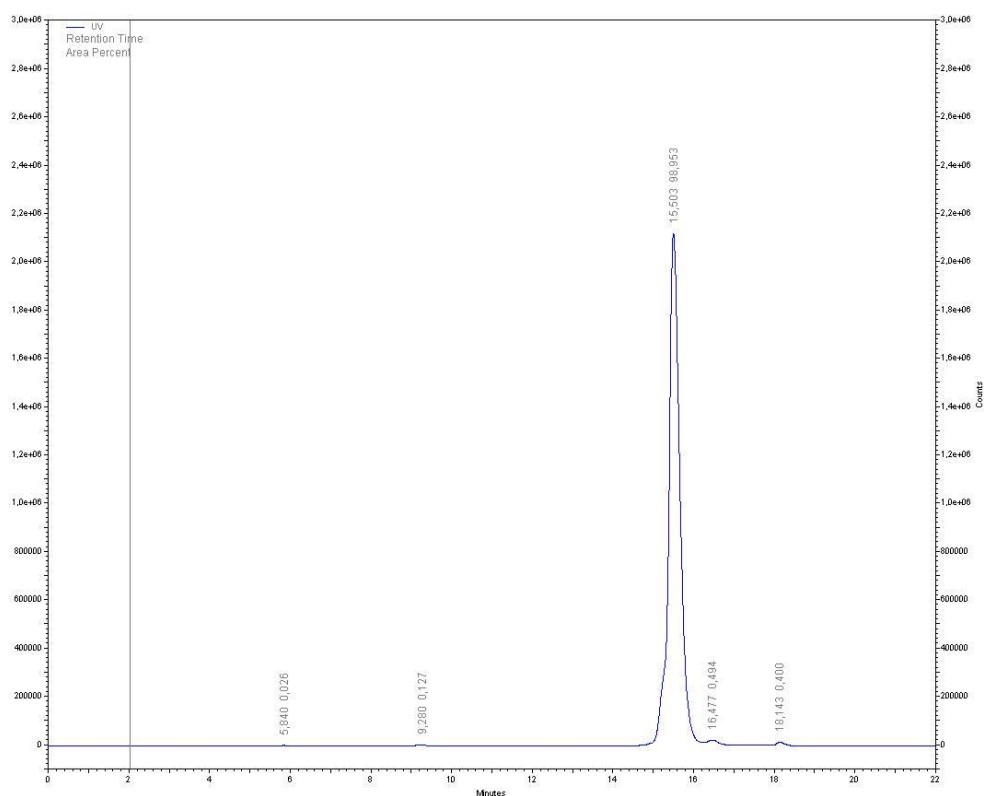


Figure S1. Analytical RP-HPLC of DOTAGA.Glu.(FAPi)₂ **7** (t_R = 15.5 min), 20-30% MeCN + 0.1% TFA (linear gradient) in 20 min, 5 mL/min: purity = 99.0%.

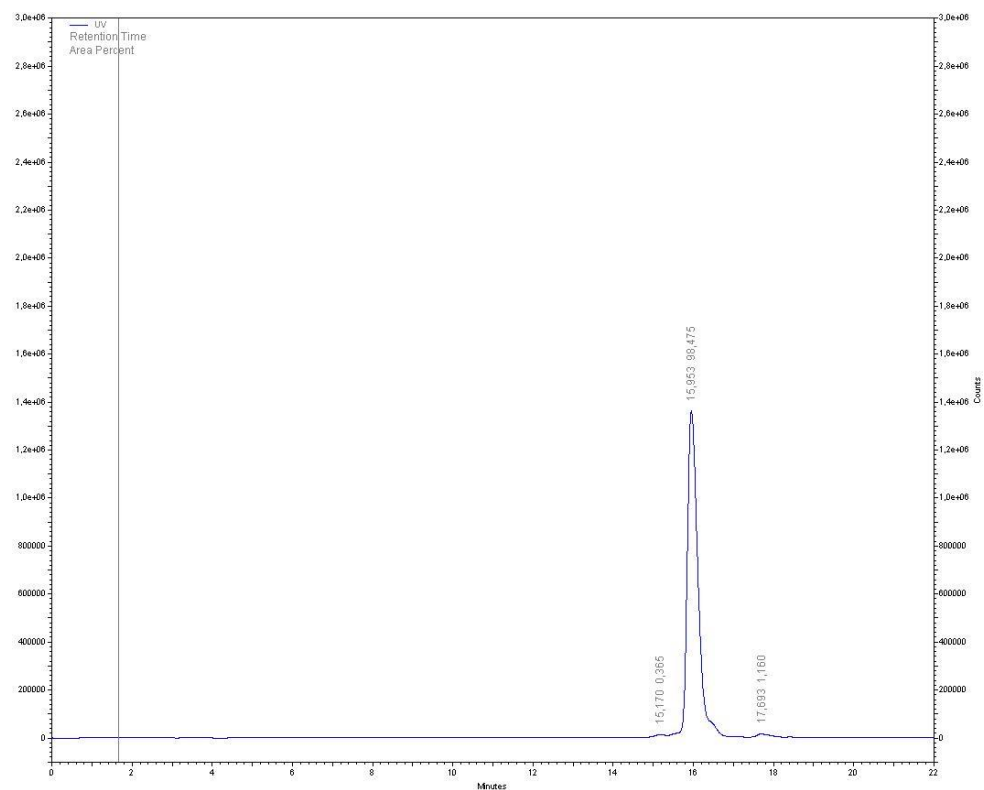


Figure S2. Analytical RP-HPLC of DO3A.Glu.(FAPi)₂ **11** (t_R = 16.0 min), 20-30% MeCN + 0.1% TFA (linear gradient) in 20 min, 5 mL/min: purity = 98.5%.

Radiosynthesis

The following Figures show additional labeling studies (reaction kinetics) as well as complex stability studies.

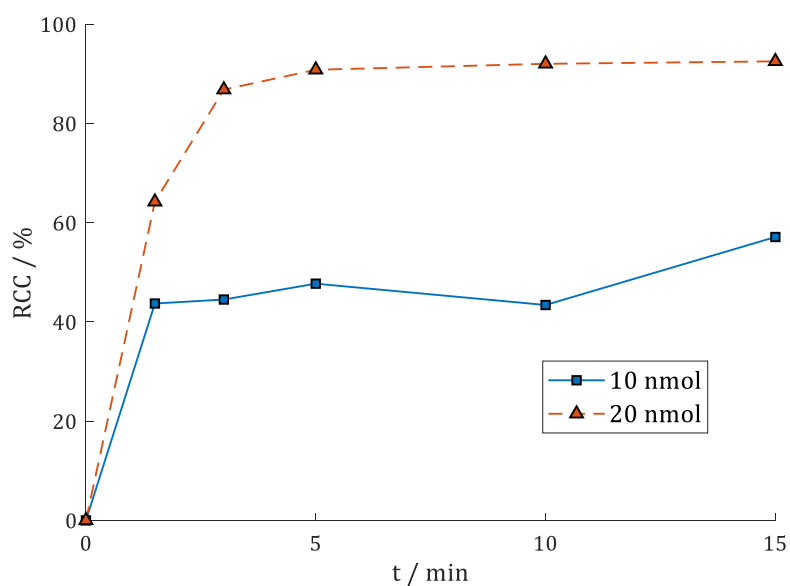


Figure S3. Reaction kinetics (radiochemical conversion (RCC) in %) of $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu.}(\text{FAPi})_2$ $^{68}\text{Ga-7}$ in 1 M AmOAc (pH = 4.5) at 95 °C with 100 MBq ^{68}Ga (n = 1).

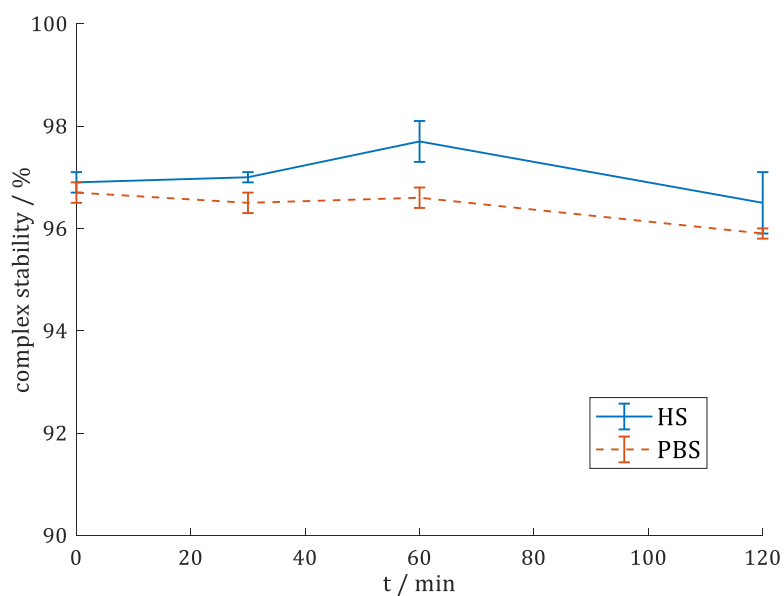


Figure S4. Complex stability of $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu.}(\text{FAPi})_2$ $^{68}\text{Ga-7}$ in human serum (HS) and phosphate-buffered saline (PBS) over 120 minutes (n = 3).

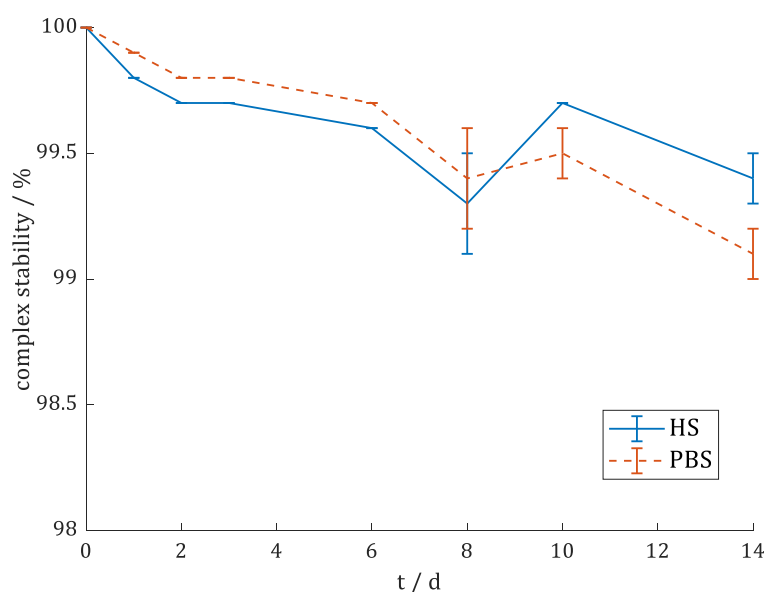


Figure S5. Complex stability of $[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu.}(\text{FAPi})_2$ $^{177}\text{Lu-7}$ in human serum (HS) and phosphate-buffered saline (PBS) over 14 days ($n = 3$).

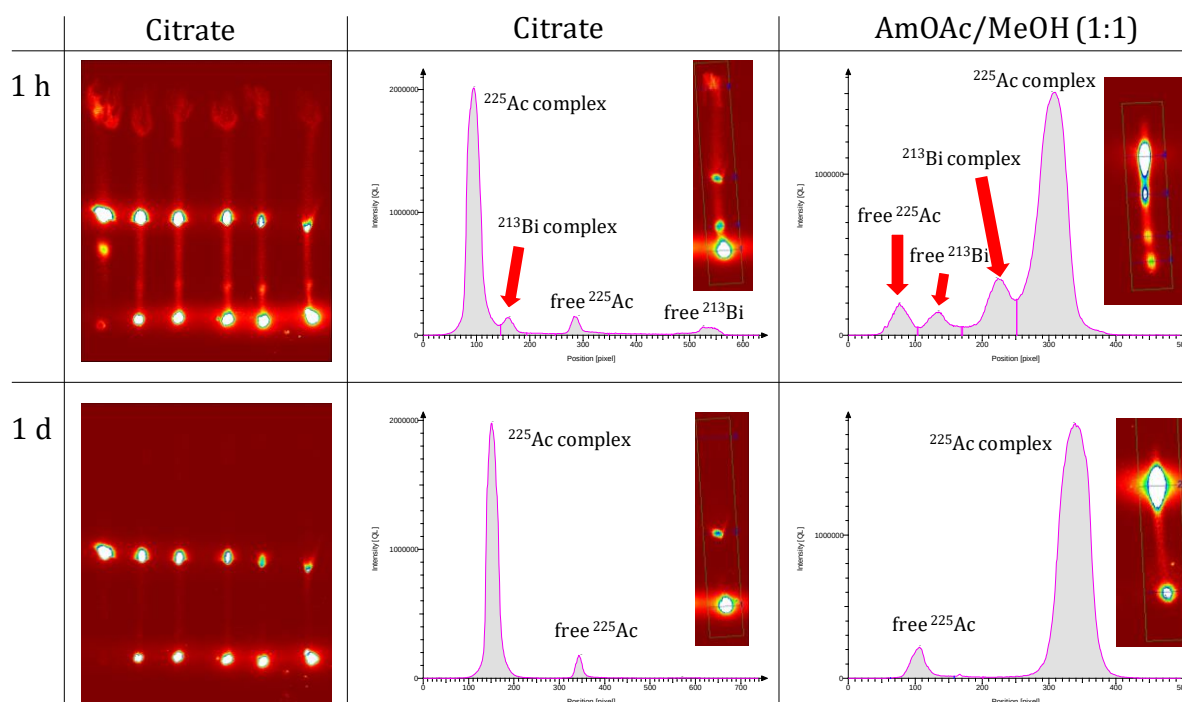


Figure S6. Exemplary reaction kinetic of $[^{225}\text{Ac}]\text{Ac-DOTAGA.Glu.}(\text{FAPi})_2$ $^{225}\text{Ac-7}$ at 95 °C analyzed with 0.1 M citrate buffer ($\text{pH} = 4.0$) radio-TLC from 0–15 min (left to right). Exemplary profiles of citrate radio-TLC after 60 minutes at 95 °C are shown in the middle. The corresponding 1 M AmOAc ($\text{pH} = 4.0$)/MeOH (1:1) radio-TLCs are shown on the right. All TLCs were imaged at different time points after 1 hour (top) and 1 day (bottom). These measurements together make clear that: $R_f(^{225}\text{Ac-complex}=\text{product } ^{225}\text{Ac-7}) = 0.0$ and $R_f(\text{free } ^{225}\text{Ac}) \approx 0.5$. The other two spots appearing in a) must be from one of the short-lived daughter nuclides, most likely ^{213}Bi since the spots are slowly decaying when measuring the TLC over several hours which fits the physical half-life and Bi is a trivalent metal that can be complexed by DOTA conjugates. Therefore, $R_f(\text{free } ^{213}\text{Bi}) > 0.7$ can be assigned to be free ^{213}Bi and $R_f(^{213}\text{Bi-complex}) = 0.1\text{--}0.2$ is considered to be the $^{213}\text{Bi-complex}$.

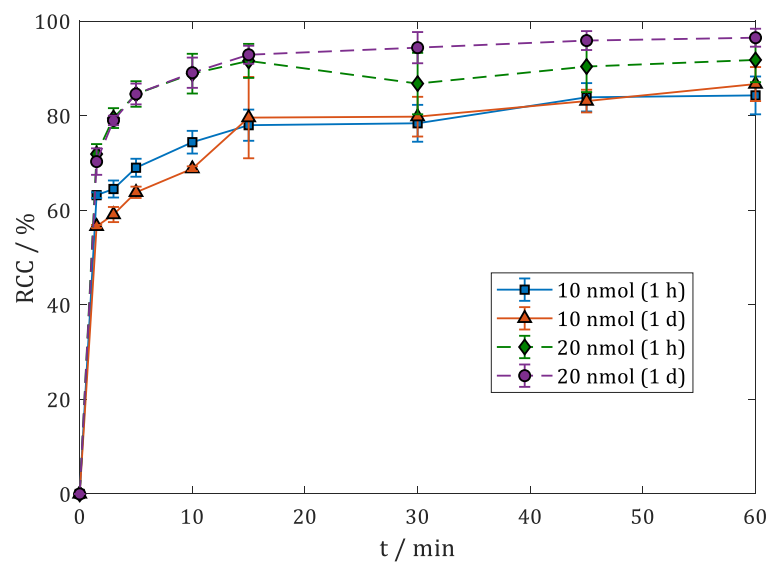


Figure S7. Reaction kinetics (radiochemical conversion (RCC) in %) of $[^{225}\text{Ac}]\text{Ac-DOTAGA.Glu.}(\text{FAPi})_2$ $^{225}\text{Ac-7}$ in 0.1 M sodium ascorbate (pH = 7.0) at 95 °C with 500 kBq ^{225}Ac (n = 2).

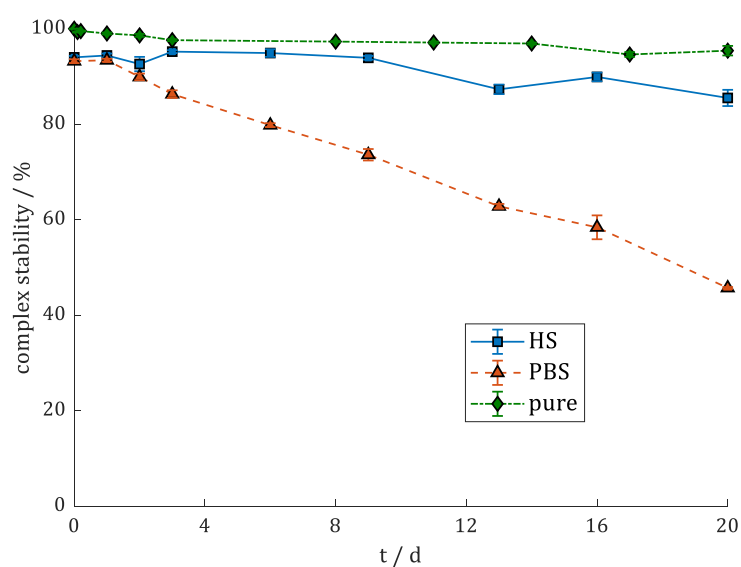


Figure S8. Complex stability of $[^{225}\text{Ac}]\text{Ac-DOTAGA.Glu.}(\text{FAPi})_2$ $^{225}\text{Ac-7}$ in human serum (HS) and phosphate-buffered saline (PBS) (0.7-0.8 MBq/mL) and final formulation ("pure", 0.2 MBq/mL) after C18 cartridge purification over 20 days (n = 3).

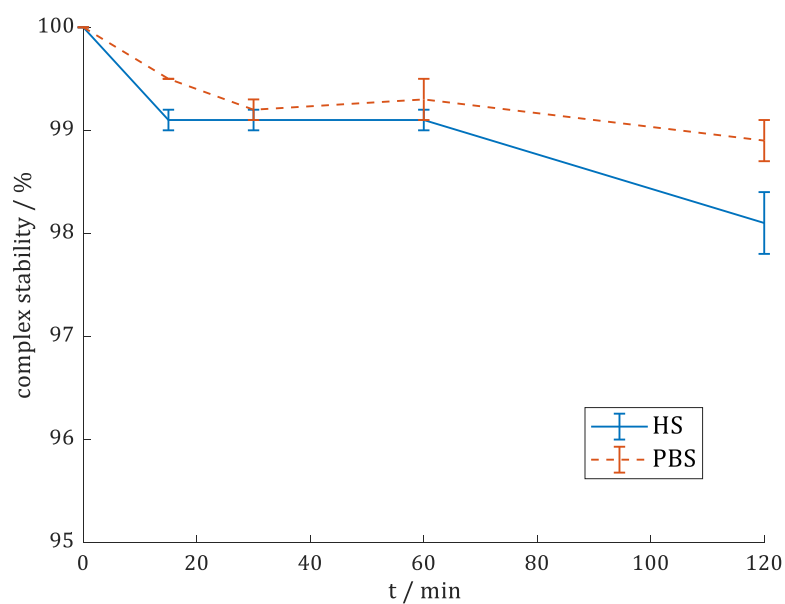


Figure S9. Complex stability of $[^{68}\text{Ga}]\text{Ga-DO3A.Glu.(FAPi)}_2$ $^{68}\text{Ga-11}$ in human serum (HS) and phosphate-buffered saline (PBS) over 120 minutes ($n = 3$).

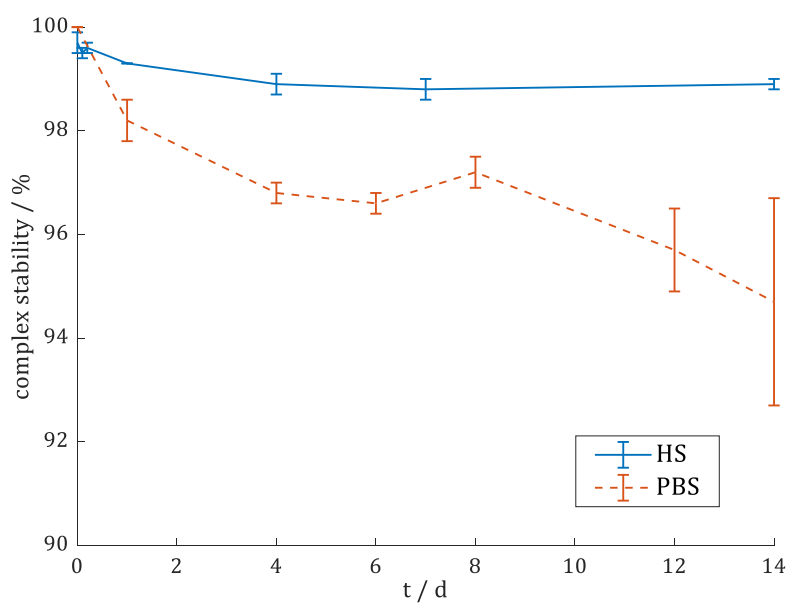


Figure S10. Complex stability of $[^{177}\text{Lu}]\text{Lu-DO3A.Glu.(FAPi)}_2$ $^{177}\text{Lu-11}$ in human serum (HS) and phosphate-buffered saline (PBS) over 14 days ($n = 3$).

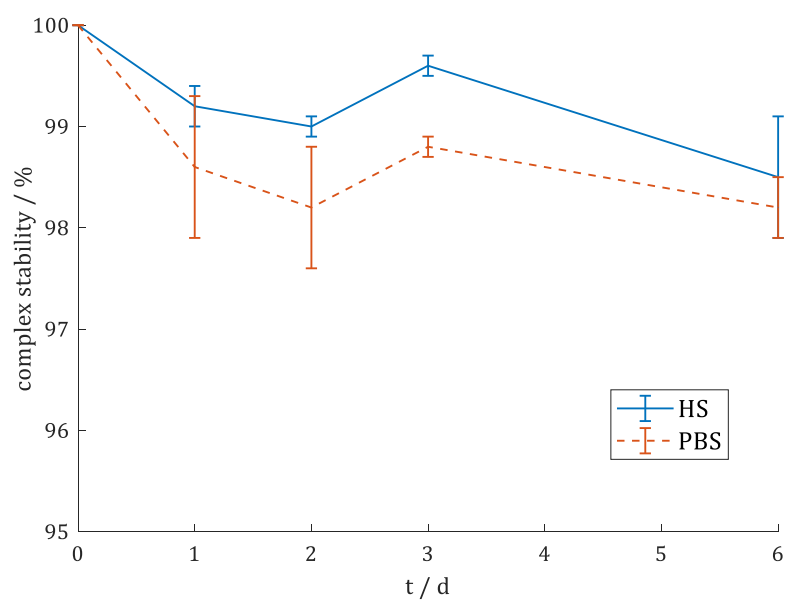


Figure S11. Complex stability of [^{90}Y]Y-DO3A.Glu.(FAPi) $_2$ ^{90}Y -**11** in human serum (HS) and phosphate-buffered saline (PBS) over 6 days ($n = 3$).

Radio-HPLC

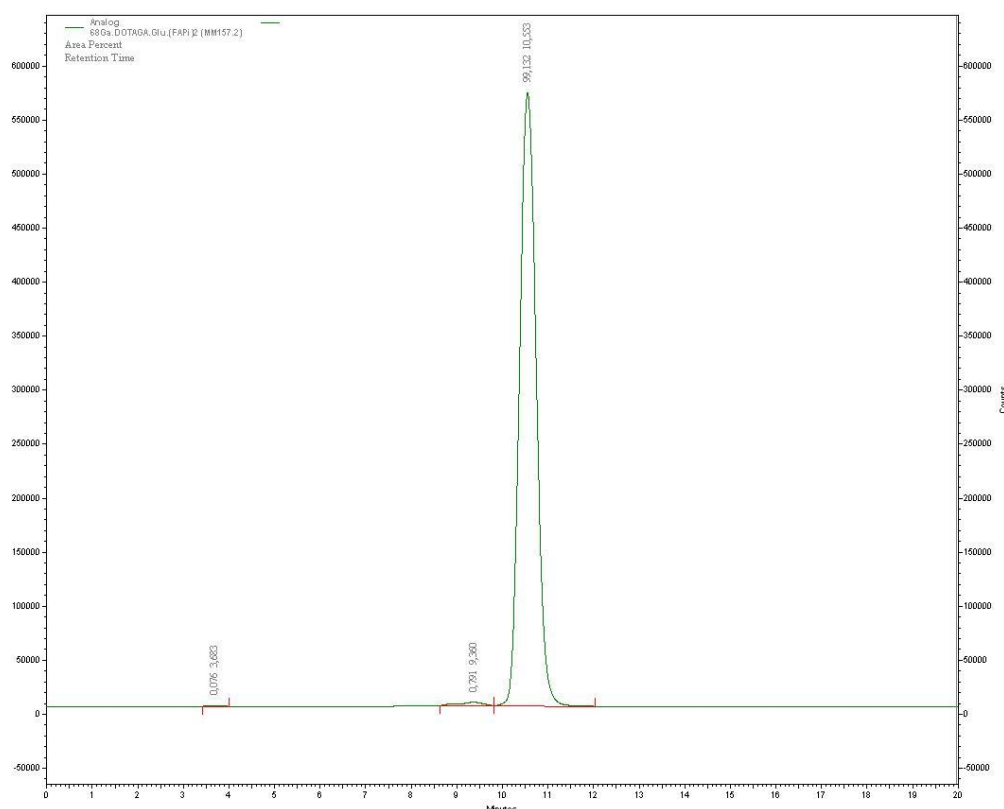


Figure S12. Analytical Radio-HPLC of $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu.}(\text{FAPi})_2$ $^{68}\text{Ga-7}$ ($t_R = 10.5$ min, 40 nmol **7**, 400 MBq ^{68}Ga), 20-70% MeCN + 0.1% TFA (linear gradient) in 20 min: RCP = 99.1%.

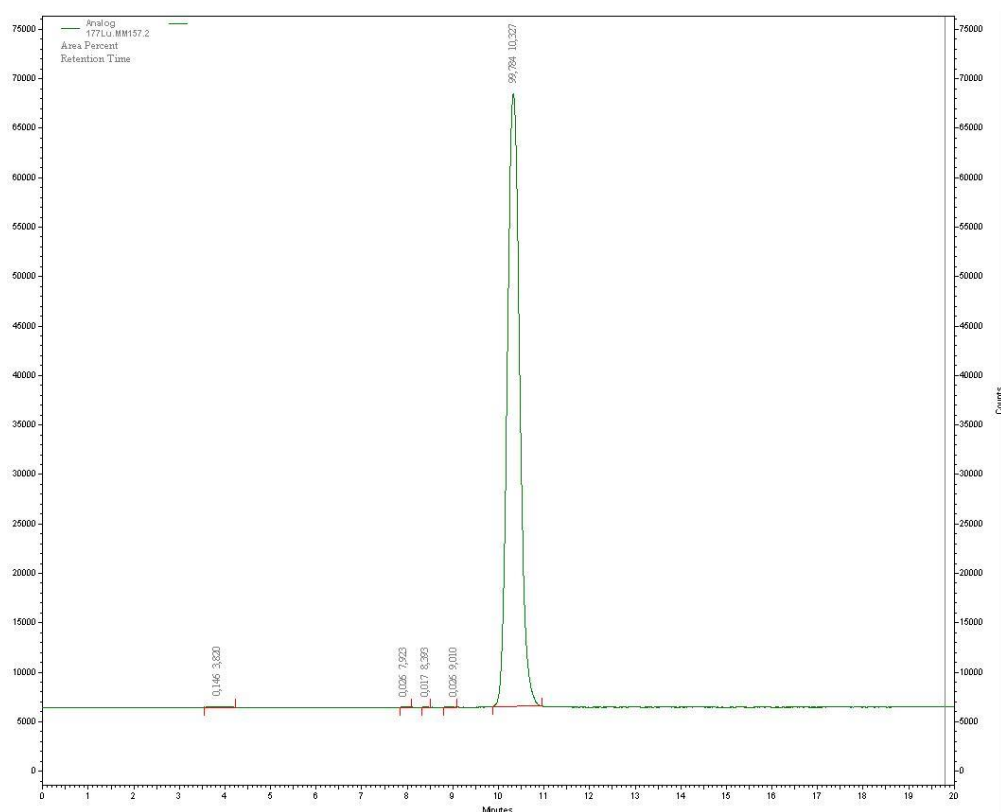


Figure S13. Analytical Radio-HPLC of $[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu.}(\text{FAPi})_2$ $^{177}\text{Lu-7}$ ($t_R = 10.3$ min, 1 nmol **7**, 100 MBq ^{177}Lu), 20-50% MeCN + 0.1% TFA (linear gradient) in 20 min: RCP = 99.8%.

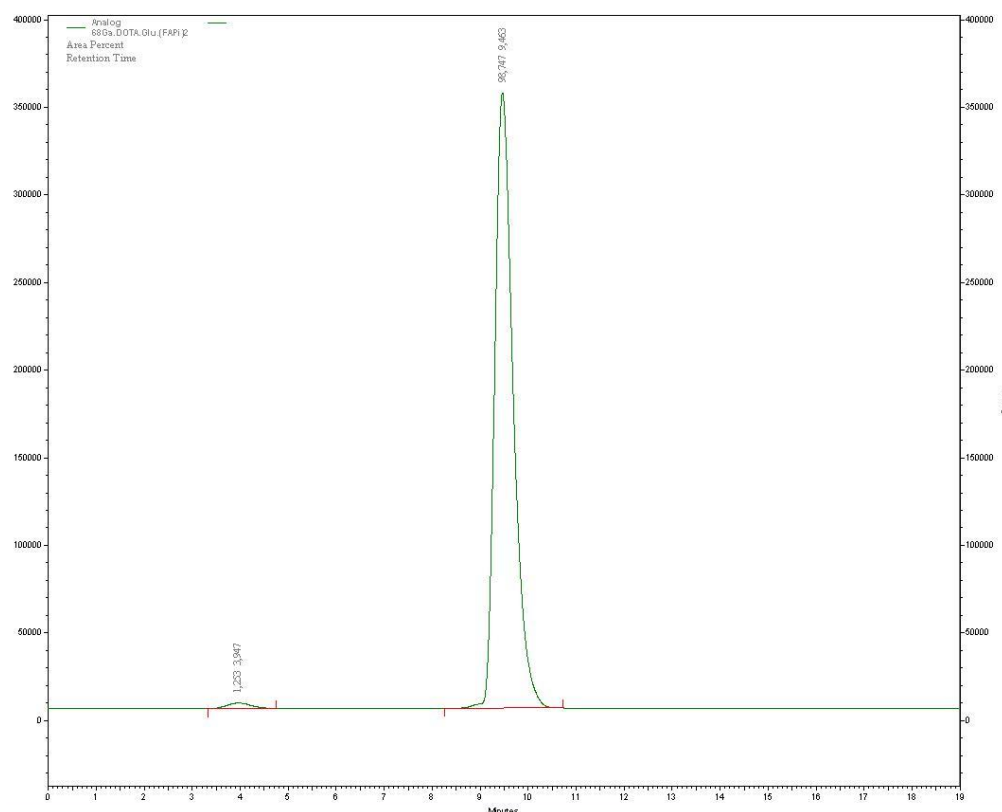


Figure S14. Analytical Radio-HPLC of $[^{68}\text{Ga}]\text{Ga-DO3A.Glu.(FAPi)}_2$ $^{68}\text{Ga-11}$ ($t_R = 9.5$ min, 20 nmol **11**, 100 MBq ^{68}Ga), 10-90% MeCN + 0.1% TFA (linear gradient) in 20 min: RCP = 98.7%.

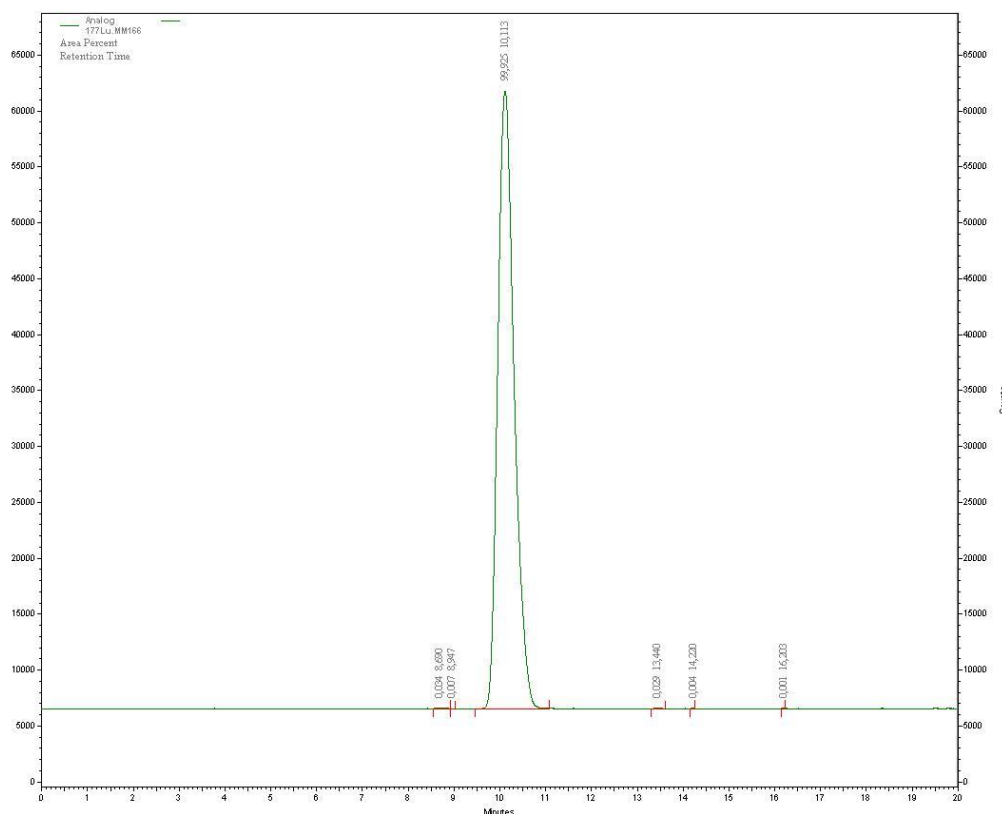


Figure S15. Analytical Radio-HPLC of $[^{177}\text{Lu}]\text{Lu-DO3A.Glu.(FAPi)}_2$ $^{177}\text{Lu-11}$ ($t_R = 10.1$ min, 2 nmol, 100 MBq ^{177}Lu), 20-50% MeCN + 0.1% TFA (linear gradient) in 10 min: RCP = 99.9%.

3.3. [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ radionuclide therapy: A new treatment option for patients with glioblastoma multiforme

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(under review)

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The following **Summary** and **Author Contributions** sections do not belong to the publication. The actual publication begins after that and was not altered in any way.

Summary

This is a case report on treatment response assessment of a 52-year old female with recurrent glioblastoma (GBM) that underwent two cycles of ^{177}Lu -FAPi dimer radioligand therapy (RLT) using ^{177}Lu -DOTAGA.Glu.(FAPi) $_2$ (2×7.4 GBq). The patient suffered from headache and balance loss.

The ^{177}Lu -FAPi dimer RLT was guided by ^{68}Ga -DOTA.SA.FAPi PET/CT and showed intense FAPi uptake in the recurrent lesion in the right parieto-temporo-occipital region pre-treatment. Because the patient showed FAP-positive lesions in PET/CT, she was considered for treatment with ^{177}Lu -DOTAGA.Glu.(FAPi) $_2$ and after the two cycles significantly reduction in FAPi uptake via ^{68}Ga -DOTA.SA.FAPi PET/CT as well as tumor lesion size reduction assessed via MRI was observed. This went along with improved patient's condition.

This case gives a first indication for ^{68}Ga -DOTA.SA.FAPi monomer-guided ^{177}Lu -DOTAGA.Glu.(FAPi) $_2$ dimer RLT as a promising treatment option for patients with recurrent glioblastoma multiforme that have exhausted all standard treatments.

Author Contributions

MARCEL MARTIN contributed to the design and conception of the study conception along with all other authors. MARCEL MARTIN synthesized the precursor used in this study. Material preparation, data acquisition, collection, and analysis were performed by Sanjana Ballal and Madhav Prasad Yadav. The first draft of the manuscript was written by Sanjana Ballal, Madhav Prasad Yadav, and Shobhana Raju and Frank Roesch finalized the manuscript. Images were processed and reported by Chandrasekhar Bal and Madhavi Tripathi. All authors including MARCEL MARTIN commented on previous versions of the manuscript. All authors read and approved the final manuscript.

[¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ radionuclide therapy: A new treatment option for patients with glioblastoma multiforme

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Abstract

A 52-year-old female case of right temporo-parietal glioblastoma presented with headache and loss of balance with evidence of multiple recurrences on MRI. Exploratory [^{68}Ga]Ga-DOTA.SA.FAPi PET/CT demonstrated fibroblast activation protein inhibitor expression in the recurrent lesion in the right parieto-temporo-occipital region following which the patient was treated with [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ treatment. After 2 cycles of [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$, follow-up [^{68}Ga]Ga-DOTA.SA.FAPi PET/CT scan showed a significant reduction in fibroblast activation protein inhibitor (FAPi) uptake and regression in the size and perilesional edema of lesion on MRI with improvement in the patient's symptoms and performance status. [^{68}Ga]Ga-DOTA.SA.FAPi monomer/[^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ dimer theranostics have potential in patients with recurrent glioblastoma multiforme when other standard-line therapeutic options are depleted.

Keywords

glioblastoma multiforme; [^{68}Ga]Ga-DOTA.SA.FAPi PET/CT; [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ therapy.

Introduction

The mainstay of treatment for GBMs is surgery, followed by radiation and chemotherapy. Despite the available treatments, due to the aggressive nature of the disease, it is associated with a poor prognosis, and treatment options are scarce. More recent evidence has found elevated levels of FAP expression in various subtypes of glioblastomas. Few studies on human gliomas discovered elevated FAP protein expression in the majority of the human gliomas of the mesenchymal subtype. They also observed a strong positive correlation between FAP expression and aggressiveness of the glioma due to its direct effect on tumor microenvironment remodeling. According to data, it has been found that FAP plays a role in facilitating the invasion of glioma cells through brain tissue by promoting the degradation of the brain parenchyma. This suggests that FAP is involved in promoting tumor cell invasion. Research has demonstrated that both tumor cells and the pro-tumorigenic microenvironment in neuroepithelial cancers exhibit increased expression of FAP [1-4]. The U87MG cell line, which naturally expresses FAP, was utilized by Pandya et al. [5] to evaluate [^{89}Zr]Zr-Df-Bz-F19 mAb, an immuno-PET radiopharmaceutical, *in vitro*. Based on their findings, FAP appears to be a promising biomarker.

The relevance of FAP expression in gliomas from literature encouraged us to exploit FAP expression for imaging and therapy of neuroepithelial cancers like GBMs using the [^{68}Ga]Ga-DOTA.SA.FAPi guided [^{177}Lu]Lu-DOTAGA,Glu.(FAPi)₂ dimer radionuclide therapy.

Case Report

A 52-year-old female presented with a right temporal space-occupying lesion craniotomy and adjuvant chemoradiotherapy in December 2017. The histopathological evaluation of the right temporoparietal lesion was reported to be Glioblastoma multiforme, IDH wild type, WHO Grade IV with Ki67 index of 10-12%, and IHC showing GFAP+, CK-, p53 wild type, IDH1 – and ATRX +. She presented with disease recurrence and underwent two subsequent craniotomies with adjuvant chemoradiotherapy. After an uneventful period of five years on temozolomide, in 2022 the patient presented with complaints of hearing problems in the right ear. As a follow-up, MRI showed residual disease, the patient underwent a redo right temporal craniotomy and tumor decompression in June 2022. In view of systemic treatment options on compassionate grounds, the patient has considered [^{68}Ga]Ga-FAPi monomer guided [^{177}Lu]Lu-FAPi dimer radionuclide therapy. The baseline [^{68}Ga]Ga-DOTA.SA.FAPi PET/CT scan of the patient shows focal intense radiotracer uptake (red arrow) in the head region in maximum intensity projection (MIP) images (a), which is localized to the right parieto-temporo-occipital region with SULpeak:7.6 (white arrow, b) and in the right cerebellar region with SULpeak:23.9 (blue arrow, c) on the axial PET/CT images. Axial T2-weighted MRI images revealed heterogeneously enhancing nodular lobulated

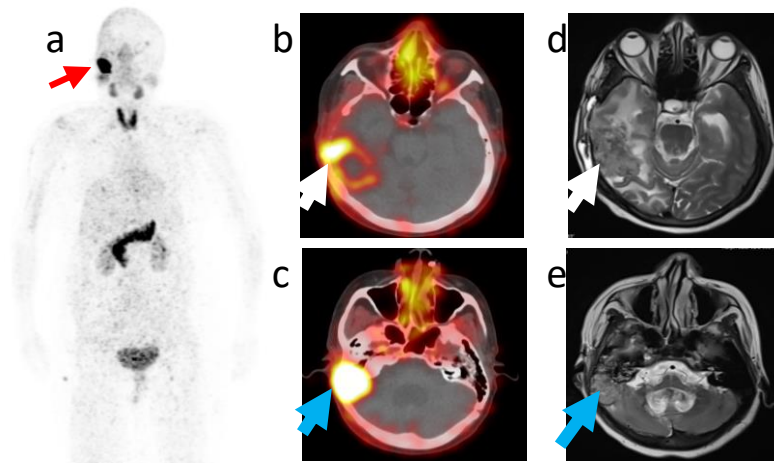
thickening of the post-op cavity wall measuring ~3.4x3.5 cm with marked perilesional edema involving the parieto-temporo-occipital region (white arrow, d). Another lobulated heterogeneously enhancing lesion contiguous with the post-op cavity was noted extending across the tentorium to involve the adjacent right cerebellum measuring ~3.2 x 2.4 x 2.8 cm) (blue arrow, e). The patient was administered 2 cycles (7.4 GBq/cycle) [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ therapy at 8 weekly intervals. Both post-therapy whole-body scan images were acquired at 24 h p.i. [green arrow, (f), (g)] which showed retention of [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ in the sites corresponding to the scans. An interim post-treatment response assessment utilizing [⁶⁸Ga]Ga-DOTA.SA.FAPi PET/CT reveals a substantial reduction in uptake of the tracer in the MIP images (red arrow, h) and a significant reduction in size and uptake of the right parieto-temporo-occipital region (SULpeak:2.5) and in the right cerebellar region (SULpeak:6.1) in the axial PET/CT images [white and blue arrows in (i) & (j), respectively]. Follow-up T2-weighted MRI axial images of the lesion confirmed a reduction in the size of the lesion and perilesional edema [white and blue arrows in (k) & (l), respectively]. No adverse events were noted during the treatment or follow-up duration of 8 months.

Evident from our findings, [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ provides a comprehensive treatment strategy that offers a new dimension of treatment strategy in patients with recurrent progressive GBMs with thin therapeutic options. The theranostic pair [⁶⁸Ga]Ga-DOTA.SA.FAPi monomer/[¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ dimer represents a successful approach for the treatment of GBM.

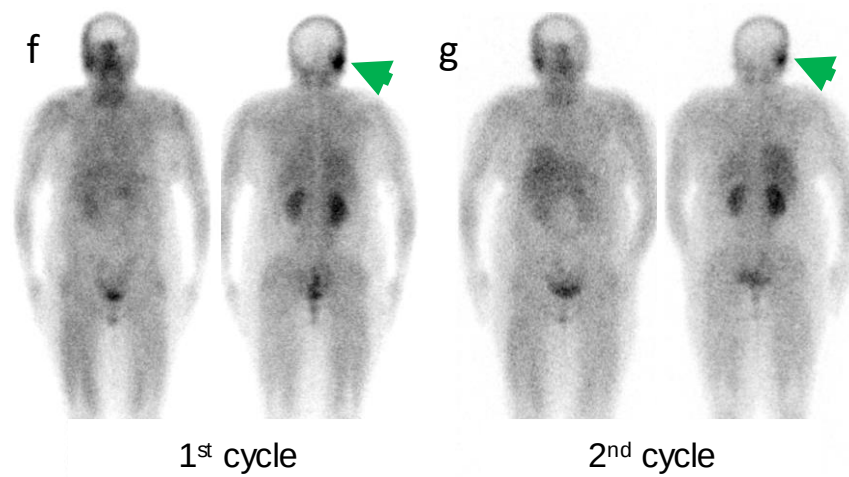
Figure & Legend

Fig 1: Baseline [⁶⁸Ga]Ga-DOTA.SA.FAPi PET/CT of the patient showing focal intense radiotracer uptake (red arrow) in the head region in maximum intensity projection (MIP) images (a), which is localized to the right parieto-temporo-occipital region and in the right cerebellar region in the axial PET/CT images (white and blue arrows in b & c respectively). Axial T2 weighted MRI images of the same lesion are noted (white and blue arrows in d & e respectively) with significant perilesional edema. Image (f) 1st cycle 7.4 GBq [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ anterior and posterior 24h p.i. post-therapy scan and Image (g) 2nd cycle 7.4 GBq [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ anterior and posterior 24h p.i. the post-therapy scan reveals retention of the therapeutic radiotracer (green arrow). Post-therapy response assessment [⁶⁸Ga]Ga-DOTA.SA.FAPi PET/CT reveals a significant reduction in uptake of the tracer in the MIP images (red arrow in h) and a reduction in size and uptake of the right parieto-temporo-occipital region and in the right cerebellar region in the axial PET/CT images (white and blue arrows in i & j respectively). Follow-up T2 weighted MRI axial images of the lesion showed a reduction in the size of the lesion and perilesional edema (white and blue arrows in k & l respectively). **The arrangement of the images has been adjusted to fit the print layout better.**

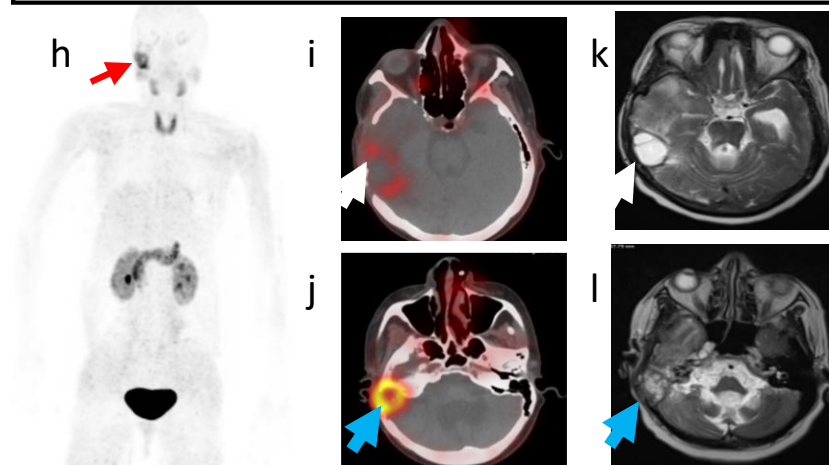
Pretherapy [^{68}Ga]Ga-DOTA.SA.FAPi
PET/CT and MRI



[^{177}Lu]Lu-DOTAGA.Glu.(FAPi)₂
24 h p.i. post-therapy images



Interim [^{68}Ga]Ga-DOTA.SA.FAPi
PET/CT and MRI



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Statements and Declarations

Competing Interests: Sanjana Ballal, Madhav P. Yadav, Shobhana Raju, Frank Roesch, Marcel Martin, Madhavi Tripathi, and Chandrasekhar Bal have no relevant financial or non-financial interests to disclose.

Data Availability: The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Disclosures: The authors have nothing to disclose.

Ethical Clearance: Ethical clearance received from the institute ethics committee of All India Institute of Medical Sciences, New Delhi [Ref No: IEC-483]

Informed Consent: Written informed consent was obtained from the patient to participate in the study, use clinical information to analyze data, and use images for the purpose of publication.

Support: The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Disclaimer: This work has not been submitted elsewhere and is not under consideration by any other journal.

Author Contributions: All authors contributed to the study's conception and design. Material preparation, data acquisition, collection, and analysis were performed by Sanjana Ballal and Madhav Prasad Yadav. The first draft of the manuscript was written by Sanjana Ballal, Madhav Prasad Yadav, and Shobhana Raju. Images were processed and reported by Chandrasekhar Bal and Madhavi Tripathi. Marcel Martin synthesized the precursor and Frank Roesch finalized the manuscript. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

3.4. FAP Inhibitor-Based Homodimers with Different Linker and Spacer Units for Application in Radioligand Therapy

In preparation for submission

Summary

Research is ongoing to improve radioligand therapy (RLT) targeting fibroblast activation protein alpha (FAP). This work follows the prior results on the second-generation FAPi homodimer DOTAGA,Glu.(FAPi)₂ with glutamic acid (Glu) as the central linker unit which led several improvements compared to the first-generation linear homodimer DOTAGA.(SA.FAPi)₂: (i) Radiolabeling: higher labeling yields with actinium-225 were achieved along with efficient labeling with gallium-68 and lutetium-177; (ii) *in vitro*: higher affinity for FAP and higher selectivity over the related enzymes PREP and the DPPs (DPP4, 8 and 9); (iii) Proof-of-concept patient study: [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ showed improved pharmacokinetic profile that might at least partially result from higher hydrophilicity while maintaining prolonged tumor retention [58].

In this work new compounds were synthesized that contained different central linker and spacer units to investigate the effect of derivatization on radiolabeling and *in vitro* properties in structure-property relationship studies to potentially improve the properties further. DO3A and DOTAGA were again used as chelators for potential theranostic application. With the compounds DO3A- and DOTAGA.NPyr.(FAPi)₂, a different central linker unit based 3-aminopyrrolidine (NPyr) was used with a central nitrogen atom. Glu was as the central linker with the compounds DOTAGA.PEG₂.Glu.(FAPi)₂, DO3A- and DOTAGA.Glu₂.(FAPi)₂ that used an additional Glu or diethylene glycol (PEG₂) as a spacer unit.

After organic synthesis of the five precursors (six steps per compound), radiolabeling with gallium-68 was investigated. The DOTAGA precursors were labeled with gallium-68 in three different buffer systems: 1 M HEPES (pH = 4.5 or 5.5), 1 M NaOAc and 1 M AmOAc (pH = 4.5). [⁶⁸Ga]Ga-DOTAGA.PEG₂.Glu.(FAPi)₂ was the only and the first FAPi homodimer from our group that showed high radiochemical conversion (RCC) rates in NaOAc or Am OAc. For all the other precursors, HEPES was the buffer of choice. High RCC > 90% was achieved for all ⁶⁸Ga-complexes except [⁶⁸Ga]Ga-DOTAGA.NPyr.(FAPi)₂ and gave ⁶⁸Ga-complexes with high radiochemical purity (RCP) > 97% after purification C18 cartridge (radio-HPLC).

The radiolabeling with lutetium-177 was carried out in 1 M HEPES (pH = 5.5) and resulted in quantitative RCC with RCP ≥ 99% (radio-HPLC) without the need of further purification. Only for [¹⁷⁷Lu]Lu-DOTAGA.PEG₂.Glu.(FAPi)₂, slightly lower RCC up to 97% was achieved.

Complex stability studies for all ⁶⁸Ga- and ¹⁷⁷Lu-labeled radiotracers in human serum (HS) and phosphate-buffered saline (PBS) were investigated over two half-lives (⁶⁸Ga: 120 min, ¹⁷⁷Lu: 14 d). ⁶⁸Ga-complexes were all > 90% stable except [⁶⁸Ga]Ga-DO3A.Glu₂.(FAPi)₂. The complex stability of the ¹⁷⁷Lu-labeled derivatives was > 98%.

The distribution coefficient *D* was determined with a shake-flask method to calculate the log*D*_{7.4} value of all radiolabeled products (⁶⁸Ga- and ¹⁷⁷Lu-complexes). Hydrophilicity was very high

($-\log D_{7.4} = 2.31 - 2.71$) and in the same range as [^{68}Ga]Ga- and [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ with the most polar new compound ($\log D_{7.4} = -2.71 \pm 0.10$) being [^{177}Lu]Lu-DOTAGA.Glu $_2$.(FAPi) $_2$.

In vitro affinity studies revealed very high FAP affinity with subnanomolar IC_{50} values for all five compounds. PREP and DPP4 selectivity was high with a selectivity index $SI > 1000$ whereas DPP8 and DPP9 was lower ($SI < 1000$). Overall, these results were all quite similar to DOTAGA.Glu.(FAPi) $_2$ and indicate that these compounds are also potentially interesting candidates for ^{177}Lu -FAP-RLT. DO3A.NPyr.(FAPi) $_2$ showed slightly higher PREP and DPP4 and the compounds DOTAGA.NPyr.(FAPi) $_2$ and DOTAGA.PEG $_2$.Glu.(FAPi) $_2$ slightly higher DPP4 selectivity. On the other hand, the influence of the new linker and spacer units seem to be not very significant with regard to the *in vitro* affinities. Future *in vivo* studies have to show if one or some of the compounds might even be superior to [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ since it is difficult to predict *in vivo* properties, especially pharmacokinetic and pharmacodynamic profile, from the *in vitro* results. However, improved labeling variability was found for [^{68}Ga]Ga-DOTAGA.PEG $_2$.Glu.(FAPi) $_2$ and might be due to the PEG $_2$ spacer unit.

Author Contributions

Frank Roesch and MARCEL MARTIN conceptualized the study. The conceptualization and planning of the *in vitro* studies was supported by Ingrid De Meester and Pieter Van der Veken. MARCEL MARTIN planned and carried out the organic synthesis as well as the radiolabeling studies and radiochemical investigations (complex stabilities, lipophilicity etc.) and subsequently analyzed the results. Sarah Stephan supported the organic synthesis and radiolabeling investigations of the compounds DOTAGA.PEG $_2$.Glu.(FAPi) $_2$, DO3A- and DOTAGA.Glu $_2$.(FAPi) $_2$. Kevin Machel supported the radiolabeling investigations of DO3A- and DOTAGA.NPyr.(FAPi) $_2$. The *in vitro* studies were carried out and analyzed by Yentl Van Rymenant, Joni De Loose and Emile Verhulst. Finally, MARCEL MARTIN interpreted all the results and drafted the manuscript.

FAP Inhibitor-Based Homodimers with Different Linker and Spacer Units for Application in Radioligand Therapy

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Abstract

Several monomeric radiopharmaceuticals targeting the fibroblast activation protein alpha (FAP) have already proven to be highly potent diagnostic PET-radiotracers in many different cancer types by addressing cancer-associated fibroblasts in the tumor microenvironment that strongly express FAP. Recent approaches with FAPi homodimers have demonstrated prolonged tumor retention and enabled a more effective radioligand therapy (RLT) with long-lived therapeutic radionuclides like lutetium-177.

Based on DOTAGA.Glu.(FAPi)₂, which already showed very promising results in previous studies including proof-of-concept patient studies, a series of new homodimeric compounds was synthesized with different linker and spacer units derived from. ⁶⁸Ga- and ¹⁷⁷Lu-radiolabeling of the new FAPi dimers showed high to quantitative radiochemical conversion and purity. Complex stability in human serum and PBS was investigated over two half-lives. All ¹⁷⁷Lu-complexes were completely stable. The ⁶⁸Ga-complex stability was > 90% in all cases except one. (DO3A.Glu₂.(FAPi)₂). All radiolabeled complexes showed high hydrophilicity ($\log D_{7.4} < -2.3$). *IC*₅₀ values were determined for FAP and the related proteases to determine *in vitro* affinity and selectivity. All compounds showed subnanomolar FAP affinity and high selectivity (> 1000) for prolyl endopeptidase (PREP) and dipeptidyl peptidase 4 (DPP4).

The results show that the new compounds are promising candidates for an efficient ¹⁷⁷Lu-RLT. Further investigations are needed to evaluate ²²⁵Ac-radiolabeling and the pharmacological properties *in vivo*.

Keywords

theranostics, radiometals, positron emission tomography (PET); FAP-targeted radioligand therapy (FAP-RLT); fibroblast activation protein alpha (FAP); cancer-associated fibroblasts (CAFs); homodimeric FAPi-based radiopharmaceuticals; FAPi dimers;

1. Introduction

Targeting the fibroblast activation protein alpha (FAP) with FAP inhibitor-based radiopharmaceuticals has emerged as a highly interesting theranostic approach in nuclear medicine over the last years. What makes this target particularly interesting is its expression pattern. FAP is absent in most adult, healthy tissue. In contrast, it is substantially expressed by activated fibroblasts such as cancer-associated fibroblasts (CAFs) in the tumor microenvironment (TME), during wound healing, at sites of inflammation or fibrosis (e.g. arthritis, liver cirrhosis) [1–4]. In most cancer types, FAP is not expressed by the tumor cells directly except in sarcomas [5], but by the TME. This specific profile makes it a universal pan-tumor target with a potentially broad application range in many different cancer types. For example, FAP is strongly expressed in over 90% of epithelial cancers such as breast, lung, colorectal, prostate and ovarian cancer amongst others [6–8].

CAFs are a substantial part of the TME, also called tumor stroma. The percentage of the TME in relation to the total tumor mass depends on the cancer type and can be up to 90% [7,9]. The TME is a complex environment around the tumor cells that, in addition to CAFs, contains inflammatory, immune, vascular cells and extracellular matrix (ECM) components [10]. The TME is essential for the tumor supply and therefore also plays a crucial role in tumor growth (metastasis, angiogenesis etc.) [1,7,10]. CAFs are known to actively promote tumor growth and metastasis directly and indirectly. CAFs produce many different cytokines, growth factors or enzymes that are released into the TME [11,12]. One important mechanism is the degradation or remodeling of the ECM that is paving the way for the tumor cell to invade into neighboring tissue [13,14]. This can also happen via the bloodstream where stromal cells such as CAFs can support the tumor cell invasion into distant metastatic sites and increase their viability and apoptosis resistance in the blood [15–17]. The serine protease FAP, also called Seprase, belongs to the S9b peptidase family along with other closely related post-proline dipeptidyl peptidases (DPPs) like DPP4, DPP8 and DPP9. It is a type II integral membrane glycoprotein and enzymatically active as a 170 kDa homodimer [1,18]. The catalytic triad consists of Ser⁶²⁴, His⁷³⁴ and Asp⁷⁰² [18–20]. FAP has unique enzymatic properties as it has dipeptidyl peptidase activity like the DPPs and additional endopeptidase activity with its endopeptidase efficiency being higher [1,21–23]. Prolyl endopeptidase (PREP) is the only other protease with the same post proline-selective endopeptidase activity [24]. Some natural substrates are known and include different neuropeptides [1] as well as α_2 -antiplasmin [25,26], a plasmin inhibitor where cleavage by FAP leads to fibrinolysis inhibition, and type I collagen [4,27], which is a major ECM component. FAP itself seems to be involved in the ECM/tissue remodeling that is known from CAFs although the exact role of FAP *in vivo* is not fully understood yet.

Furthermore, a role in epithelial carcinogenesis, tumor proliferation and angiogenesis has been discussed in studies investigating tumor cells of different cancer types [28–33].

The compound UAMC-1110 (see Figure 1a) was the first FAP inhibitor (FAPi) that showed both very high affinity for FAP and selectivity over the DPPs and PREP simultaneously [34,35] and was used in the first clinically applied FAPi-based radiopharmaceuticals in 2018 and 2019 by the group of Haberkorn from Heidelberg [36,37].

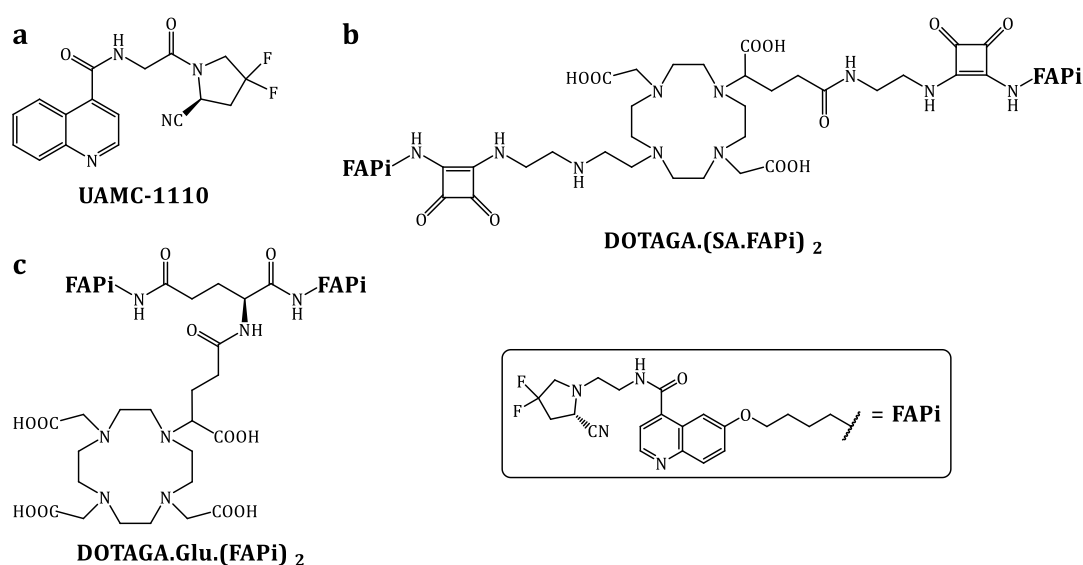


Figure 1: Structures of a) UAMC-1110, b) the first-generation linear FAPi dimer DOTAGA.(SA.FAPi)₂ and c) the second-generation branched FAPi dimer DOTAGA.Glu.(FAPi)₂.

Since then, several other FAPi monomers were published [38–41]. e.g. DOTA.SA.FAPi from our group [42,43] that was already extensively investigated in human PET studies with ⁶⁸Ga and showed fast renal clearance that led to high-contrast PET images in different cancer types 10 min post-injection (p.i.) [44,45]. While this early time-point imaging suits clinical routine requirements in PET/CT diagnosis very well, this could turn into a disadvantage for radioligand therapy (RLT). All these FAPi-based monomers so far suffered from short tumor retention time of several hours only. Long tumor retention appears to be an important requirement for efficient RLT with more long-lived radiometals like ¹⁷⁷Lu ($t_{1/2}$ = 6.7 d) or targeted alpha-particle therapy with ²²⁵Ac ($t_{1/2}$ = 10.0 d) to deposit a sufficient radiation dose to the tumor for optimized therapeutic effect. Currently, there are several strategies under investigation to extend tumor retention of CAF-targeting radiopharmaceuticals such as using modified cyclic peptides with high FAP affinity [46–48] or adding an albumin-binding moiety to a monomeric FAPi-based scaffold [49–53].

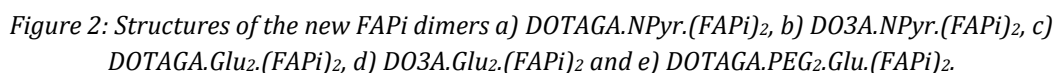
In parallel, the tumor retention was improved drastically by synthesizing FAPi dimers with two identical FAPi targeting vectors (two functionalized UAMC-1110 units) such as the first-generation linear homodimer DOTAGA.(SA.FAPi)₂ as published by Moon et al. (see Figure 1b) in

2021. The first clinical data of [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$ were presented by Ballal et al. at the SNMMI annual meeting in 2021 [54,55]. The therapeutic effectiveness and safety of [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$ was investigated, first and foremost in radioiodine-refractory differentiated thyroid cancer (RR-DTC) patients, and was found to be very promising [54,56,57]. [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$ showed similar accumulation profiles to the diagnostic PET/CT counterpart [^{68}Ga]Ga-DOTA.SA.FAPi but remained at the tumor lesions for several days [56,57]. The excretion of [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$ is rather slow and predominantly via hepatobiliary pathway. Yet, the properties could be improved substantially with the next-generation dimer DOTAGA.Glu.(FAPi) $_2$ (see Figure 1c) [58]. [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ showed higher hydrophilicity, superior *in vitro* affinity and selectivity as well as faster off-target pharmacokinetics and improved pharmacodynamics. The radiation dose to the critical healthy organs such as the colon could be reduced compared to [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$ [58].

In the meanwhile, other groups also synthesized dimeric FAPi-based radiopharmaceuticals such as DOTA-2P(FAPi) $_2$ [59], BiOncoFAP-DOTAGA [60] and BiOncoFAP-11 [61] and all confirmed that the concept of prolonging tumor retention can be achieved with FAPi homodimers. Additionally, anti-tumor efficacy could also be demonstrated by treating FAP-positive HT-1080 tumor bearing mice with [^{177}Lu]Lu-BiOncoFAP-DOTAGA [60].

The present study consists of new compounds with a similar design as DOTAGA.Glu.(FAPi) $_2$ as depicted in Figure 2a–e.

This work is focused on structure-property relationship studies to investigate if substituting or adding different linker and spacer units compared to DOTAGA.Glu.(FAPi) $_2$ will have a beneficial effect on (i) the radiolabeling properties and (ii) the *in vitro* properties that will ultimately effect (iii) the pharmacological (pharmacokinetic and pharmacodynamic) properties *in vivo*.



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2. Materials and Methods

2.1. Chemicals and Equipment

All basic chemicals were purchased from Sigma-Aldrich and Merck (Darmstadt, Germany), TCI (Eschborn, Germany), ABCR (Karlsruhe, Germany), Alfa Aesar by Thermo Fisher Scientific (Kandel, Germany), Acros Organics (Schwerte, Germany) and VWR (Bruchsal, Germany). Dry solvents were bought from Acros Organics, Alfa Aesar by Thermo Fischer Scientific. Merck as well as deuterated solvents from Deutero (Kastellaun, Germany). The chelators DOTA-tri(^tBu)ester (2-(4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)acetic acid) and DOTA-GA(^tBu)₄ (5-(*tert*-butoxy)-5-oxo-4-(4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)pentanoic acid) were obtained from CheMatech (Dijon, France). (*S*)-6-(4-Aminobutoxy)-*N*-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide, trifluoroacetate salt (FAPi-NH₂*TFA) was purchased from KE Biochem Co. (Shanghai, China).

Thin-layer chromatography (TLC) plates coated with silica gel 60 F254 from Merck were used. For analysis they were investigated via UV lamp ($\lambda = 254$ and 366 nm) as well as staining with potassium permanganate. Silica 60 (0.040-0.063 mm particle size) was used for column chromatography.

Nuclear magnetic resonance (NMR) spectra were recorded in deuterated solvents on an Avance II 400 spectrometer (400 MHz, 5 mm BBFO sample head with z-gradient and ATM, SampleXPress 60 sample changer). MestReNova 14.2.0 software from Mestrelab Research (Santiago de Compostela, Spain) was used to analyze the spectra.

Liquid chromatography coupled with electrospray ionization mass spectrometry (ESI-LC/MS) was performed using a LC/MS system from Shimadzu (Japan) with CBM-20A system controller, SIL-20A HT autosampler, two LC-20AD pump modules, CTO-20 AC column oven. The UV signal was measured with SPD-M20A photodiode array detector and the mass spectrum with a LC/MS-2020 mass spectrometer. The separation was realized with a Eurosphere II 100-5 C18 column (150x4 mm) from Knauer (Berlin, Germany) and linear gradient of acetonitrile (MeCN)/water (HPLC grade) + 0.1% formic acid (HFO).

High-pressure liquid chromatography coupled with electrospray ionization mass spectrometry (ESI-HPLC-MS) measurements were performed using an Agilent Technologies G6545A Q-ToF with electron spray ionization coupled to a 1260 Infinity II HPLC system (Agilent Technologies, Santa Clara, USA) with G7111B 1260 Quaternary Pump, G7129A 1260 Vial Sampler, and G7116A Multicolumn Thermostat. Separation was performed using an Agilent Poroshell 120 EC-C8 column (2.1x100 mm, 2.7 μ m) with H₂O + 2% MeCN / MeCN + 2% H₂O + 0.05% HFO and a flow rate of 0.1 mL/min.

Semi-preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) was performed using LaChrom HPLC (7000 series) from Merck Hitachi with L-7100 pump, L-7400 UV detector ($\lambda = 254$ nm), a D-7000 interface and autosampler. Separation was performed using a Phenomenex (Aschaffenburg, Germany) Synergi Max-RP C18 column (250x10 mm, 4 μ m) and linear gradient of MeCN/H₂O + 0.1% trifluoacetic acid (TFA) and a flow rate of 5 mL/min.

For radiolabeling only chemicals of the highest purity (TraceSelect if available) were used. Radio-TLCs were developed using 0.1 M or 0.25 M citrate buffer (pH = 4.0). Additional TLCs were developed using 1 M ammonium acetate (AmOAc, pH = 4.0)/methanol (MeOH) (1:1). All radio-TLCs were evaluated using a CR-35 Bio Test-Imager from Duerr-ndt (Bietigheim-Bissingen, Germany) and AIDA software from Elysia-Raytest (Straubenhardt, Germany). Analytical radio-HPLC was performed also using a Merck Hitachi LaChrom HPLC (7000 series). Separation was performed using Phenomenex Luna C18 column (250x4.6 mm, 5 μ m) and linear gradient of MeCN/H₂O + 0.1% TFA and flow rate of 1 mL/min. The radio-HPLC is equipped with a Ramona analog radio detector from Elysia-Raytest. Its energy window was set to 100-1200 keV for ⁶⁸Ga and 100-250 keV for ¹⁷⁷Lu.

2.2. Organic Synthesis

2.2.1. Synthesis of DOTAGA.NPyr.(FAPi)₂ and DO3A.NPyr.(FAPi)₂

Dibenzyl (R)-2,2'-((1-(tert-butoxycarbonyl)pyrrolidin-3-yl)azanediyl)-diacetate (3, Boc-NPyr(OBzl)₂)

(S)-1-Boc-3-aminopyrrolidine **1** (1.07 g, 5.74 mmol, 1.00 eq) and *N,N*-diisopropylethylamine (DIPEA, 1.5 mL) were dissolved in MeCN (6 mL). After 60 min, a solution of benzyl bromoacetate **2** (1.74 g, 7.55 mmol, 1.32 eq) in MeCN (6 mL) was slowly added and stirred at room temperature (RT) for another two hours. MeCN was removed under reduced pressure. Subsequent column chromatography (CHCl₃/MeOH (30:1) + 1% triethylamine (TEA)) yielded Boc-NPyr(OBzl)₂ **3** (1.31 g, 2.71 mmol, 47%) as a colorless oil. ¹H-NMR (400 MHz, CDCl₃) δ = 7.42 – 7.28 (m, 10H), 5.13 (s, 4H), 3.76 – 3.38 (m, 7H), 3.21 (td, *J* = 10.6, 6.7 Hz, 1H), 3.16 – 3.03 (m, 1H), 2.08 – 1.94 (m, 1H), 1.83 – 1.69 (m, 1H), 1.44 (s, 9H). MS (ESI⁺): *m/z* (%) = 383.2 (45, [M-Boc+H]⁺), 483.2 (100, [M+H]⁺), 484.2 (30, [M+H]⁺), calculated for C₂₇H₃₄N₂O₆: 482.24 [M].

(R)-2,2'-((1-(tert-Butoxycarbonyl)pyrrolidin-3-yl)azanediyl)diacetic acid (4, Boc-NPyr)

Boc-NPyr(OBzl)₂ **3** (1.21 g, 2.51 mmol, 1.00 eq) was dissolved in dry MeOH (8 mL) and palladium on carbon (10 wt% Pd, 53 mg, 50 μ mol, 0.02 eq) was added. It was stirred at RT for two days under hydrogen atmosphere. It was filtered through Celite® and washed with MeOH. The solvent was removed *in vacuo*. Boc-NPyr **4** (643 mg, 2.13 mmol, 85%) was obtained as a colorless solid. ¹H-NMR (400 MHz, MeOD) δ = 3.75 – 3.45 (m, 6H), 3.35 (s, 1H), 3.31 – 3.11 (m, 2H), 2.15 – 2.05

(m, 1H), 1.95 – 1.79 (m, 1H), 1.46 (s, 9H). MS (ESI⁺): m/z (%) = 247.0 (100, [M-^tBu+H]⁺), 303.1 (36, [M+H]⁺), 605.3 (23, [2M+H]⁺), calculated for C₁₃H₂₂N₂O₆: 302.15 [M].

tert-Butyl (*R*)-3-(bis(2-((4-((2-((*S*)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-2-oxoethyl)amino)pyrrolidine-1-carboxylate (**5**, Boc-NPyr.(FAPi)₂) Boc-NPyr **4** (30.2 mg, 100 μmol, 1.00 eq), 1-hydroxybenzotriazole (HOBt, 36.0 mg, 266 μmol, 2.66 eq) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC*HCl, 50.0 mg, 260 μmol, 2.60 eq) were dissolved in dry *N,N*-dimethylformamide (DMF, 3 mL) and stirred under argon atmosphere at 30 °C for 60 minutes. Then, a solution of FAPi-NH₂*TFA (110 mg, 202 μmol, 2.02 eq) and DIPEA (51.0 μL, 300 μmol, 3.00 eq) in DMF (2 mL) was added and the solution was stirred at 30 °C for another 3.5 hours. HOBt (8.5 mg, 62.9 μmol, 0.63 eq) and EDC*HCl (12.0 mg, 62.6 μmol, 0.63 eq) was then added, followed by a solution of FAPi-NH₂*TFA (25.0 mg, 45.8 μmol, 0.46 eq) and DIPEA (17.0 μL, 100 μmol, 1.00 eq) in dry DMF (1 mL) 30 minutes later. After stirring at 30 °C overnight, the additions of HOBt (8.5 mg, 62.9 μmol, 0.63 eq), EDC*HCl (12.0 mg, 62.6 μmol, 0.63 eq), and 30 minutes later of FAPi-NH₂*TFA (16.0 mg, 29.3 μmol, 0.29 eq) with DIPEA (17.0 μL, 100 μmol, 1.00 eq) in dry DMF (1 mL) were repeated one more time. Stirring continued for another five hours at 30 °C and then the solvent was removed *in vacuo*. After column chromatography (CHCl₃/MeOH/TEA, 100:7.5–10:1) Boc-NPyr.(FAPi)₂ **5** (102 mg, 90.3 μmol, 90%) was obtained as a yellow oil. MS (ESI⁺): m/z (%) = 358.6 (86, [M-^tBu+H]³⁺), 372.2 (58, [M-^tBu+MeCN+H]³⁺), 377.3 (100, [M+H]³⁺), 390.3 (68, [M+MeCN+H]³⁺), 515.3 (36, [M-Boc+H]²⁺), 537.5 (8, [M-^tBu+H]²⁺), 565.5 (84, [M+H]²⁺), 1129.6 (28, [M+H]⁺), 1130.6 (17, [M+H]⁺), calculated for C₅₅H₆₄F₄N₁₂O₁₀: 1128.48 [M].

6,6'-((((2,2'-(((*R*)-Pyrrolidin-3-yl)azanediyl)bis(acetyl))bis(azanediyl))bis(butane-4,1-diyl))bis(oxy))bis(*N*-(2-((*S*)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide) (**6**, NPyr.(FAPi)₂)

Boc-NPyr.(FAPi)₂ **5** (102 mg, 90.3 μmol) was stirred with Milli-Q® water (50 μL), triisopropylsilane (TIPS, 50 μL), and TFA (1.9 mL) at RT for one hour. Then, MeOH (10 mL) was added and removed again *in vacuo* which was repeated five times. A yellow oil was obtained which was used in the next step without further purification. MS (ESI⁺): m/z (%) = 344.1 (100, [M+H]³⁺), 357.6 (45, [M+MeCN+H]³⁺), 515.5 (18, [M+H]²⁺), 1029.5 (3, [M+H]⁺), calculated for C₅₀H₅₆F₄N₁₂O₈: 1028.43 [M].

2,2',2''-(10-(4-((*R*)-3-(Bis(2-((4-((2-((*S*)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-2-oxoethyl)amino)pyrrolidin-1-yl)-1-carboxy-4-oxobutyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (**8**, DOTAGA.NPyr.(FAPi)₂)

DOTAGA(*t*Bu)₄ **7** (50.0 mg, 71.3 μmol, 1.43 eq), *N*-hydroxysuccinimide (NHS, 16.4 mg, 142 μmol, 2.84 eq) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 54.1 mg, 143 μmol, 2.86 eq) were dissolved in dry DMF (1 mL) and shaken overnight at 30 °C under argon atmosphere. NHS (8.2 mg, 71.2 μmol, 1.42 eq) and HBTU (27.0 mg, 71.2 μmol, 1.42 eq) were added again. Four hours later, a solution of NPyr.(FAPi)₂ **6** (51.5 mg, 50.0 μmol, 1.00 eq) and DIPEA (40 μL, 235 μmol, 4.70 eq) in dry DMF (1 mL) was added. Stirring continued for two days at 40 °C and then all solvents were completely removed *in vacuo*. A yellow oil was obtained and used without further purification.

25 μL of Milli-Q® water, 50 μL of TIPS, 100 μL MeCN and 1 mL of TFA (TFA/MeCN/TIPS/H₂O, 100:10:5:2.5) was added and the solution was stirred at RT for 5 hours. Then the solvents were removed under reduced pressure via co-distillation with MeOH (3x 10 mL). The crude product was purified by semi-preparative RP-HPLC (25–27% MeCN in 20 min, *t_R* = 18–20 min). 8.3 mg (5.6 μmol, 11%) DOTAGA.NPyr.(FAPi)₂ **8** were obtained as a yellow solid (purity = 99.6%: cf. analytical RP-HPLC in Figure S1). MS (ESI⁺): *m/z* (%) = 372.6 (100, [M+H]⁴⁺), 382.9 (38, [M+MeCN+H]⁴⁺), 496.6 (76, [M+H]³⁺), 744.4 (5, [M+H]²⁺), calculated for C₆₉H₈₆F₄N₁₆O₁₇: 1486.63 [M].

*tri-tert-Butyl 2,2',2''-(10-(2-((2,5-dioxopyrrolidin-1-yl)oxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (10, DOTA(*t*Bu)₃-NHS)*

DOTA-tris(*tert*-butyl ester) **9** (573 mg, 1.00 mmol, 1.00 eq) and HBTU (417 mg, 1.10 mmol, 1.10 eq) were dissolved in dry MeCN (12 mL) and dry DMF (5 mL) and then NHS (127 mg, 1.10 mmol, 1.10 eq) was added under argon atmosphere. After stirring at 30 °C for 6 hours, HBTU (190 mg, 501 μmol, 0.50 eq) and NHS (57.5 mg, 500 μmol, 0.50 eq) were added and stirring was continued overnight. After all solvents were removed *in vacuo*, purification by column chromatography (dichloromethane (DCM)/MeOH, 10:1) gave **10** as a colorless solid (643 mg, 961 μmol, 96%). ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 2.97 – 2.77 (m, 12H), 2.66 – 2.02 (m, 16H), 1.44 (s, 27H). MS (ESI⁺): *m/z* (%) = 335.7 (100, [M+H]²⁺), 670.4 (50, [M+H]⁺), 671.4 (18, [M+H]⁺), calculated for C₃₂H₅₅N₅O₁₀: 669.39 [M].

*2,2',2''-(10-(2-((*R*)-3-(Bis(2-((4-((*S*)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-2-oxoethyl)amino)pyrrolidin-1-yl)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (11, DO3A.NPyr.(FAPi)₂)*

DOTA(*t*Bu)₃-NHS **10** (33.5 mg, 50.0 μmol, 1.25 eq) and NPyr.(FAPi)₂ **6** (41.2 mg, 40.0 μmol, 1.00 eq) were dissolved in dry DMF (1 mL) and DIPEA (50 μL) was added. The reaction was stirred under argon atmosphere at 40 °C for three days and then all solvents were completely removed *in vacuo*. A yellow oil was obtained which was directly used without further purification.

After addition of 50 μ L Milli-Q® water, 50 μ L TIPS, and 1.5 mL TFA (TFA/TIPS/H₂O, 94:3:3) it was stirred at RT for 12 hours. MeOH (10 mL) was added removed again *in vacuo* which was repeated five times. The crude product was purified by semi-preparative RP-HPLC (21–22% MeCN in 20 min, t_R = 18.5–19.5 min). DO3A.NPyr.(FAPi)₂ **11** (5.6 mg, 4.0 μ mol, 10%) was obtained as a yellow solid (purity = 83.2%: cf. analytical RP-HPLC in Figure S2). MS (ESI⁺): m/z (%) = 354.6 (95, [M+H]⁴⁺), 364.8 (59, [M+MeCN+H]⁴⁺), 472.6 (100, [M+H]³⁺), 708.6 (13, [M+H]²⁺), 1415.5 (5, [M+H]⁺), calculated for C₆₆H₈₂F₄N₁₆O₁₅: 1414.61 [M].

2.2.2. Synthesis of DOTAGA.Glu₂.(FAPi)₂ and DO3A.Glu₂.(FAPi)₂

Dibenzyl ((S)-4-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-(tert-butoxy)-5-oxopentanoyl)-L-glutamatetri-tert-Butyl (14, Fmoc-Glu(O^tBu).Glu(OBzl)₂)

Fmoc-Glu-O^tBu **13** (400 mg, 940 μ mol, 1.00 eq) was dissolved in dry DMF (2 mL). DIPEA (200 μ L, 1.18 mmol, 1.26 eq) and 1-[bis(dimethylamino)methylene]-1H-1,2,3-triazolo[4,5-b]pyridinium 3-oxide hexafluorophosphate (HATU, 393 mg, 1.03 mmol, 1.10 eq) was added and the solution was stirred under argon atmosphere at RT. After one hour, a solution of dibenzyl glutamate **12** (385 mg, 1.18 mmol, 1.26 eq) and DIPEA (352 μ L, 2.07 mmol, 2.17 eq) in dry DMF (1 mL) was added and stirred overnight at RT. HATU (357 mg, 939 μ mol, 1.00 eq) and DIPEA (156 μ L, 917 μ mol, 0.97 eq) was added again. After three more days of stirring at RT, HATU (357 mg, 939 μ mol, 1.00 eq) and one hour later a solution of dibenzyl glutamate **12** (154 mg, 470 μ mol, 0.50 eq) and DIPEA (160 μ L, 941 μ mol, 1.00 eq) in DMF (0.5 mL) was added. The solvent was removed under reduced pressure after another day at RT and the product was purified by column chromatography (cyclohexane/ethyl acetate (CH/EA), 3:1). Fmoc-Glu(O^tBu).Glu(OBzl)₂ **14** (657 mg, 894 μ mol, 95%) was obtained as a yellowish solid. ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 7.76 (dd, J = 7.5, 0.9 Hz, 2H), 7.60 (d, J = 7.4 Hz, 2H), 7.43 – 7.36 (m, 2H), 7.36 – 7.27 (m, 12H), 6.50 (d, J = 7.7 Hz, 1H), 5.54 (d, J = 8.1 Hz, 1H), 5.14 (s, 2H), 5.07 (s, 2H), 4.67 (td, J = 7.8, 5.0 Hz, 1H), 4.45 – 4.34 (m, 2H), 4.27 – 4.17 (m, 2H), 2.53 – 2.32 (m, 2H), 2.31 – 2.14 (m, 4H), 2.11 – 1.81 (m, 2H), 1.46 (s, 9H). MS (ESI⁺): m/z (%) = 679.2 (27, [M-^tBu+H]⁺), 680.3 (11, [M-^tBu+H]⁺), 735.5 (100, [M+H]⁺), 736.2 (15, [M+H]⁺), calculated for C₄₃H₄₆N₂O₉: 734.32 [M].

((S)-4-((((9H-fluoren-9-yl)methoxy)carbonyl)amino)-5-(tert-butoxy)-5-oxopentanoyl)-L-glutamic acid (15, Fmoc-Glu(O^tBu).Glu)

Fmoc-Glu(O^tBu).Glu(OBzl)₂ **14** (200 mg, 272 μ mol, 1.00 eq) was dissolved in dry tetrahydrofuran (THF, 5 mL) and palladium on carbon (10 wt% Pd, 29.0 mg, 27.2 μ mol, 0.10 eq) was added. The suspension was stirred overnight under hydrogen atmosphere and filtered through Celite®. The residue was washed with THF and the solvent was removed under reduced pressure. Fmoc-Glu(O^tBu).Glu **15** (150 mg, 270 μ mol, 99%) was obtained as a colorless solid. ¹H-NMR (400 MHz,

DMSO- d_6): δ [ppm] = 8.32 (s, 1H), 8.11 (d, J = 7.7 Hz, 1H), 7.93 – 7.83 (m, 2H), 7.76 – 7.67 (m, 2H), 7.46 – 7.28 (m, 4H), 6.87 (s, 1H), 4.35 – 4.09 (m, 3H), 3.99 – 3.70 (m, 2H), 2.31 – 2.16 (m, 5H), 2.01 – 1.88 (m, 1H), 1.83 – 1.67 (m, 1H), 1.49 – 1.39 (m, 1H), 1.39 (s, 9H). MS (ESI⁺): m/z (%) = 499.05 (57, [M-^tBu+H]⁺), 500.2 (11, [M-^tBu+H]⁺), 555.3 (100, [M+H]⁺), 556.2 (21, [M+H]⁺), calculated for C₂₉H₃₄N₂O₉: 554.23 [M].

tert-Butyl (2*S*,7*R*)-2-((((9*H*-fluoren-9-yl)methoxy)carbonyl)amino)-10-((4-((4-((2-((*S*)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-7-((4-((4-((2-((*S*)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)carbamoyl)-5,10-dioxodecanoate (**16**, Fmoc-Glu(O^tBu).Glu.(FAPi)₂)

Fmoc-Glu(O^tBu).Glu **15** (33.3 mg, 60.0 μ mol, 1.00 eq), HOBT (20.4 mg, 151 μ mol, 2.52 eq) and EDC·HCl (28.8 mg, 150 μ mol, 2.50 eq) were dissolved in dry DMF (2.5 mL) and stirred under argon atmosphere at RT for one hour. FAPi-NH₂·TFA (65.4 mg, 120 μ mol, 2.00 eq) and DIPEA (31.0 μ L, 182 μ mol, 3.03 eq), dissolved in dry DMF (0.5 mL), was added and stirred at RT overnight. HOBT (7.8 mg, 57.7 μ mol, 0.96 eq) and EDC·HCl (11.4 mg, 59.5 μ mol, 0.99 eq) was added, followed by a solution of FAPi-NH₂·TFA (16.5 mg, 30.2 μ mol, 0.50 eq) and DIPEA (10.0 μ L, 58.8 μ mol, 0.98 eq) in DMF (0.5 mL) 30 minutes later. One day later, HOBT (3.9 mg, 28.8 μ mol, 0.48 eq) and EDC·HCl (5.7 mg, 29.7 μ mol, 0.50 eq) and after one more hour, additional FAPi-NH₂·TFA (16.5 mg, 30.2 μ mol, 0.50 eq) and DIPEA (5.0 μ L, 29.4 μ mol, 0.49 eq) in DMF (0.5 mL), was added. This step was repeated again the next day. After the final addition the reaction solution was stirred for one more day and then the solvent was subsequently removed under reduced pressure. Column chromatography (CHCl₃/MeOH, 10:1) yielded Fmoc-Glu(O^tBu).Glu.(FAPi)₂ **16** (86.7 mg, 62.8 μ mol, 79%) as a yellowish solid. MS (ESI⁺): m/z (%) = 461.3 (32, [M+H]³⁺), 691.5 (100, [M+H]²⁺), 692.3 (12, [M+H]²⁺), 1382.0 (12, [M+H]⁺), calculated for C₇₁H₇₆F₄N₁₂O₁₃: 1380.56 [M].

tert-Butyl (2*S*,7*R*)-2-amino-10-((4-((4-((2-((*S*)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-7-((4-((4-((2-((*S*)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)carbamoyl)-5,10-dioxodecanoate (**17**, Glu(O^tBu).Glu.(FAPi)₂)

Fmoc-Glu(O^tBu).Glu.(FAPi)₂ **16** (72.2 mg, 52.3 μ mol, 1.00 eq) was dissolved in dry DMF (1 mL) and piperidine (100 μ L) was added. The solution was stirred at RT for 90 min. Then, the solvents were completely removed under reduced pressure. Glu(O^tBu).Glu.(FAPi)₂ **17** was obtained as a yellow oil in quantitative yield and used directly in the next step without further purification. MS (ESI⁺): m/z (%) = 387.1 (99, [M+H]³⁺), 580.4 (37, [M+H]²⁺), 1159.3 (4, [M+H]⁺), calculated for C₅₆H₆₆F₄N₁₂O₁₁: 1158.49 [M].

2,2',2''-(10-(4-(((S)-4-(((S)-1,5-Bis((4-((4-((2-((S)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-1,5-dioxopentan-2-yl)amino)-1-carboxy-4-oxobutyl)amino)-1-carboxy-4-oxobutyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (**18**, DOTAGA.Glu₂.(FAPi)₂)

DOTAGA(^tBu)₄ **7** (36.3 mg, 45.5 μmol, 1.25 eq) and HATU (27.7 mg, 72.9 μmol, 2.00 eq) was dissolved in dry DMF (700 μL), DIPEA (9.3 μL, 54.7 μmol, 1.50 eq) was added and stirred under argon atmosphere at RT for 60 minutes. Glu(O^tBu).Glu.(FAPi)₂ **16** (42.2 mg, 36.4 μmol, 1.00 eq) and DIPEA (12.4 μL, 72.9 μmol, 2.00 eq), dissolved in dry DMF (500 μL) and dry MeCN (500 μL), was added and stirred at RT overnight. The solvents were removed *in vacuo* and the residue was dissolved in 25 μL Milli-Q® water, 25 μL TIPS, 50 μL MeCN and 900 μL TFA (TFA/MeCN/TIPS/H₂O, 90:5:2.5:2.5) and stirred at RT for 6 hours. The solvents were removed via co-distillation with MeOH (3x 10 mL). Subsequent semipreparative RP-HPLC (23–25% MeCN in 20 min, *t_R* = 15–16 min) yielded DOTAGA.Glu₂.(FAPi)₂ **18** (9.1 mg, 5.8 μmol, 16%) as a yellow solid (purity = 97.8%: cf. analytical RP-HPLC in Figure S3). MS (ESI⁺): *m/z* (%) = 391.1 (78, [M+H]⁴⁺), 401.2 (19, [M+MeCN+H]⁴⁺), 521.3 (100, [M+H]³⁺), 781.8 (6, [M+H]²⁺), 1561.7 (3, [M+H]⁺). calculated for C₇₁H₈₈F₄N₁₆O₂₀: 1560.63 [M].

2,2',2''-(10-(2-(((S)-4-(((S)-1,5-Bis((4-((4-((2-((S)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-1,5-dioxopentan-2-yl)amino)-1-carboxy-4-oxobutyl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (**19**, DO3A.Glu₂.(FAPi)₂)

DOTA(^tBu)₃-NHS **9** (26.0 mg, 38.9 μmol, 1.00 eq) and Glu(O^tBu).Glu.(FAPi)₂ **16** (45.1 mg, 38.9 μmol, 1.00 eq) was dissolved in dry DMF (1.5 mL) and DIPEA (15.0 μL, 88.2 μmol, 2.27 eq) was added. After stirring at 40 °C and under argon atmosphere for three days, DOTA(^tBu)₃-NHS **9** (6.5 mg, 9.7 μmol, 0.25 eq) was added and after another six hours at 40 °C the solvent was removed under reduced pressure.

25 μL Milli-Q® water, 25 μL TIPS, 50 μL MeCN and 900 μL TFA (TFA/MeCN/TIPS/H₂O, 90:5:2.5:2.5) was added and stirred for 6 hours at RT. The solvents were removed via co-distillation with MeOH (3x 10 mL). After semipreparative RP-HPLC purification (22–23% MeCN in 20 min, *t_R* = 13.5–14.5 min) DO3A.Glu₂.(FAPi)₂ **19** (6.0 mg, 4.0 μmol, 10%) was obtained as a yellow solid (purity = 94.9%: cf. analytical RP-HPLC in Figure S4). MS (ESI⁺): *m/z* (%) = 373.1 (84, [M+H]⁴⁺), 497.2 (100, [M+H]³⁺), 745.7 (5, [M+H]²⁺), calculated for C₆₈H₈₄F₄N₁₆O₁₈: 1488.61 [M].

2.2.3. Synthesis of DOTAGA.PEG₂.Glu.(FAPi)₂

Dibenzyl (1-(9H-fluoren-9-yl)-3-oxo-2,7,10-trioxa-4-azatridecan-13-oyl)-L-glutamatetri-tert-Butyl (21, Fmoc-PEG₂.Glu(OBzl)₂)

Fmoc-N-amido-dPEG₂ acid **20** (450 mg, 1.13 mmol, 1.00 eq), HBTU (470 mg, 1.24 mmol, 1.10 eq), and HOBt (168 mg, 1.24 mmol, 1.10 eq) were dissolved in dry DMF (9 mL) and DIPEA (240 μ L, 1.41 mmol, 1.25 eq) was added. The colorless solution was stirred under argon atmosphere at RT for one hour. Then, dibenzyl glutamate **12** (461 mg, 1.41 mmol, 1.25 eq) dissolved in dry DMF (3 mL) and DIPEA (422 μ L, 2.48 mmol, 2.19 eq) was added. After one day at RT, the reaction was completed and the solvent was removed under reduced pressure. The yellowish oil was purified by column chromatography (DCM/MeOH, 100:2) to yield Fmoc-PEG₂.Glu(OBzl)₂ **21** (795 mg, 1.12 mmol, 99%) as a colorless oil. ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 7.75 (d, J = 7.5 Hz, 2H), 7.59 (d, J = 7.5 Hz, 2H), 7.43 – 7.26 (m, 14H), 6.91 (d, J = 8.0 Hz, 1H), 5.52 (t, J = 5.8 Hz, 1H), 5.12 (s, 2H), 5.07 (d, J = 2.5 Hz, 2H), 4.70 (td, J = 8.0, 5.1 Hz, 1H), 4.39 (d, J = 7.1 Hz, 2H), 4.21 (t, J = 7.0 Hz, 1H), 3.77 – 3.63 (m, 2H), 3.60 – 3.30 (m, 6H), 2.80 (s, 2H), 2.54 – 2.28 (m, 4H), 2.28 – 2.16 (m, 1H), 2.05 – 1.93 (m, 1H). MS (ESI⁺): m/z (%) = 709.4 (100, [M+H]⁺), 710.2 (15, [M+H]⁺), calculated for C₄₁H₄₄N₂O₉: 708.30 [M].

(1-(9H-Fluoren-9-yl)-3-oxo-2,7,10-trioxa-4-azatridecan-13-oyl)-L-glutamic acid (22, Fmoc-PEG₂.Glu)

Fmoc-PEG₂.Glu(OBzl)₂ **21** (196 mg, 276 μ mol, 1.00 eq) was dissolved in dry THF (2 mL) and palladium on carbon (10 wt% Pd, 30.0 mg, 28.2 μ mol, 0.10 eq) was added. It was then stirred under hydrogen atmosphere at RT for 24 hours. The suspension was filtered through Celite®, the residue was washed with THF and the solvent was removed under reduced pressure. Fmoc-PEG₂.Glu **22** (122 mg, 231 μ mol, 84%) was obtained as a colorless oil and used in the next step without further purification. ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 7.76 (d, J = 7.0 Hz, 2H), 7.62 – 7.48 (m, 2H), 7.43 – 7.27 (m, 4H), 6.98 (s, 1H), 5.01 (s, 1H), 4.69 – 4.55 (m, 1H), 4.47 – 4.31 (m, 2H), 4.28 – 4.18 (m, 1H), 3.99 – 3.27 (m, 8H), 2.80 (s, 2H), 2.62 – 2.38 (m, 3H), 2.32 – 2.20 (m, 2H), 2.12 – 1.99 (m, 1H). MS (ESI⁺): m/z (%) = 529.3 (100, [M+H]⁺), 530.2 (12, [M+H]⁺), calculated for C₂₇H₃₂N₂O₉: 528.21 [M].

(9H-Fluoren-9-yl)methyl ((S)-19-((4-((2-((S)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)-11-((4-((2-((S)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)carbamoyl)-9,14-dioxo-3,6-dioxa-10,15-diazanonadecyl)carbamate (23, Fmoc-PEG₂.Glu.(FAPi)₂)

Fmoc-PEG₂.Glu **22** (32.0 mg, 60.5 μ mol, 1.00 eq), HOBt (20.4 mg, 151 μ mol, 2.50 eq) and EDC·HCl (28.8 mg, 150 μ mol, 2.48 eq) were dissolved in dry DMF (1 mL) and stirred at RT under argon

atmosphere. After one hour, a solution of FAPi-NH₂*TFA (65.4 mg, 120 µmol, 1.98 eq), DIPEA (30.0 µL, 176 µmol, 2.91 eq), in dry DMF (500 µL) was added. Three hours later, HOBt (7.8 mg, 57.7 µmol, 0.95 eq) and EDC*HCl (11.4 mg, 59.5 µmol, 0.98 eq) was added. Another 30 minutes later, additional FAPi-NH₂*TFA (16.5 mg, 30.2 µmol, 0.50 eq) with DIPEA (10.0 µL, 58.8 µmol, 0.97 eq) in dry DMF (500 µL) was added and the stirring at RT was continued overnight. After another addition of HOBt (3.9 mg, 28.9 µmol, 0.48 eq) and EDC*HCl (5.7 mg, 29.7 µmol, 0.49 eq) and four more hours at RT, the reaction was terminated by removing the solvent under reduced pressure. Purification by column chromatography (CHCl₃/MeOH, 10:1) yielded Fmoc-PEG₂.Glu.(FAPi)₂ **23** (79.1 mg, 58.4 µmol, 97%) as a yellow solid. MS (ESI⁺): m/z (%) = 452.5 (31, [M+H]³⁺), 678.5 (100, [M+H]²⁺), 679.3 (13, [M+H]²⁺), 1355.9 (9, [M+H]⁺), calculated for C₆₉H₇₄F₄N₁₂O₁₃: 1354.54 [M].

(S)-2-(3-(2-(2-Aminoethoxy)ethoxy)propanamido)-N¹,N⁵-bis(4-((4-((2-((S)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)pentanediamide
(**24**, PEG₂.Glu.(FAPi)₂)

Fmoc-PEG₂.Glu.(FAPi)₂ **23** (67.0 mg, 49.4 µmol, 1.00 eq) was dissolved in dry DMF (1 mL) and piperidine (100 µL) was added. The yellowish solution was stirred at RT for two hours and then the solvents were removed under reduced pressure. PEG₂.Glu.(FAPi)₂ **24** was obtained as a yellow oil in quantitative yield and used directly in the next step without further purification. MS (ESI⁺): m/z (%) = 378.4 (100, [M+H]³⁺), 567.4 (26, [M+H]²⁺), 1133.4 (3, [M+H]⁺), calculated for C₅₄H₆₄F₄N₁₂O₁₁: 1132.48 [M].

2,2',2''-(10-((16S)-1-Carboxy-24-((4-((2-((S)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)-16-((4-((4-((2-((S)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)carbamoyl)-4,14,19-trioxo-8,11-dioxo-5,15,20-triazatetracosyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid
(**25**, DOTAGA.PEG₂.Glu.(FAPi)₂)

DOTAGA(tBu)₄ **7** (21.0 mg, 30.0 µmol, 1.36 eq), HATU (12.2 mg, 32.2 µmol, 1.46 eq) and DIPEA (5.1 µL, 30.0 µmol, 1.36 eq) was dissolved in dry DMF (1 mL) and stirred at 30 °C and under argon atmosphere for 60 minutes. A solution of PEG₂.Glu.(FAPi)₂ **24** (24.9 mg, 22.0 µmol, 1.00 eq) and DIPEA (5.1 µL, 30.0 µmol, 1.36 eq) in dry DMF (500 µL) was added and stirred at RT overnight. HATU (6.1 mg, 16.0 µmol, 0.73 eq) was added once more and the reaction was stirred for two more days at 30 °C. The solvents were removed under reduced pressure.

The residual yellow oil was dissolved in 25 µL Milli-Q® water, 50 µL TIPS, 100 µL MeCN and 1 mL TFA (TFA/MeCN/TIPS/H₂O, 100:10:5:2.5) and stirred at RT for 6 hours. The solvents were removed via co-distillation with MeOH (3x 10 mL). After semipreparative RP-HPLC (26–28% MeCN in 20 min, *t_R* = 18.5–20.0 min) yielded DOTAGA.PEG₂.Glu.(FAPi)₂ **25** (7.3 mg, 4.6 µmol,

21%) as a yellow solid (purity = 97.2%: cf. analytical RP-HPLC in Figure S5). MS (ESI⁺): m/z (%) = 398.7 (93, [M+H]⁴⁺), 531.3 (100, [M+H]³⁺), 796.2 (8, [M+H]²⁺), 1591.9 (3, [M+H]⁺), calculated for C₇₃H₉₄F₄N₁₆O₂₀: 1590.68 [M].

2.3. Radiosynthesis

2.3.1. ⁶⁸Ga-Radiolabeling

Gallium-68 was eluted from a ITM (Isotope Technologies Munich SE, Munich, Germany) ⁶⁸Ge/⁶⁸Ga-generator with 5 mL of 0.05 M HCl (TraceSelect). Cationic post processing was carried out according to the procedure developed by EPPARD et al. [62]. Precursor **8**, **11**, **18**, **19** or **25** (2.5–20 nmol, 2.5–20 µL 1 mM stock solution) was added to 400 µL buffer. Three different buffer systems were used: 1 M 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES pH = 4.5 or 5.5). 1 M ammonium acetate (AmOAc, pH = 4.5) or 1 M sodium acetate (NaOAc, pH = 4.5). Finally, the generator eluate (N5 solution: 0.9 M HCl in 90% EtOH) with 50–100 MBq ⁶⁸Ga was added and the reaction was carried out at 95 °C. Reaction controls were taken at different time points to investigate reaction kinetics analyzed via radio-TLC with 0.1 M citrate buffer (pH = 4.0) as the mobile phase. Additional radio-TLCs (1 M AmOAc (pH = 4.0)/MeOH (1:1)) and radio-HPLC were carried out for the last reaction control to double-check radiochemical conversion (RCC) and purity (RCP).

2.3.2. ¹⁷⁷Lu-Radiolabeling

[¹⁷⁷Lu]LuCl₃ in 0.04 M HCl was purchased from ITM. 400 µL 1 M HEPES (pH = 5.5) buffer solution and 2–10 nmol (2.0–10 µL 1 mM stock solution) precursor **8**, **11**, **18** or **25** was added to 50 MBq ¹⁷⁷Lu and heated at 95 °C. Reaction controls were taken at different time points to investigate reaction kinetics analyzed via radio-TLC with 0.1 M citrate buffer (pH = 4.0) as the mobile phase. Additional 1 M AmOAc (pH = 4.0)/MeOH (1:1) radio-TLCs and radio-HPLC were carried out to double-check radiochemical conversion (RCC) and purity (RCP).

2.3.3. Complex Stability Measurements

The ⁶⁸Ga-labeled products were purified via Sep-Pak® Light C18 cartridge after radiolabeling. The cartridge was conditioned with 2 mL ethanol and 5 mL Milli-Q® water. The reaction solution was passed through the cartridge. The reaction vial was rinsed once with buffer solution or TraceSelect H₂O and passed through the cartridge, too. After washing with TraceSelect H₂O (1 mL) the product was eluted with 1 mL ethanol or ethanol/water (1:1).

The ¹⁷⁷Lu-labeled products were used without any purification and the reaction solution was directly used to investigate complex stability.

Ca. 10 MBq of the labeled tracer was incubated in 0.5 mL human serum (HS) and phosphate-buffered saline (PBS) at 37 °C for 120 minutes (^{68}Ga) and 14 days (^{177}Lu), respectively. All investigations were carried out in triplicate ($n = 3$). The complex stability was determined via radio-TLC with 0.1 M citrate buffer ($\text{pH} = 4.0$).

2.3.4. Determination of $\log D_{7.4}$ (Lipophilicity Measurement)

The ^{68}Ga -labeled products were purified via Sep-Pak® Light C18 cartridge after radiolabeling as described before. The ^{177}Lu -labeled products were used without any purification. The $\log D_{7.4}$ value was determined by diluting 5–10 MBq of the labeled tracer solution to 700 μL with PBS ($n = 4$). 1-Octanol (700 μL) was added to each solution and shaken vigorously for 1–2 minutes (1500 rpm), followed by centrifugation for 1–2 minutes. 400 μL of the organic and aqueous phase were separated into a new Eppendorf tube, respectively. Samples of 3 μL (PBS) or 6 μL (1-octanol) were taken and pipetted onto a TLC plate. Most activity was in the aqueous PBS phase, so PBS (300 μL) and 1-octanol (700 μL) was added to it. The procedure (shaking, centrifuging, separating, taking samples) was repeated twice. The TLC plate was imaged (exposure time: 5–10 min) and the integral of each spot (octanol phase: I_o , aqueous PBS phase: I_{PBS}) was determined. The $\log D_{7.4}$ value was calculated using equation (1) where the different volumes of $V_o = 6 \mu\text{L}$ und $V_{\text{PBS}} = 3 \mu\text{L}$ were taken into account.

$$\log D_{7.4} = \log \left(\frac{I_o}{2 \cdot I_{\text{PBS}}} \right) \quad \text{Equation (1)}$$

Only the values of the second and third extraction were considered in the calculation of the final $\log D_{7.4}$ value.

2.4. In Vitro-Inhibition Assays (IC_{50} Measurements)

DPP4 was purified from human seminal plasma as described previously [63]. DPP8 (dipeptidyl peptidase VIII) and DPP9 (dipeptidyl peptidase IX) were expressed and purified as described previously as well as rhFAP (recombinant human fibroblast activation protein) [64,65]. Human recombinant PREP (prolyl endopeptidase) was expressed and purified as described before [64]. IC_{50} measurements for FAP and PREP were carried out as described before [43,65].

To briefly summarize, Z-Gly-Pro-7-amino-4-methylcoumarine (AMC, Bachem) at a final concentration of 50 μM at $\text{pH} = 8.0$ in 0.05 M Tris-HCl buffer, 1 mg/mL BSA and 140 mM NaCl was used as the substrate for FAP. N-succinyl-Gly-Pro-AMC (Bachem) was used for PREP. Here, a final concentration of 250 μM at $\text{pH} = 7.4$ in 0.1 M K-phosphate, 1 mM EDTA, 1 mM DTT and 1 mg/mL BSA buffer was used. IC_{50} measurements for DPP4, DPP8 and DPP9 were performed using Ala-Pro-pNA as the substrate at the respective concentrations of 25 μM (DPP4), 300 μM (DPP8) and

150 μ M (DPP9) at pH = 7.4 in 0.05 M HEPES-NaOH buffer with 0.1% Tween-20, 0.1 mg/mL BSA and 150 mM NaCl) as published before [43,66].

The new inhibitors were pre-incubated with the enzyme for 15 minutes at 37 °C, afterwards the substrate was added and the velocities of AMC release were measured kinetically at λ_{ex} = 380 nm and λ_{em} = 465 nm (405 nm for *p*NA) for at least 10 minutes at 37 °C. At least eight concentrations of inhibitors were tested for each inhibitor and the measurements were carried out in triplicate ($n = 3$). The final DMSO concentration was kept constant during the experiment. The measurements were carried out using an Infinite 200 from Tecan Group Ltd. (Männedorf, Switzerland) and the data were processed with Magellan software. Data fitting was performed using a non-linear fitting model in Grafit 7.4.

3. Results

3.1. Organic Synthesis

3.1.1. Synthesis of DOTAGA.NPyr.(FAPi)₂ and DO3A.NPyr.(FAPi)₂

DOTAGA.NPyr.(FAPi)₂ **8** and DO3A.NPyr.(FAPi)₂ **11** were synthesized according to Figure 3 starting from (*S*)-1-Boc-3-aminopyrrolidine **1**.

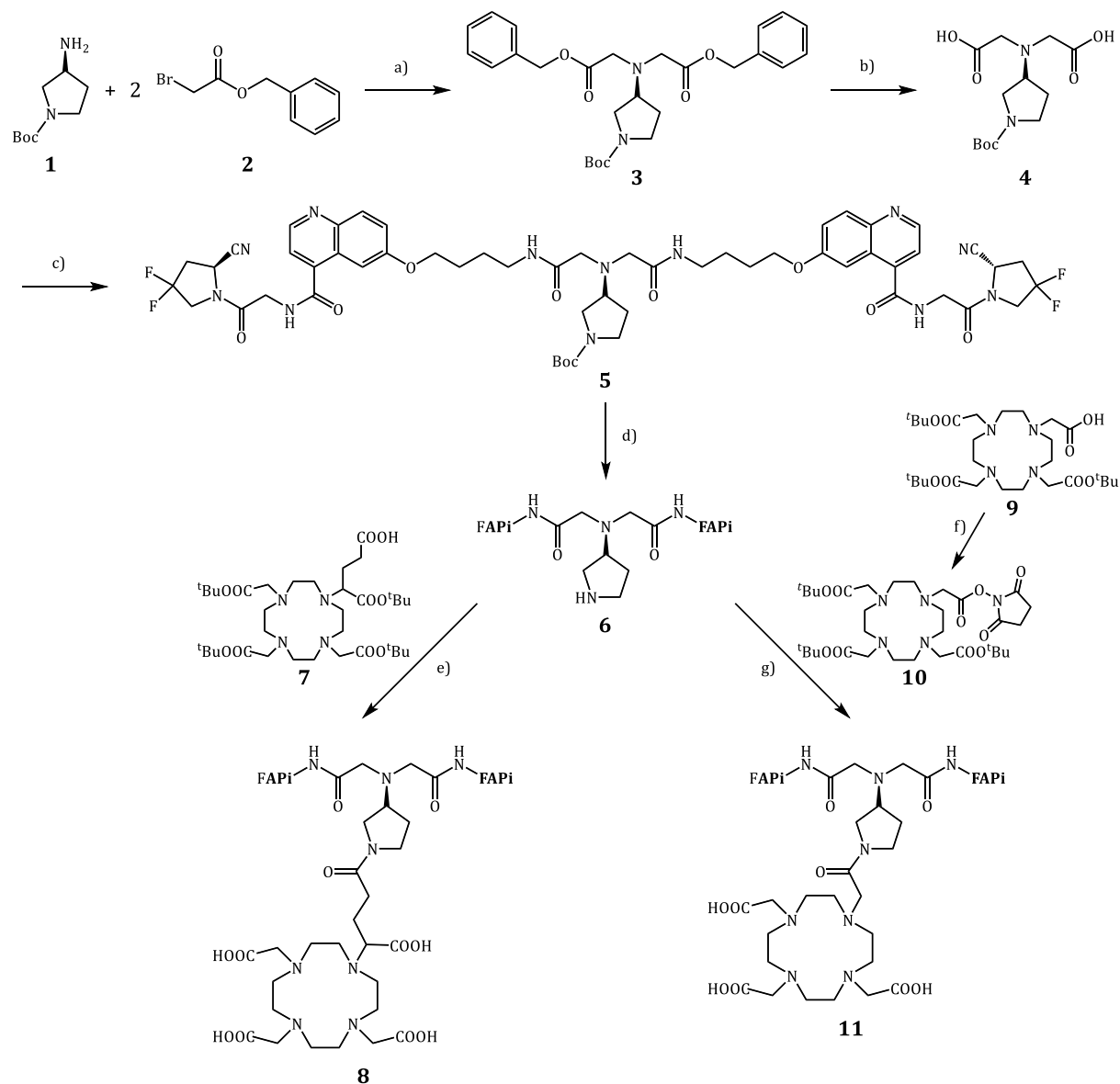


Figure 3: Synthesis of DOTAGA.NPyr.(FAPi)₂ **8** and DO3A.NPyr.(FAPi)₂ **11**: a) DIPEA, MeCN, RT, 2 h, 47%; b) H₂, Pd/C, MeOH, RT, 2 d, 85%; c) 2 **FAPi-NH₂***TFA, HOBt, EDC*HCl, DIPEA, DMF, 30 °C, 3 d, 90%; d) TFA/TIPS/H₂O (95:2.5:2.5), RT, 1 h, 100%; e) 1. NHS, HBTU, DMF, 30 °C, 1 d; 2. DIPEA, DMF, 40 °C, 2 d; 3. TFA/MeCN/TIPS/H₂O (100:10:5:2.5), RT, 5 h, 11% (over 3 steps); f) NHS, HBTU, MeCN, DMF, 30 °C, 1 d, 96%; g) 1. DIPEA, DMF, 40 °C, 3 d; 2. TFA/TIPS/H₂O (94:3:3), RT, 12 h, 10% (over 2 steps).

Boc-NPyr(OBzl)₂ **3** was synthesized with benzyl bromoacetate **2**. Only 1.32 eq **2** were used because this synthesis was actually carried out to obtain the monosubstituted derivative Boc-NPyr(OBzl) which was isolated with a yield of 35% while the yield of Boc-NPyr(OBzl)₂ **3** was 47%. Subsequently, the benzyl ester groups were transformed into free carboxylic acids under reductive conditions with hydrogen and palladium on carbon as a catalyst. FAPi-NH₂ was coupled twice to the dicarboxylic acid Boc-NPyr **4** with HOBt, EDC·HCl, DIPEA in dry DMF and slight excess of the amine FAPi-NH₂·TFA (2.75 eq) analogously to our previous works (2.5 – 3.0 eq) [58]. After cleavage of the Boc-protecting group with TFA/TIPS/H₂O (95:2.5:2.5) NPyr.(FAPi)₂ **6** was obtained. The chelators DOTAGA(*t*Bu)₄ **7** and DOTA-tris(*tert*-butyl ester) **9** were transformed into the corresponding NHS esters with HBTU. DOTA(*t*Bu)₃-NHS **10** was synthesized in bigger scale and isolated whereas DOTAGA(*t*Bu)₄-NHS was formed *in situ* before they were coupled to the amine **6**, respectively. Finally, the *tert*-butyl esters were cleaved with TFA to yield DOTAGA.NPyr.(FAPi)₂ **8** (11% over 3 steps) and DO3A.NPyr.(FAPi)₂ **11** (10% over 2 steps), respectively. The overall yield was 4% over 6 steps for both compounds.

3.1.2. Synthesis of DOTAGA.Glu₂.(FAPi)₂ and DO3A.Glu₂.(FAPi)₂

Two different glutamate derivatives were used at the start of the synthesis of DOTAGA.Glu₂.(FAPi)₂ **18** and DO3A.Glu₂.(FAPi)₂ **19** which is shown in Figure 4.

Dibenzyl glutamate **12** is the starting material for the central linker unit and was coupled to Fmoc-Glu-O^{*t*}Bu **13**, which is used as a polar spacer unit between central linker and chelator, with HBTU and HOBt and resulted in a very high yield (95%). Subsequent reductive cleavage of both benzyl ester groups yielded Fmoc-Glu(O^{*t*}Bu).Glu **15** in almost quantitative yield to enable double coupling of the amine FAPi-NH₂ to the free carboxylic acid groups under similar conditions as described before (3.0 eq amine) [58]. Then the Fmoc protecting group was cleaved using 10% (v/v) piperidine in DMF to yield the FAPi dimer intermediate Glu(O^{*t*}Bu).Glu.(FAPi)₂ **17**.

After chelator coupling and subsequent deprotection of the carboxylic acids, final RP-HPLC purification yielded DOTAGA.Glu₂.(FAPi)₂ **18** and DO3A.Glu₂.(FAPi)₂ **19** with a yield of 16% and 10%, and an overall yield of 12% and 7%, respectively.

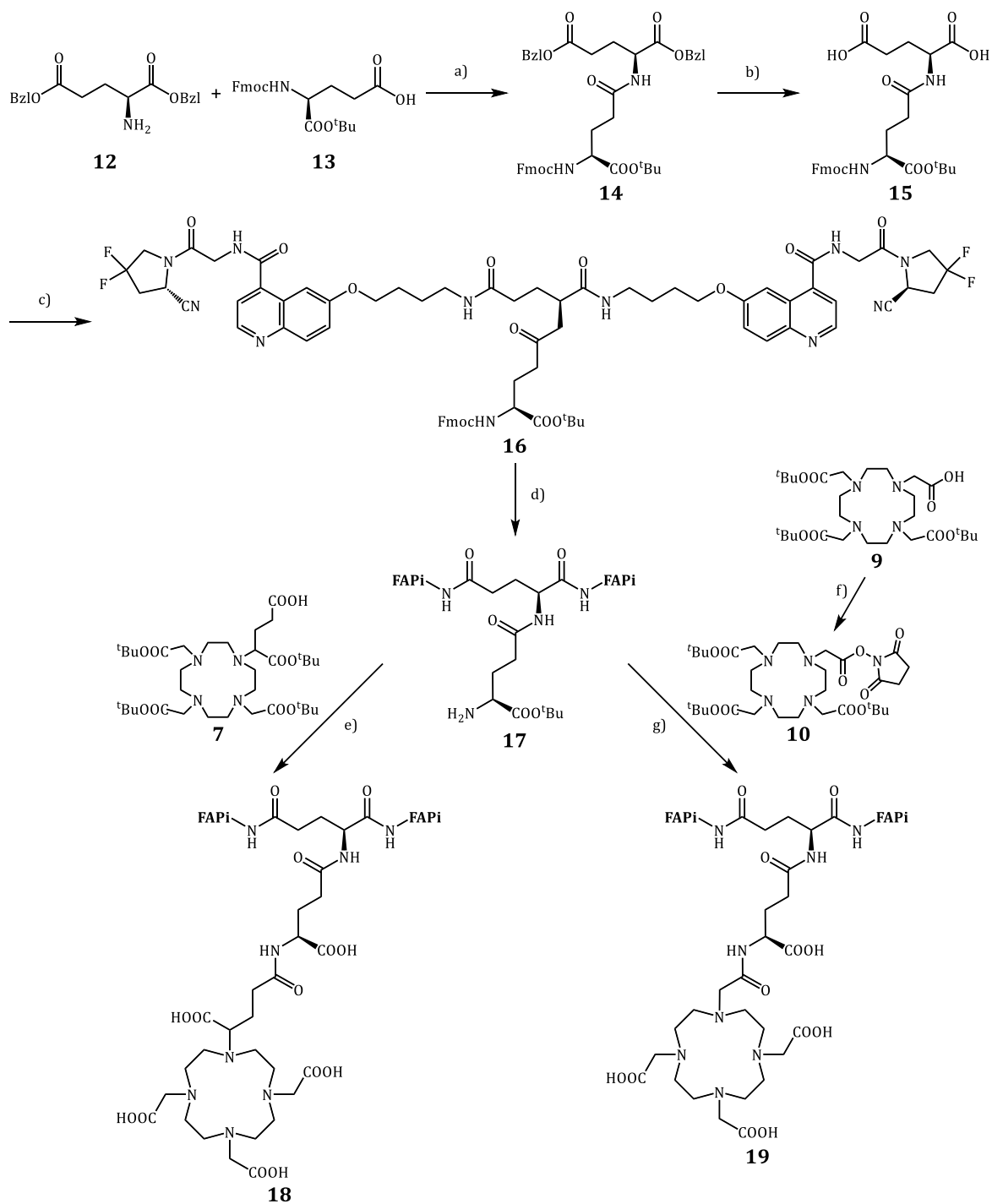


Figure 4: Synthesis of DOTAGA.Glu₂.(FAPi)₂ **18 and DO3A.Glu₂.(FAPi)₂ **19**:** a) HBTU, HOBT, DIPEA, DMF, RT, 5 d, 95%; b) H₂, Pd/C, THF, RT, 1 d, 99%; c) 2 **FAPi-NH₂***TFA, HOBT, EDC*HCl, DIPEA, DMF, RT, 5 d, 79%; d) Piperidine (10% v/v), DMF, RT, 90 min, 100%; e) 1. HATU, DIPEA, DMF, RT, 1 h; 2. DIPEA, DMF, RT, 1 d; 3. TFA/MeCN/TIPS/H₂O (90:5:2.5:2.5), RT, 6 h, 16% (over 3 steps); f) NHS, HBTU, MeCN, DMF, 30 °C, 1 d, 96%; g) 1. DIPEA, DMF, 40 °C, 3 d; 2. TFA/MeCN/TIPS/H₂O (90:5:2.5:2.5), RT, 5 h, 10% (over 2 steps).

3.1.3. Synthesis of DOTAGA.PEG₂.Glu.(FAPi)₂

DOTAGA.PEG₂.Glu.(FAPi)₂ was synthesized from dibenzyl glutamate **12** which was used as the central linker unit again. A PEG₂ unit acted as a spacer unit between central linker and chelator, starting with Fmoc-N-amido-dPEG2 acid **20**. The complete synthesis scheme is depicted in Figure 5.

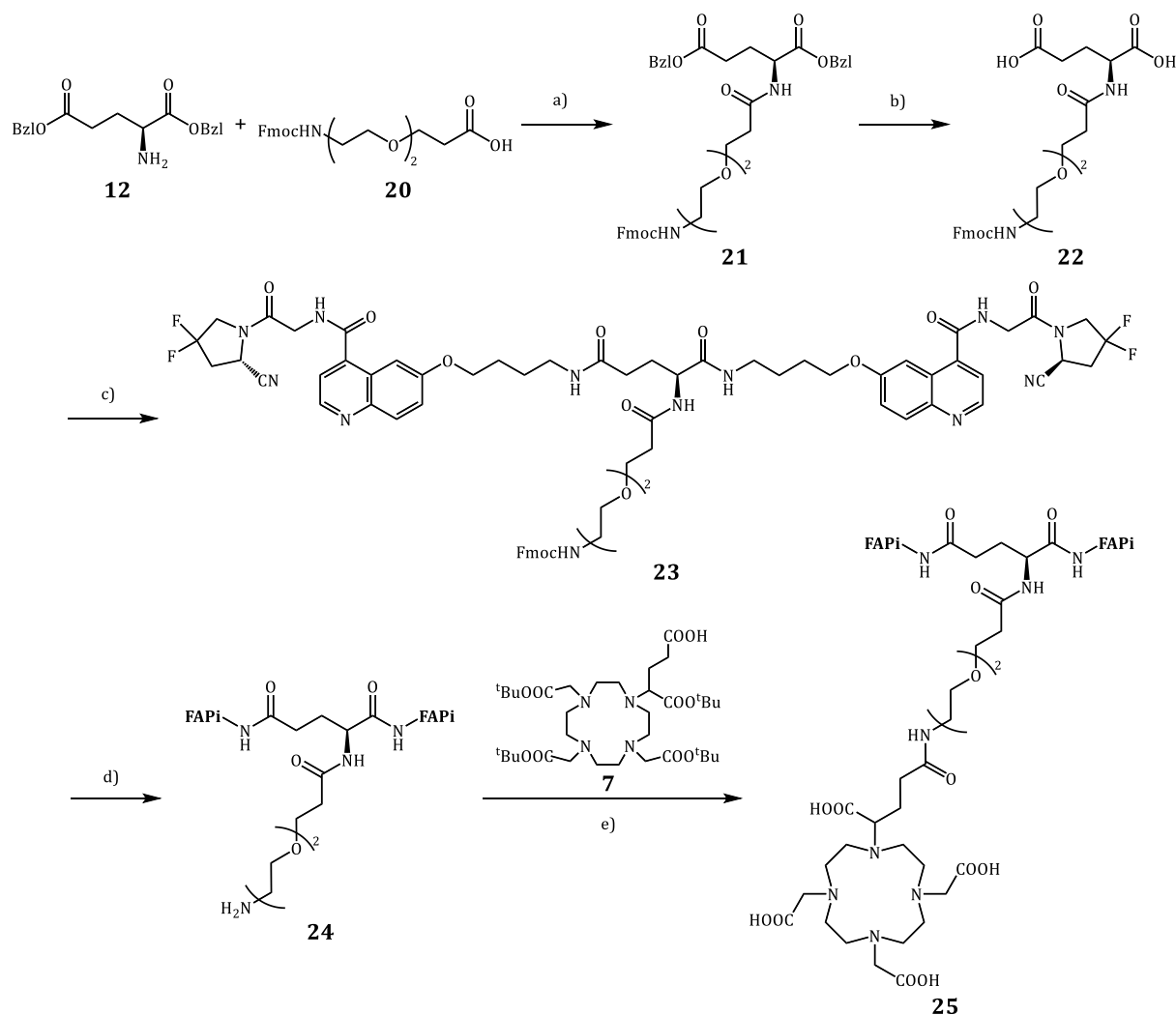


Figure 5: Synthesis of DOTAGA.PEG₂.Glu.(FAPi)₂ **25**: a) HBTU, HOBT, DIPEA, DMF, RT, 1 d, 99%; b) H₂, Pd/C, THF, RT, 1 d, 84%; c) 2 **FAPi-NH₂·TFA**, HOBT, EDC·HCl, DIPEA, DMF, RT, 1 d, 97%; d) Piperidine (10% v/v), DMF, RT, 2 h, 100%; e) 1. HATU, DIPEA, DMF, 30 °C, 45 min; 2. DIPEA, DMF, RT-30 °C, 3 d; 3. TFA/MeCN/TIPS/H₂O (100:10:5:2.5), RT, 6 h, 21% (over 3 steps).

The coupling of **12** and **20** with HBTU and HOBT yielded Fmoc-PEG₂.Glu(OBzl)₂ **21** in practically quantitative yields (99%). Analogously to the previous synthesis, the benzyl esters were transformed into free carboxylic acids with hydrogen and palladium on carbon to allow the subsequent double coupling with FAPi-NH₂ under similar conditions as described before (2.5 eq amine, HOBT, EDC·HCl, DIPEA in dry DMF) [58] to obtain Fmoc-PEG₂.Glu.(FAPi)₂ **23** in very high yields (97%). Deprotection of the amino group with 10% (v/v) piperidine in DMF yielded the FAPi dimer intermediate PEG₂.Glu.(FAPi)₂ **24**. DOTAGA(tBu)₄ **7** was coupled to it with HATU and after

final deprotection with TFA/MeCN/TIPS/H₂O (100:10:5:2.5) and RP-HPLC DOTAGA.PEG₂.Glu.(FAPi)₂ **25** was obtained with a yield of 21% (over 3 steps) and an overall yield of 17% (6 steps).

3.2. Radiosynthesis

The precursors are all DO3A or DOTAGA derivatives. The bifunctional macrocyclic chelator DOTA and its derivatives need elevated temperatures for efficient labeling [67–69]. Therefore, all labeling reactions were carried out at 95 °C and no other temperatures were tried.

3.2.1. [⁶⁸Ga]Ga-DOTAGA.NPyr.(FAPi)₂ (**68Ga-8**)

Previous studies showed that radiolabeling of FAPi homodimers generally resulted in higher yields when 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES) was used as a buffer compared to ammonium acetate (AmOAc) or sodium acetate (NaOAc) [54,58].

Labeling of DOTAGA.NPyr.(FAPi)₂ **8** was tested in the three different buffer systems: 1 M HEPES (pH = 5.5), 1 M AmOAc (pH = 4.5) and 1 M NaOAc (pH = 4.5). The reaction kinetics with 5 nmol **8** and ca. 50 MBq gallium-68 are shown in Figure 6a and show that the labeling only worked in HEPES.

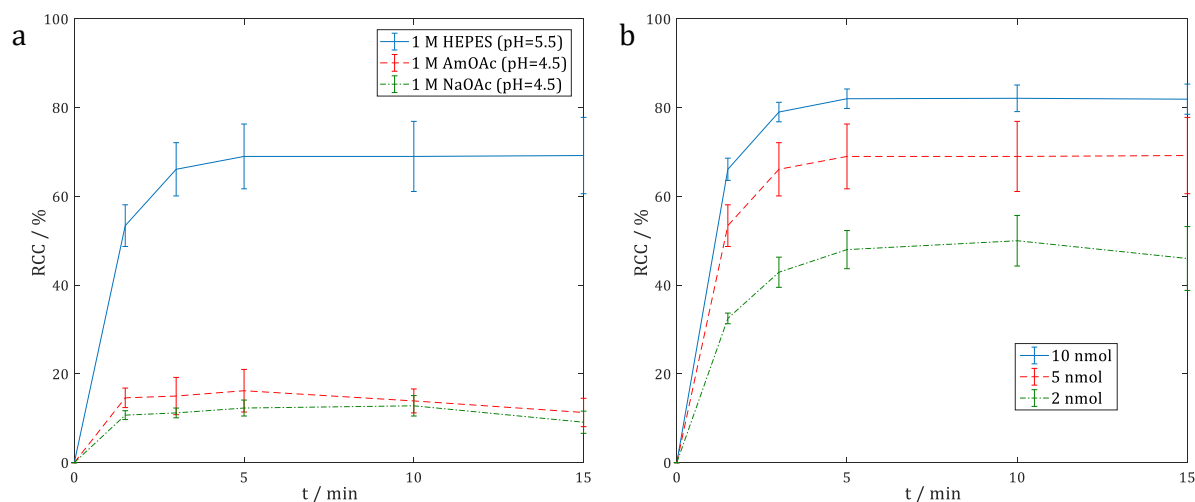


Figure 6: Reaction kinetics (radiochemical conversion (RCC) in %) of [⁶⁸Ga]Ga-DOTAGA.NPyr.(FAPi)₂ **68Ga-8** at 95 °C with 50 MBq ⁶⁸Ga, 400 μL buffer solution and a) 5 nmol DOTAGA.NPyr.(FAPi)₂ **8** in 1 M HEPES (pH = 5.5), 1 M AmOAc (pH = 4.5) or 1 M NaOAc (pH = 4.5); b) 10, 5 or 2.5 nmol precursor **8** in 1 M HEPES (pH = 5.5). Reactions were carried out three times in AmOAc and NoOAc (n = 3) and four times in HEPES (n = 4), respectively.

Different precursor amounts (10, 5 and 2.5 nmol **8**) were investigated (see Figure 6b). The radiochemical conversion (RCC) did not increase after five minutes and reached 82.0 ± 2.2% (10 nmol). Radio-HPLC (RCP = 82.3%) gave confirming results as shown exemplary in Figure S15 while the

RCC was slightly higher ($RCC = 89.2 \pm 4.7\%$) with the second TLC system 1 M AmOAc ($pH = 4.0$)/MeOH (1:1). RCP > 97% was obtained after purification with C18 cartridge (cf. Figure S16).

Labeling at different pH (1 M HEPES ($pH = 4.5$)) did not lead to significantly different RCCs with different precursor amounts **8** (20, 10, 5 nmol) and 100 MBq ^{68}Ga (cf. Figure S29). The same RCC was observed with 10 nmol ($RCC = 82.1 \pm 2.0\%$ after 5 min). A slight improvement could be achieved with 20 nmol ($RCC = 85.3 \pm 2.4\%$ after 10 min), but still the RCC was not quantitative.

The complex stability was determined in human serum (HS) and phosphate-buffered saline (PBS) in triplicate each ($n = 3$) by incubating ca. 10 MBq of ^{68}Ga -**8** in 0.5 mL of HS and PBS at 37 °C for 120 min, respectively (≈ 20 MBq/mL). It was analyzed via radio-TLC and proved to be mostly stable over two half-lives, with $93.4 \pm 0.2\%$ and $91.8 \pm 0.7\%$ in HS and PBS after 120 minutes, respectively (see Figure S6).

3.2.2. $[^{177}\text{Lu}]\text{Lu-DOTAGA.NPyr.}(FAPi)_2$ (^{177}Lu -**8**)

8 was labeled with ca. 50 MBq ^{177}Lu in 1 M HEPES buffer ($pH = 5.5$) at 95 °C and gave excellent results with different precursor amounts as can be seen in Figure 7.

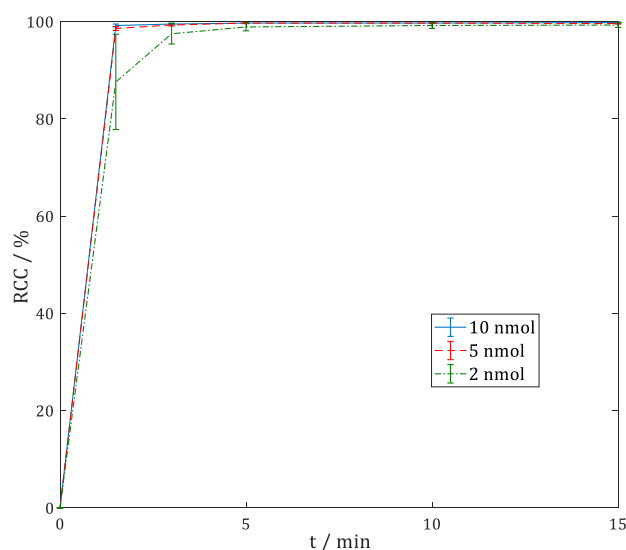


Figure 7: Reaction kinetics (radiochemical conversion (RCC) in %) of $[^{177}\text{Lu}]\text{Lu-DOTAGA.NPyr.}(FAPi)_2$ ^{177}Lu -**8** at 95 °C with 10, 5 or 2 nmol DOTAGA.NPyr.(FAPi)₂ **8** and 50 MBq ^{177}Lu in 400 µL in 1 M HEPES ($pH = 5.5$), with $n = 3$ for every labeling condition.

RCCs > 99% could be reached after 1.5 min (10 nmol), 3 min (5 nmol) and 10 min (2 nmol) which was supported by both radio-TLC systems as well as radio-HPLC (RCP = 99.4%, cf. Figure S17) without any further purification needed.

The complex stability of $[^{177}\text{Lu}]\text{Lu-DOTAGA.NPyr.}(FAPi)_2$ ^{177}Lu -**8** was investigated similarly to the ^{68}Ga -complex ^{68}Ga -**8**. The incubation in HS and PBS (≈ 20 MBq/mL) at 37 °C was carried out for

14 days. ^{177}Lu -**8** showed no degradation and the complex was stable over 2 half-lives (> 99% in HS and PBS; cf. Figure S7).

3.2.3. $[^{68}\text{Ga}]\text{Ga-DO3A.NPyr.}(\text{FAPi})_2$ (^{68}Ga -**11**)

Different precursor amounts of DO3A.NPyr.(FAPi)₂ **11** (20, 10, 5 nmol) were labeled with ca. 100 MBq ^{68}Ga in 1 M HEPES (pH = 4.5) as depicted in Figure 8.

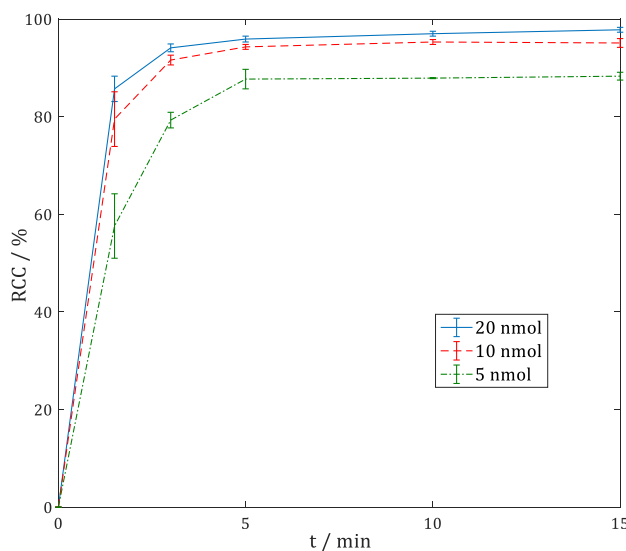


Figure 8: Reaction kinetics (radiochemical conversion (RCC) in %) of $[^{68}\text{Ga}]\text{Ga-DO3A.NPyr.}(\text{FAPi})_2$ ^{68}Ga -**11** at 95 °C with 20, 10 or 5 nmol DO3A.NPyr.(FAPi)₂ **11** and 100 MBq ^{68}Ga in 400 µL 1 M HEPES (pH = 4.5). All reactions were carried out in triplicate (n = 3).

High RCCs of $97.0 \pm 0.5\%$ (20 nmol), $95.3 \pm 0.5\%$ (10 nmol) and $87.9 \pm 0.1\%$ (5 nmol) were observed after 10 minutes that can also be confirmed via radio-HPLC (cf. Figure S18). The small amounts of free gallium-68 could be separated with C18 cartridge to give ^{68}Ga -**11** in 99.6% radiochemical purity (cf. Figure S19).

The complex stability was >95% in HS and PBS after 120 minutes (see Figure S8).

3.2.4. $[^{177}\text{Lu}]\text{Lu-DO3A.NPyr.}(\text{FAPi})_2$ (^{177}Lu -**11**)

Figure 9 shows the reaction kinetics of $[^{177}\text{Lu}]\text{Lu-DO3A.NPyr.}(\text{FAPi})_2$ ^{177}Lu -**11** in 1 M HEPES buffer (pH = 5.5).

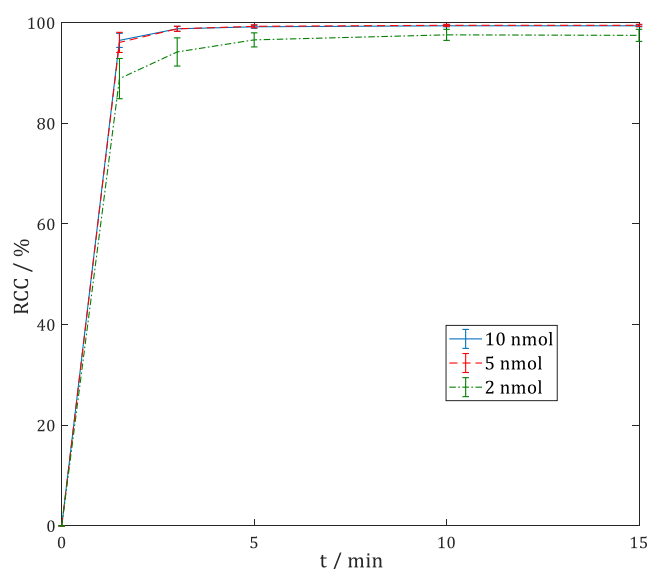


Figure 9: Reaction kinetics (radiochemical conversion (RCC) in %) of $[^{177}\text{Lu}]\text{Lu-DO3A.NPyr.}(\text{FAPi})_2$ **11** at 95 °C with 10, 5 or 2 nmol DO3A.NPyr.(FAPi)₂ **8** and 50 MBq ^{177}Lu in 400 µL in 1 M HEPES (pH = 5.5). All reactions were carried out in triplicate (n = 3).

Quantitative RCCs > 99% could be observed with 10 and 5 nmol **11** after 5 minutes. High yields were also observed with just 2 nmol **11** ($97.6 \pm 1.1\%$ after 10 min) and a quantitative RCP via radio-HPLC (cf. Figure S20).

Practically no decomposition could be observed when $^{177}\text{Lu-11}$ was incubated in HS and PBS at 37 °C for 14 days with ca. 20 MBq/mL (stability > 99% in HS and > 98% in PBS, cf. Figure S9).

3.2.5. $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu}_2(\text{FAPi})_2$ (**$^{68}\text{Ga-18}$**)

The three different buffers (HEPES, AmOAc, NaOAc) were tested with 5 nmol DOTAGA.Glu₂.(FAPi)₂ **18**. As with $^{68}\text{Ga-8}$ the labeling did not work in AmOAc and NaOAc but in HEPES as shown in Figure 10a.

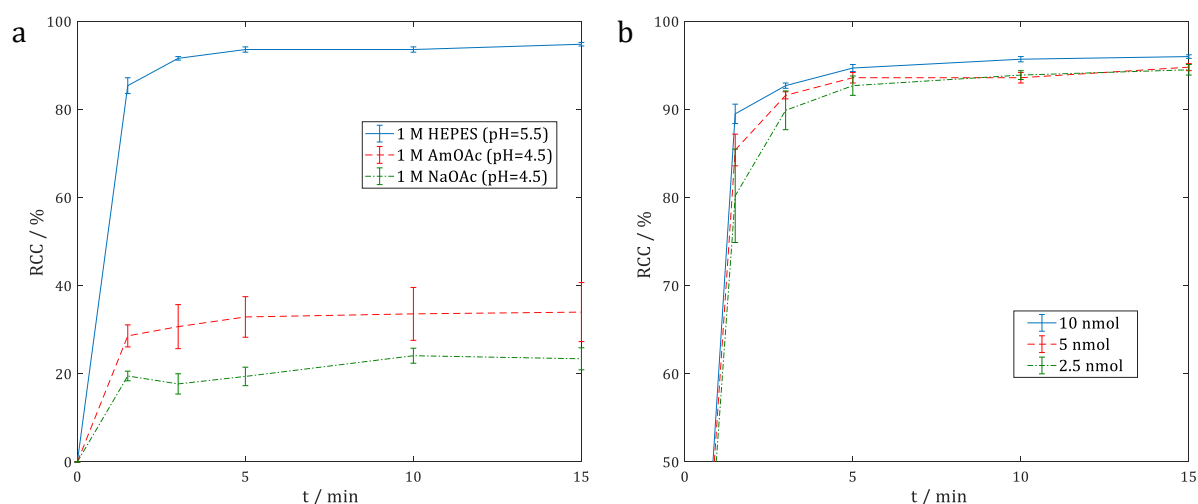


Figure 10: Reaction kinetics (radiochemical conversion (RCC) in %) of $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu}_2(\text{FAPi})_2$ **$^{68}\text{Ga-18}$** at 95 °C with 50 MBq ^{68}Ga , 400 µL buffer solution and a) 5 nmol DOTAGA.Glu₂.(FAPi)₂ **18** in 1 M HEPES

(pH = 5.5), 1 M AmOAc (.5) or 1 M NaOAc (pH = 4.5) and b) 10, 5 or 2.5 nmol precursor **18** in 1 M HEPES (pH = 5.5). All reactions were carried out in triplicate ($n = 3$).

The amount of precursor **18** was varied and Figure 10b shows that the same RCC could be achieved with 2.5 nmol ($\text{RCC} = 94.5 \pm 0.6\%$) than with 5 nmol ($94.8 \pm 0.4\%$). Even slightly higher RCCs could be achieved with 10 nmol ($\text{RCC} = 96.0 \pm 0.2\%$). The yields determined via MeOH/AmOAc (1:1) radio-TLC ($\text{RCC} > 99\%$) were higher whereas the RCP was slightly lower in radio-HPLC ($\text{RCP} = 92.9\%$, cf. Figure S21). The RCP could be improved to 97.6% after C18 purification (cf. Figure S22).

After incubation for 120 minutes in HS and PBS the complex stability of ^{68}Ga -**18** was $92.1 \pm 1.0\%$ and $95.0 \pm 1.5\%$, respectively (see Figure S10).

3.2.6. [^{177}Lu]Lu-DOTAGA.Glu₂.(FAPi)₂ (^{177}Lu -**18**)

^{177}Lu -**18** was labeled in 1 M HEPES buffer (pH = 5.5) and Figure 11 shows the respective reaction kinetics.

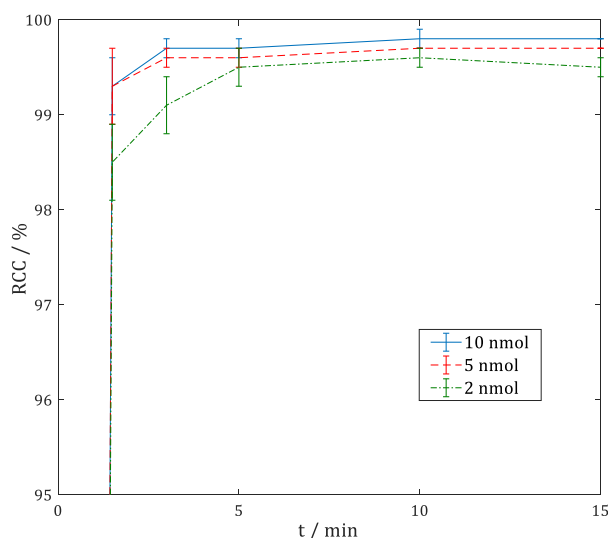


Figure 11: Reaction kinetics (radiochemical conversion (RCC) in %) of [^{177}Lu]Lu-DOTAGA.Glu₂.(FAPi)₂ ^{177}Lu -**18** at 95 °C with 10, 5 or 2 nmol DOTAGA.Glu₂.(FAPi)₂ **18** and 50 MBq ^{177}Lu in 400 μL in 1 M HEPES (pH = 5.5) with $n = 3$ for every labeling conditions.

Quantitative $\text{RCC} > 99\%$ was obtained after 1.5 minutes (10 and 5 nmol) and after 3 minutes (2 nmol), respectively, which was confirmed via AmOAc/MeOH radio-TLC and radio-HPLC ($\text{RCP} = 99.3\%$, cf. Figure S23).

The complex stability was around 99% in HS and PBS after 14 days (see Figure S11).

3.2.7. [^{68}Ga]Ga-DO3A.Glu₂.(FAPi)₂ (**^{68}Ga -19**)

The ^{68}Ga -labeling of DO3A.Glu₂.(FAPi)₂ **19** was tested with different precursor amounts (20, 10, 5 nmol) and 100 MBq ^{68}Ga in 1 M HEPES (pH = 4.5) and the reaction kinetics are shown in Figure 12.

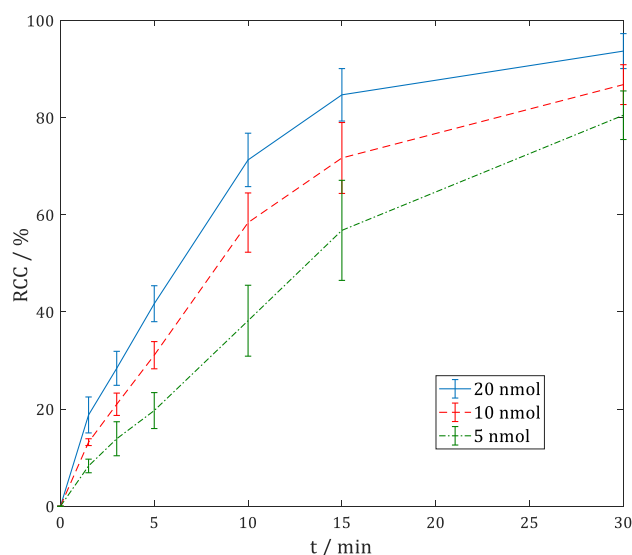


Figure 12: Reaction kinetics (radiochemical conversion (RCC) in %) of [^{68}Ga]Ga-DO3A.Glu₂.(FAPi)₂ **^{68}Ga -19** at 95 °C with 20, 10 or 5 nmol DO3A.Glu₂.(FAPi)₂ **19** and 100 MBq ^{68}Ga in 400 μL 1 M HEPES (pH = 4.5), ($n = 3$).

The reaction kinetics were rather slow. After 30 minutes, RCCs > 80% could be achieved with 5 or 10 nmol and high RCC = $93.7 \pm 3.6\%$ with 20 nmol **19**. Similar RCP = 95.2% was obtained via radio-HPLC (see Figure S24) that could be improved to 98.3% after purification (cf. Figure S25). The complex was not completely stable and showed slight release of gallium-68. The stability was $87.6 \pm 1.4\%$ in PBS and $79.4 \pm 2.7\%$ in HS after 120 minutes (cf. Figure S12).

3.2.8. [^{68}Ga]Ga-DOTAGA.PEG₂.(FAPi)₂ (**^{68}Ga -25**)

Surprisingly, DOTAGA.PEG₂.(FAPi)₂ **25** showed a different behavior than all the previously described FAPi dimers when labeled with gallium-68. There was no significant difference when 10 nmol **25** were labeled with ca. 50 MBq ^{68}Ga in the different buffer systems HEPES, AmOAc and NaOAc (see Figure 13a) and RCC of around 95% was found in all cases after 5–15 minutes.

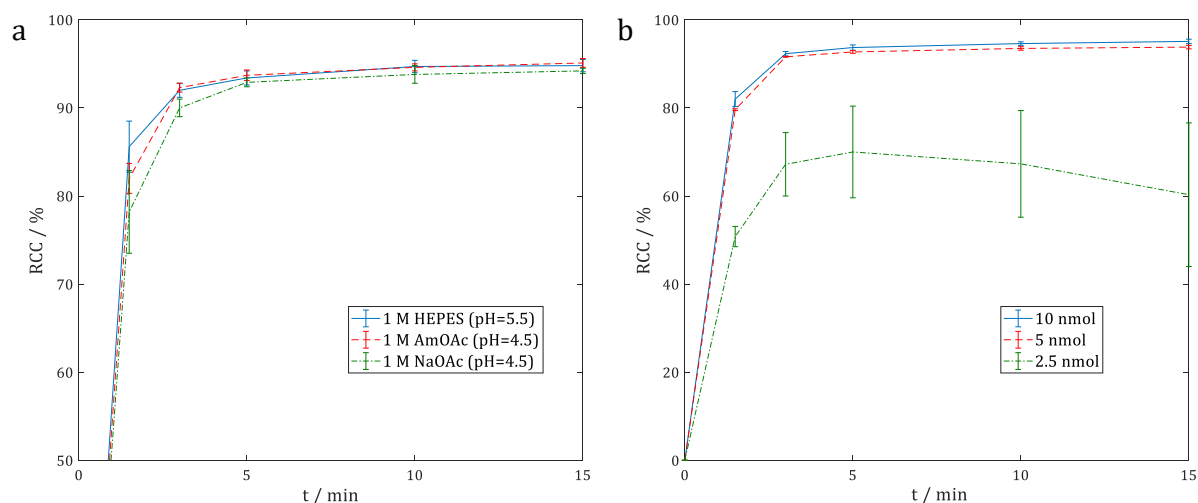


Figure 13: Reaction kinetics (radiochemical conversion (RCC) in %) of $[^{68}\text{Ga}]\text{Ga-DOTAGA.PEG}_2.\text{Glu.}(\text{FAPi})_2$ **68Ga-25** at 95 °C with 50 MBq ^{68}Ga , 400 μL buffer solution and a) 10 nmol DOTAGA.PEG₂.Glu.(FAPi)₂ **25** in 1 M HEPES (pH = 5.5), 1 M AmOAc (pH = 4.5) or 1 M NaOAc (pH = 4.5) and b) 10, 5 or 2.5 nmol precursor **25** in 1 M AmOAc (pH = 4.5). All reactions were carried out in triplicate (n = 3).

Subsequently, smaller precursor amounts were further tested in 1 M AmOAc (pH = 4.5) and as it turned out 5 nmol **25** also gave high RCC > 90% after 3 minutes and RCC = 93.5 ± 0.4% after 10 minutes (see Figure 13b). 2.5 nmol was not enough to be labeled efficiently.

Smearing was observed on the citrate radio-TLC slightly above baseline that in some cases manifested itself as a second spot ($R_f \approx 0.1$, cf. Figure S30a). The integral was determined to be ca. 3% on average (range: 2–5%). Radio-HPLC gave confirming results regarding RCC/RCP (RCP = 93.1%, cf. Figure S26) but no additional peak with a significant peak integral of more than 1% was observed via radio-HPLC that would fit to the smearing on citrate radio-TLC. The only other peak with > 1% integral was the peak of the remaining free gallium-68 (3.7%, cf. Figure S26). 1 M AmOAc/MeOH (1:1) radio-TLC did not show a second spot either and RCCs = 97–99%, (cf. Figure S30b) were obtained with this method.

$[^{68}\text{Ga}]\text{Ga-DOTAGA.PEG}_2.\text{Glu.}(\text{FAPi})_2$ **68Ga-25** could be obtained in high RCP = 98.7% as determined via radio-HPLC (see Figure S27) after C18 cartridge purification.

No substantial decomposition in PBS (complex stability = 97.4 ± 0.7%) and slight decomposition in HS (complex stability = 94.6 ± 0.2%) was observed after incubation at 37 °C after 120 minutes, respectively (see Figure S13).

3.2.9. $[^{177}\text{Lu}]\text{Lu-DOTAGA.PEG}_2.\text{Glu.}(\text{FAPi})_2$ (**$^{177}\text{Lu-25}$**)

Labeling of DOTAGA.PEG₂.Glu.(FAPi)₂ **25** was carried out analogously to the other ^{177}Lu -derivatives in 1 M HEPES buffer (pH = 5.5) and the reaction kinetics are depicted in Figure 14.

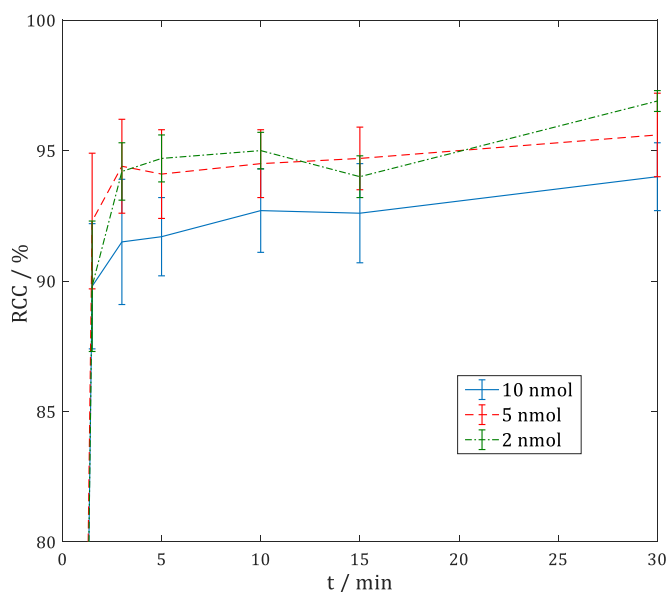


Figure 14: Reaction kinetics (radiochemical conversion (RCC) in %) of $[^{177}\text{Lu}]\text{Lu-DOTAGA.PEG}_2\text{Glu.(FAPi)}_2$ $^{177}\text{Lu-25}$ at 95 °C with 10, 5 or 2 nmol DOTAGA.PEG₂Glu.(FAPi)₂ **25** and 50 MBq ^{177}Lu in 400 µL in 1 M HEPES (pH = 5.5). All reactions were carried out in triplicate (n = 3).

No free lutetium-177 could be observed after 3–5 minutes with all precursor amounts which proves efficient and fast labeling. The RCC was > 90% for all precursor amounts directly at the first time point. But smearing slightly above the baseline was observed on the citrate radio-TLC similar to ^{68}Ga -radiolabeling ($R_f \approx 0.05\text{--}0.10$, cf. Figure S30c). The integral was determined to be ca. 5% on average (range: 2–10%) which tended to decrease with longer reaction time and to be less with lower precursor amounts and vice versa. Therefore, the analysis as depicted in Figure 13 resulted in higher RCC for 2 nmol than for 10 nmol and no quantitative RCCs could be achieved. No second spot was observed via the second radio-TLC system 1 M AmOAc (pH = 4.0)/MeOH (1:1, cf. Figure S30d) and via radio-HPLC. Figure S28 shows an exemplary radio-HPLC chromatogram where an almost quantitative RCP = 98.8% was obtained of a sample directly taken from the reaction solution and without any purification.

No significant release of lutetium-177 was observed in the complex stability studies that were conducted in HS and PBS over 14 days (see Figure S14). The stability was > 98% in both media.

3.2.10. Lipophilicity

The lipophilicity was investigated experimentally via shake-flask method for all radiolabeled compounds and distribution coefficient D in PBS and 1-octanol was investigated (n = 4). The $\log D_{7.4}$ values were calculated with equation (1) by determining the integral of each spot on the radio-TLC. Table 1 gives an overview over the different $\log D_{7.4}$ values of the new compounds as well as from previous works [42,54,58].

Table 1: $\log D_{7.4}$ values of different labeled FAPI-based radiopharmaceuticals. The values for the literature compounds are taken from Moon et al.[42] ($[^{68}\text{Ga}]\text{Ga-DOTA.SA.FAPi}$), Moon et al.[54] ($[^{68}\text{Ga}]\text{Ga-DOTAGA.(SA.FAPi)}_2$) and Martin et al. [58] ($[^{68}\text{Ga}]\text{Ga-}$ and $[^{177}\text{Lu}]\text{Lu-DO3A-}$, and $-\text{DOTAGA.Glu.(FAPi)}_2$).

Complex	$\log D_{7.4}$
$[^{68}\text{Ga}]\text{Ga-DOTA.SA.FAPi}$	-2.68 ± 0.06
$[^{68}\text{Ga}]\text{Ga-DOTAGA.(SA.FAPi)}_2$	-2.02 ± 0.06
$[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu.(FAPi)}_2$	-2.48 ± 0.05
$[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu.(FAPi)}_2$	-2.77 ± 0.10
$[^{68}\text{Ga}]\text{Ga-DO3A.Glu.(FAPi)}_2$	-2.08 ± 0.07
$[^{177}\text{Lu}]\text{Lu-DO3A.Glu.(FAPi)}_2$	-1.77 ± 0.10
$[^{68}\text{Ga}]\text{Ga-DOTAGA.NPyr.(FAPi)}_2$ $^{68}\text{Ga-8}$	-2.52 ± 0.11
$[^{177}\text{Lu}]\text{Lu-DOTAGA.NPyr.(FAPi)}_2$ $^{177}\text{Lu-8}$	-2.57 ± 0.14
$[^{68}\text{Ga}]\text{Ga-DO3A.NPyr.(FAPi)}_2$ $^{68}\text{Ga-11}$	-2.43 ± 0.10
$[^{177}\text{Lu}]\text{Lu-DO3A.NPyr.(FAPi)}_2$ $^{177}\text{Lu-11}$	-2.31 ± 0.06
$[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu}_2.\text{(FAPi)}_2$ $^{68}\text{Ga-18}$	-2.46 ± 0.08
$[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu}_2.\text{(FAPi)}_2$ $^{177}\text{Lu-18}$	-2.71 ± 0.10
$[^{68}\text{Ga}]\text{Ga-DO3A.Glu}_2.\text{(FAPi)}_2$ $^{68}\text{Ga-19}$	-2.59 ± 0.13
$[^{68}\text{Ga}]\text{Ga-DOTAGA.PEG}_2.\text{(FAPi)}_2$ $^{68}\text{Ga-25}$	-2.54 ± 0.06
$[^{177}\text{Lu}]\text{Lu-DOTAGA.PEG}_2.\text{(FAPi)}_2$ $^{177}\text{Lu-25}$	-2.48 ± 0.10

All investigated radiolabeled complexes showed high hydrophilicity. The values do not vary drastically between the different compounds and the $\log D_{7.4}$ value is around -2.5 (range: $-\log D_{7.4} = 2.31 - 2.71$) for all compounds which is comparable to $\text{DOTAGA.Glu.(FAPi)}_2$.

3.3. In Vitro-Inhibition Assays

The precursors were tested for their *in vitro* affinity for FAP, and the related proteases PREP, DPP4, DPP8 and DPP9. The measured IC_{50} values for FAP are summarized in Table 2 as well as the selectivity index (*SI*) as the ratio of IC_{50} (related protease) to IC_{50} (FAP) of the respective FAP inhibitor sample. The exact values of the IC_{50} values for the related proteases are summarized in Table S1.

Table 2: IC_{50} (FAP) value and selectivity index (*SI*) regarding the related proteases PREP, DPP4, DPP8 and DPP9 of different FAP inhibitors. The data for [^{nat} Lu]Lu- and DOTAGA.Glu.(FAPi)₂ are taken from Martin et al. [58], for [^{nat} Lu]Lu- and DOTAGA.(SA.FAPi)₂ from Moon et al. [54] and for the initial FAP inhibitor UAMC-1110 from Jansen et al. [35] and Moon et al. [54].

Compound	IC_{50} / nM	<i>SI</i>			
	FAP	PREP/FAP	DPP4/FAP	DPP8/FAP	DPP9/FAP
DOTAGA.NPyr.(FAPi) ₂ (8)	0.50 ± 0.03	1454	5312	211	702
DO3A.NPyr.(FAPi) ₂ (11)	0.48 ± 0.04	3899	8187	437	417
DOTAGA.Glu ₂ .(FAPi) ₂ (18)	0.48 ± 0.06	1306	2854	84	478
DO3A.Glu ₂ .(FAPi) ₂ (19)	0.31 ± 0.18	1308	3288	237	415
DOTAGA.PEG ₂ .(FAPi) ₂ (25)	0.37 ± 0.03	1351	6581	63	159
DOTAGA.Glu.(FAPi) ₂	0.26 ± 0.04	2269	4577	112	319
[^{nat} Lu]Lu- DOTAGA.Glu.(FAPi) ₂	0.33 ± 0.02	1292	1967	661	557
DOTAGA.(SA.FAPi) ₂	0.92 ± 0.06	424	435	456	174
[^{nat} Lu]Lu- DOTAGA.(SA.FAPi) ₂	1.54 ± 0.15	364	409	266	117
UAMC-1110	0.43 ± 0.02	4186	23256	23256	10930

The new compounds all show very high affinity for FAP with subnanomolar IC_{50} (FAP) values. The selectivity for PREP and DPP4 is also high for all compounds (> 1000). The respective *SI*s for DPP8 and DPP9 versus FAP are generally lower than for PREP and DPP4 (< 1000). Overall, the results are quite similar to DOTAGA.Glu.(FAPi)₂.

4. Discussion

4.1. Organic Synthesis

The amide coupling of the chelator DOTAGA(*t*Bu)₄ **7** was carried out with HATU to obtain the derivatives DOTAGA.(Glu)₂.(FAPi)₂ **18** and DOTAGA.PEG₂.(FAPi)₂ **25** as it was found to be superior compared to other coupling reagents in previous similar reactions [58]. In contrast, coupling of **7** to the intermediate NPyr.(FAPi)₂ **6** with HATU did not show any product formation as monitored via TLC and LC/MS. In this case, the NHS ester was synthesized *in situ* with HBTU as an additional coupling reagent similar to the route used for DO3A derivatives in this work. The only difference was that the DOTAGA-NHS ester was not isolated due to the smaller scale reaction, although this is principally possible with NHS esters like DOTA(*t*Bu)₃-NHS **10**. The overall yield of DOTAGA.NPyr.(FAPi)₂ **8** and DO3A.NPyr.(FAPi)₂ **11** was 4% over 6 steps for both compounds.

The low yields in the final steps might have two reasons. While the secondary amine **6** is supposed to be electronically more reactive than a primary amine due to the additional electron donating group (EDG), here the pyrrolidine ring it is sterically hindered which could be the dominating factor with these rather large molecules. Secondly, there was no analytical method that could give quick and useful results during the final reaction with TFA (TLC: no clear spots with smearing, LC/MS with C18 column: *tert*-butyl ester protected derivatives cannot or hardly be seen, HPLC/MS with C8 column: results took several days and (partly) deprotected chelators cannot or hardly be seen with C8 column). Therefore, the reaction was stopped blindly which is definitely not optimal. This can be improved but would need better analytical options/capacities to give useful and quick results during reaction. Alternatively, a complete identification of all the different peaks during RP-HPLC purification might show that the reaction was not complete and the crude product should have been taken up in TFA/TIPS/H₂O (95:2.5:2.5) again to continue the reaction. This was not realized because it would have resulted in extensive LC/MS measurement times. On the other hand, the longer reaction time of **11** compared to **8** (12 vs. 5 hours) did not show an increased yield (11% vs. 10% over 3 steps), so it is not certain that an increased reaction time would have resulted in higher yields.

The overall yields of synthesizing DOTAGA.Glu₂.(FAPi)₂ **18**, DO3A.Glu₂.(FAPi)₂ **19** and DOTAGA.PEG₂.Glu.(FAPi)₂ **25** were 12%, 7% and 17%, respectively. At least for the DOTAGA derivatives **18** and **25**, this is significantly higher than for **8** (4%). The yield for the DO3A derivatives **11** and **19** was not very high in both cases. The synthesis of DO3A.PEG₂.Glu.(FAPi)₂ was tried several times under the same conditions but did not yield any product. In past similar reactions, low conversion rates were observed when DOTA-tris(*tert*-butyl ester) **9** was activated *in situ* with coupling reagents such as HATU, HBTU, EDC·HCl and HOBT (including various

combination of these coupling reagents). Nevertheless, further investigations with other coupling reagents could lead to improved coupling and therefore higher overall yields might be achieved. In the respective final step, the cleavage of the *tert*-butyl esters, a side product NP was identified via LC/MS several times that was found to be 74.1 Da heavier than the respective unprotected precursor M ($[\text{NP}+\text{H}]^+ = [\text{M}+74.1+\text{H}]^+$). MeCN (5%) was added to TFA/TIPS/H₂O (95:2.5:2.5). This was tried to reduce the emergence of the side product NP which is believed to be a cyano group being converted into a secondary *tert*-butyl amide group. Instead, the excess MeCN should react with its cyano group and suppress the side reaction.

The final deprotection reaction step for DOTAGA.Glu₂.(FAPi)₂ **18** was carried out once without adding MeCN. TFA/TIPS/H₂O (95:2.5:2.5) was used instead. In this case the yield over the last three steps was only without MeCN 5% compared to 16% with MeCN. It has to be noted that this is a combined yield over two steps and other factors could have also played a role here. And for the two synthesized DO3A compounds **11** and **19**, there was no difference although **19** was deprotected with and **11** without MeCN. Nonetheless, this should be further investigated in the future to optimize yields of (homodimeric) FAPi compounds.

4.2. Radiosynthesis

Previous works have already shown that ⁶⁸Ga- and ¹⁷⁷Lu-labeling efficiency of FAPi dimers is superior in HEPES buffer than in AmOAc or NaOAc [54,58].

Despite that, all three buffer systems were investigated for the ⁶⁸Ga-labeling of the DOTAGA derivatives **8**, **18** and **25**. With DOTAGA.NPyr.(FAPi)₂ **8** and DOTAGA.Glu₂.(FAPi)₂ **18** the expected results were observed. They did not label in 1 M AmOAc (pH = 4.5) and 1 M NaOAc (pH = 4.5). RCCs of around 80% for **8** and 95% **18** were observed with 10 nmol and 5 nmol precursor in 1 M HEPES (pH = 5.5), respectively. Labeling of ⁶⁸Ga-**8** in at lower pH (1 M HEPES, pH = 4.5) gave very similar RCCs that could be slightly improved to around 85% of with higher precursor amount (20 nmol). Ethanol is known to improve labeling efficiency of many DOTA derivatives like DOTA-TOC [62,70] but still the RCC was not quantitative. Steric hindrance should not be the reason for this since the DO3A analogue **11** showed better radiolabeling properties.

Surprisingly, [⁶⁸Ga]Ga-DOTAGA.PEG₂.Glu.(FAPi)₂ ⁶⁸Ga-**25** was the first FAPi dimer of our group that can be equally labeled in all three buffer systems (RCC > 95%). Subsequently, the precursor amount was optimized in AmOAc and RCC = 93–94% could also be reached with 5 nmol **25**. Perhaps this can be addressed to the longer spacer between chelator and the rest of the molecule that minimizes steric hindrance and therefore shows higher flexibility towards different labeling conditions (buffer, pH etc.).

Despite achieving good conversion rates (RCC > 90%) after heating for 30 minutes, the labeling kinetics of [^{68}Ga]Ga-DO3A.Glu₂.(FAPi)₂ **^{68}Ga -19** were rather slow. For this reason and because only limited amount of [^{177}Lu]LuCl₃ was available, **19** was not labeled with lutetium-177.

All ^{177}Lu -radiolabeling reactions were carried out in 1 M HEPES (pH = 5.5) and showed very similar results with fast and quantitative complexation of lutetium-177 (resp. [^{177}Lu]LuCl₃) that resulted in very high RCC/RCY. Even with 2 nmol precursor the labeling reached quantitative RCC after a maximum of 5 minutes. 2 nmol per 50 MBq ^{177}Lu corresponds to 40 nmol/GBq. The precursor amount can perhaps be optimized even further to 20 or even 10 nmol/GBq since slightly longer reaction time do not lead to decreased RCY with the relatively long-lived lutetium-177 ($t_{1/2}$ = 6.7 d). To obtain robust results, those labeling studies should be carried out with bigger activity amounts (at least 200 MBq ^{177}Lu per labeling) because adding very small amounts of precursor (< 2 nmol) to the reaction solution can easily result in big relative errors. This could not be realized yet due to the limited amount of [^{177}Lu]LuCl₃ that was available. That was also the reason why no other buffer systems like 1 M AmOAc (pH = 4.5) were tested for ^{177}Lu -labeling. No purification (e.g. C18 cartridge) was needed. Nevertheless, RCP $\geq$ 99% via radio-HPLC and radio-TLCs were achieved, except the citrate radio-TLCs for [^{177}Lu]Lu-DOTAGA.PEG₂.Glu.(FAPi)₂ **^{177}Lu -25**.

The citrate radio-TLCs of both ^{68}Ga - and ^{177}Lu -labelings of DOTAGA.PEG₂.Glu.(FAPi)₂ **25** showed some activity slightly above the baseline ($R_f \approx 0.05$ – 0.10) that depending on the sample looked like smearing or an additional weak spot. The integral was ca. 3% on average (range: 2–5%) for **^{68}Ga -25** and ca. 5% (range: 2–10%) for **^{177}Lu -25** before purification, respectively. The other two analytic methods (radio-TLC with AmOAc/MeOH (1:1) and radio-HPLC) did not show a side product formation. If a side product would have formed that can be clearly identified on a TLC plate as a second spot with a different R_f value this should definitely be separated and resolved via HPLC. HPLC generally has much higher separation power compared to TLC and the gradient was chosen relatively shallow (20 – 30% in 20 min).

A very slight smearing ($R_f < 0.05$) could also be observed in some cases with other DOTAGA derivatives like [^{177}Lu]Lu-DOTAGA.NPyr.(FAPi)₂ **^{177}Lu -8** (cf. Figure S30e) and [^{177}Lu]Lu-DOTAGA.Glu₂.(FAPi)₂ **^{177}Lu -18** (cf. Figure S30f). DOTAGA derivatives have an additional fourth carboxylic acid groups. If all of them are deprotonated, for example because they are part of the chelate complex, the molecule has a net charge of -1 . If one group is not part of the complex an equilibrium between protonated (net charge: 0) and deprotonated (net charge: -1) form exists. A different complex formation of the different precursors **8**, **18** and **25** with 7 donor ligands ($4x\text{N} + 3x\text{COO}^-$) or 8 donor ligands ($4x\text{N} + 4x\text{COO}^-$) might lead to a different ratio of protonated to deprotonated form because protonation only takes place if the carboxylic acid group is not coordinated to the central trivalent metal ion. That might result in the observed behavior on the TLC although no specific reason can be given why this should be more pronounced with **^{177}Lu -25**

than with the other DOTAGA derivatives **8** and **18**. Different citrate buffer were tested as mobile phases, pH = 4 in various concentrations (0.1 M, 0.25 M, 0.5 M) and 0.1 M with different pH values (pH = 2, 3, 4, 5), but did not lead to any significant differences regarding the second spot.

Beyond just quantifying the lipophilicity of a compound, the $\log D_{7.4}$ value is also supposed to be a first indicator that might help to estimate the pharmacologic behavior *in vivo*. Prior studies suggest that hydrophilic properties are preferred for FAP-targeting radiopharmaceuticals since they are usually cleared faster from non-target tissues [42,44,54,56–58,60].

The first generation linear FAPi dimer DOTAGA.(SA.FAPi)₂ was not as hydrophilic as the original FAPi monomer DOTA.SA.FAPi and slower hepatobiliary excretion was observed [54,56,57]. The next generation FAPi dimer DOTAGA.Glu.(FAPi)₂ showed faster pharmacokinetics. The faster excretion pattern observed compared to DOTAGA.(SA.FAPi)₂ was probably due to its higher hydrophilicity to at least some extent [58]. Substitution patterns with different linker and spacer units were synthesized to investigate whether the pharmacologic properties can be further improved. For example, a second glutamate unit was introduced as a spacer with a free carboxylic acid that is supposed to increase hydrophilicity of compound **18** and **19** with the additional Glu spacer as well as its labeled derivatives. Of course, the additional carboxylic acid also changes the net charge of the labeled molecule when a deprotonation at physiological pH = 7.4 is assumed. Even with this additional polar/charged functional group the $\log D_{7.4}$ value did not decrease significantly. The lowest $\log D_{7.4}$ value was found for [¹⁷⁷Lu]Lu-DOTAGA.Glu₂.(FAPi)₂ ¹⁷⁷Lu-**18** (-2.71 ± 0.10) which was in the same range as [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ (-2.77 ± 0.10) from previous works [58]. The least hydrophilic compounds seemed to be the labeled complexes of DO3A.NPyr.(FAPi)₂ **11**. But overall, all radiolabeled complexes showed high hydrophilicity. Additional labeling studies with actinium-225 might complete the picture of the new homodimeric FAPi compounds as they are potentially interesting for FAP-targeted alpha-particle therapy (FAP-TAT). Unfortunately, the currently very limited access of [²²⁵Ac]AcCl₃ did not allow for ²²⁵Ac-radiolabeling investigations until now.

4.3. In Vitro-Inhibition Assays

Figure 15 depicts the results of the *in vitro* affinity studies where the IC_{50} value for FAP is plotted against the selectivity index (SI) versus PREP (Figure 15a) and DPP4 (Figure 15b) based on the values from Table 2.

Subnanomolar IC_{50} (FAP) values were determined for all compounds. On top of the very high affinity, high selectivity ($SI > 1000$) was for FAP over the related proteases PREP and DPP4. DO3A.NPyr.(FAPi)₂ **11** showed higher selectivity for both enzymes than DOTAGA.Glu.(FAPi)₂ while the other compounds showed slightly decreased selectivity for PREP and slightly lower or higher selectivity for DPP4. Overall, the values are all in the same order of magnitude.

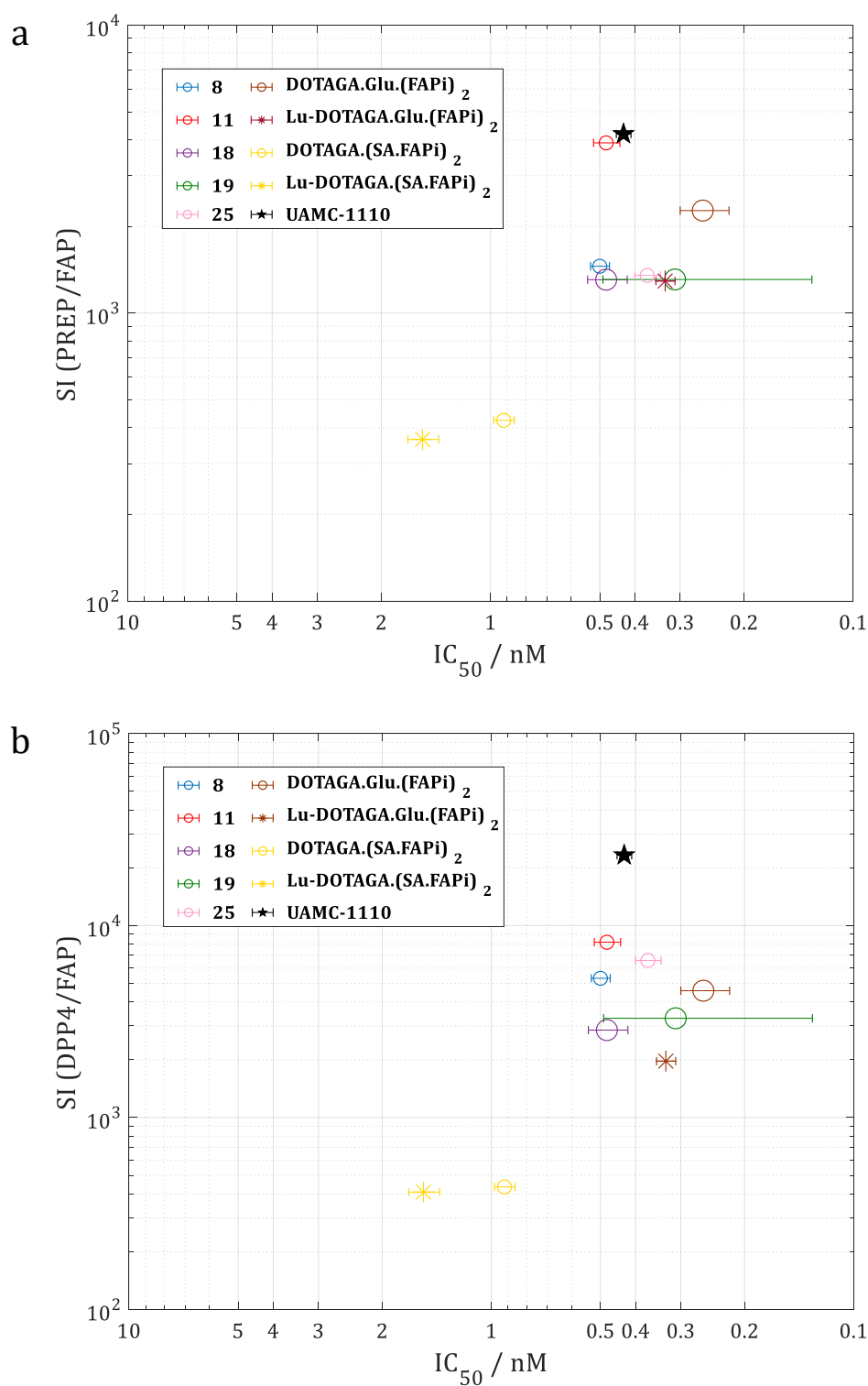


Figure 15: FAP affinity and selectivity. IC_{50} value for FAP plotted against a) the selectivity index (SI) for FAP over PREP and b) SI for FAP over DPP4 with $SI = IC_{50}(\text{PREP or DPP4}) / IC_{50}(\text{FAP})$. The symbol "o" stands for the precursor and "*" for the Lu-complex.

The respective SI of DPP8 and DPP9 versus FAP are lower (< 1000) and partially < 100 (DPP8 selectivity of **18** and **25**) but again, the DPP8- and DPP9-selectivity of DOTAGA.Glu.(FAPi)₂ is similar. It has to be noted that the selectivity for DPP8 and DPP9 is less critical because these proteases have found to be located in the cytosol and/or the nucleus only [71–74]. Therefore,

binding can only take place after the molecule has been internalized into the cell. It might even be hypothesized that once a FAPi dimer is internalized the higher affinity for DPP8 and DPP9 and the stronger binding to those proteases could lead to a decreased externalization rate which would fit with the prolonged tumor retention that is observed with all FAPi dimers *in vivo*. To investigate this hypothesis the internalization and externalization of FAPi dimers has to be investigated. This is currently being tested by our cooperation partners. Secondly, further cell internalization assays would be needed with cells that express FAP and additionally are DPP8/9-positive or -negative. If the hypothesis might be substantiated in future studies, one could compare this to the metabolic trapping of [¹⁸F]F-FDG, but in this case a term like (intra)cellular or enzymatic trapping would be more appropriate.

No obvious leading candidate with clearly improved *in vitro* properties compared to DOTAGA.Glu.(FAPi)₂ can be picked from this selection of compounds. Several compounds showed slightly improved selectivity. DOTAGA.PEG₂.Glu.(FAPi)₂ **25**, DO3A.NPyr.(FAPi)₂ **8** and DOTAGA.NPyr.(FAPi)₂ **11** showed improved DPP4 selectivity and **8** had improved PREP selectivity on top of that. None of the other compounds can be excluded either since all of them show good *in vitro* properties that are similar to DOTAGA.Glu.(FAPi)₂.

It is possible that some of the compounds may demonstrate improved pharmacologic properties, faster clearance from non-target tissues and/or higher and prolonged tumor uptake even when compared to DOTAGA.Glu.(FAPi)₂. This has to be evaluated *in vivo* for the ¹⁷⁷Lu-labeled compounds in the next step. The challenge with current xenograft models for FAP-targeting radiopharmaceuticals goes back to the very basic. FAP is a marker for CAFs that are expressed in the TME and not by the tumor cells directly. Tumor cells artificially transfected to express FAP do not reflect the complex composition of a real tumor with tumor cells along with stromal cells. Therefore, the significance of such studies is limited and should be handled and interpreted with caution as the pharmacologic *in vivo* behavior of a compound in humans might deviate substantially from its murine behavior.

5. Conclusion

Five new FAPi homodimers were synthesized and the radiolabeling properties with gallium-68 and lutetium-177 were evaluated. For the DOTAGA derivatives DOTAGA.NPyr.(FAPi)₂ **8**, DOTAGA.Glu₂.(FAPi)₂ **18** and DOTAGA.PEG₂.(FAPi)₂ **25**, ⁶⁸Ga-radiolabeling was tested in three different buffer systems. As generally with FAPi dimers, using HEPES as a buffer seems to accelerate labeling reaction and led to higher radiochemical conversions (RCCs). Only **25** could also be labeled well in ammonium or sodium acetate buffer. High RCCs (> 90%) were achieved with all precursors except **8** with high radiochemical purity (RCP > 97%) after purification with C18 cartridge.

Quantitative and fast labeling could be achieved with ¹⁷⁷Lu in HEPES buffer with all precursors. Only with ¹⁷⁷Lu-**25**, slightly lower yields (94–97% RCC after 30 minutes at 95 °C) were obtained with citrate radio-TLC in contrast to AmOAc/MeOH radio-TLC and radio-HPLC (both > 99% RCC and RCP, respectively). The reason for this discrepancy is not quite clear and might come from smearing due to protonation equilibrium but could not be fixed by altering concentrations or pH value of the citrate buffer.

Complex stability in human serum and phosphate-buffered saline was > 90% for all ⁶⁸Ga-complexes after two hours, except for ⁶⁸Ga-**19**, and > 98% for all ¹⁷⁷Lu-complexes.

All compounds showed similar and high hydrophilicity ($-\log D_{7.4} = 2.31 - 2.71$) as well as very high affinity with subnanomolar *IC*₅₀ values for FAP. The selectivity for FAP over PREP and DPP4 was generally high (*SI* > 1000) and lower for DPP8 and DPP9 (*SI* < 1000). The different linker and spacer units seem to not have significant effect on FAP affinity and no drastic changes in the selectivity over the related proteases were observed in the mutual comparison with DOTAGA.Glu.(FAPi)₂. Nevertheless, it is possible that one or some of the new FAPi homodimers may show an improved pharmacologic profile that will make the radioligand therapy with FAPi-based homodimers even more efficient.

Overall, the new FAPi dimers did show radiolabeling and *in vitro* properties similar to DOTAGA.Glu.(FAPi)₂. Higher radiolabeling variability was found with [⁶⁸Ga]Ga-DOTAGA.PEG₂.(FAPi)₂ ⁶⁸Ga-**25** and might result from the additional long PEG₂ spacer unit. All the compounds, for example DO3A.NPyr.(FAPi)₂ **11** or DOTAGA.PEG₂.(FAPi)₂ **25**, seem to be suitable FAPi-radiotracers for further *in vivo* investigations.

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Conflicts of interest

Marcel Martin and Frank Roesch have filed a patent (WO/2022/258637) on the new compounds presented in this publication.

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Supporting Information

Organic Synthesis - Analytical RP-HPLC

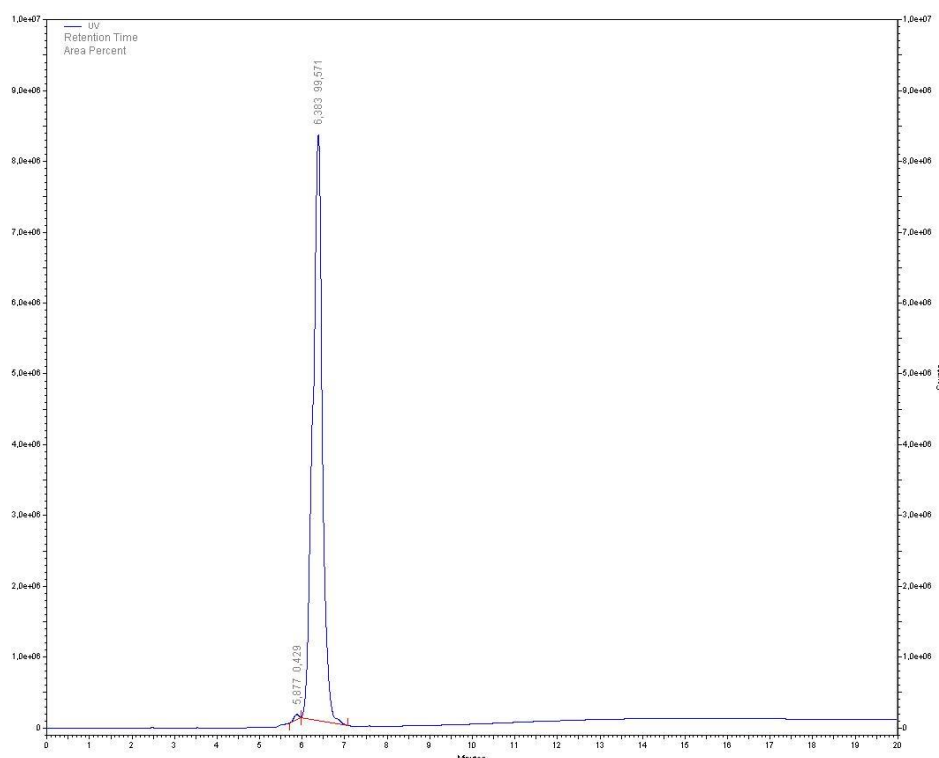


Figure S1: Analytical RP-HPLC of DOTAGA.NPyr.(FAPi)₂ **8** (t_r = 6.4 min), 30-40% MeCN + 0.1% TFA (linear gradient) in 20 min, 5 mL/min: purity = 99.6%.

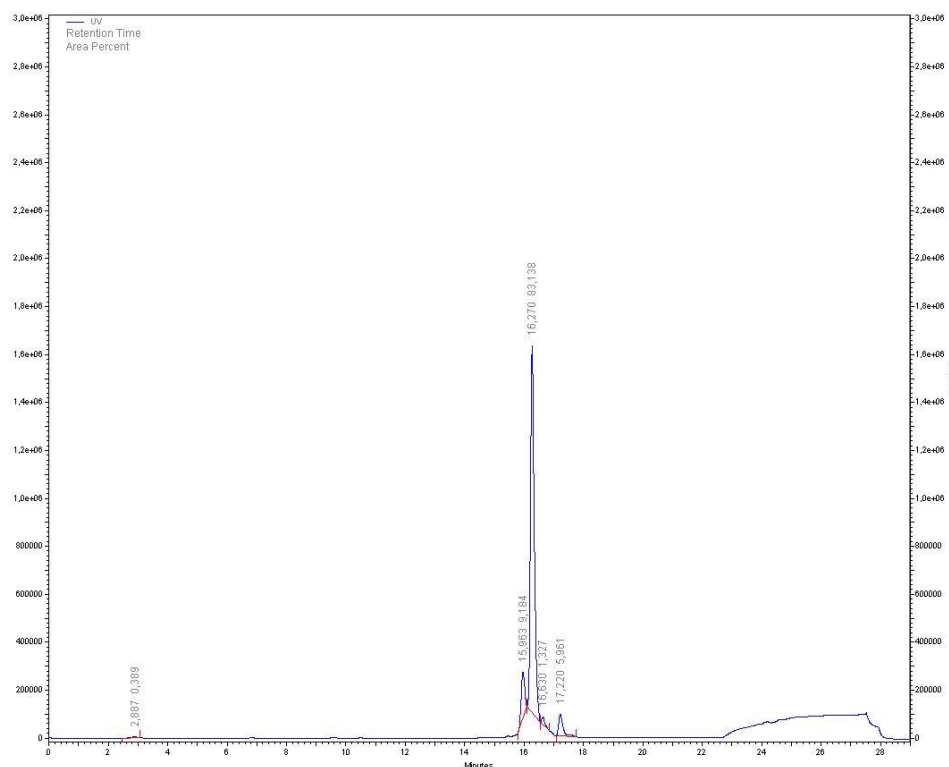


Figure S2: Analytical RP-HPLC of DO3A.NPyr.(FAPi)₂ **11** (t_r = 16.3 min), 25-35% MeCN + 0.1% TFA (linear gradient) in 20 min, 5 mL/min: purity = 83.2%.

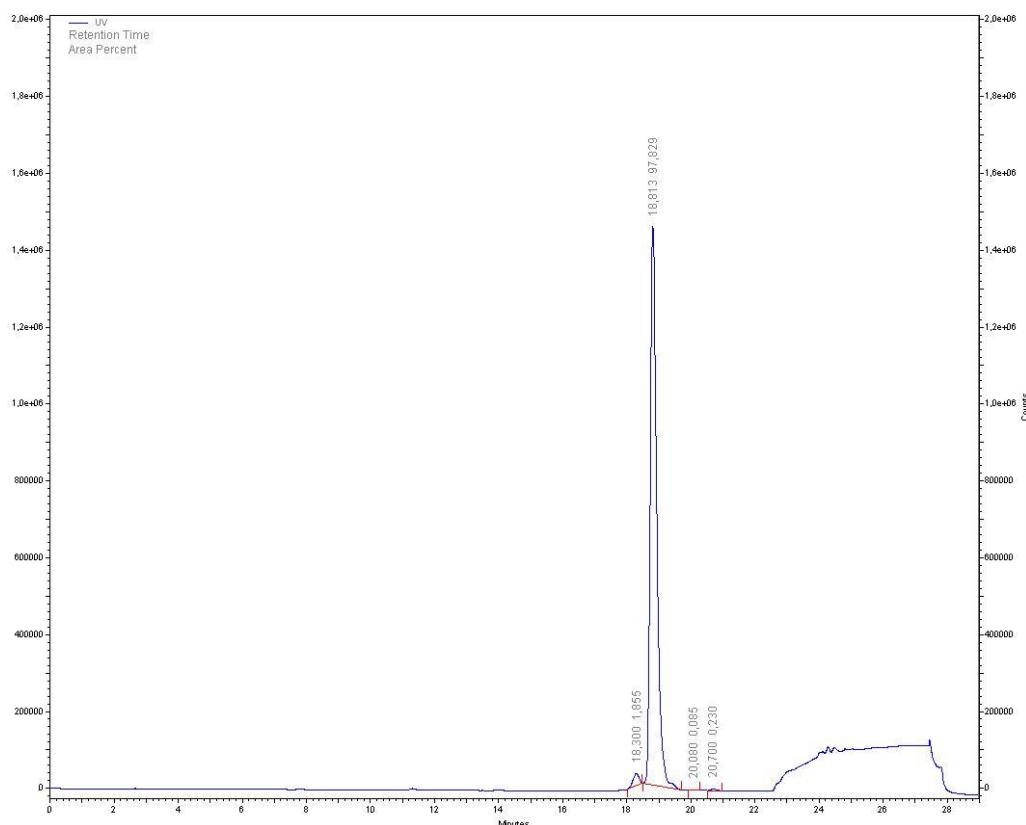


Figure S3: Analytical RP-HPLC of DOTAGA.Glu₂.(FAPi)₂ **18** (t_R = 18.8 min), 20-30% MeCN + 0.1% TFA (linear gradient) in 20 min, 5 mL/min: purity = 97.8%.

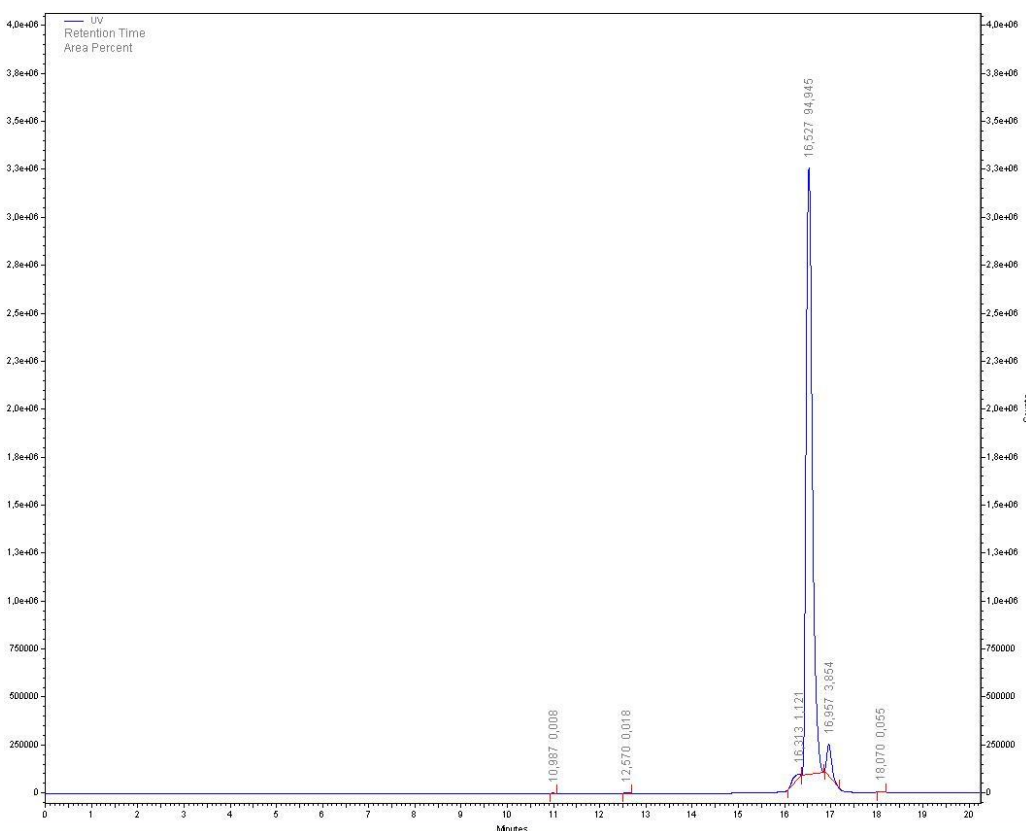


Figure S4: Analytical RP-HPLC of DO3A.Glu₂.(FAPi)₂ **19** (t_R = 16.5 min), 25-35% MeCN + 0.1% TFA (linear gradient) in 20 min, 5 mL/min: purity = 94.9%.

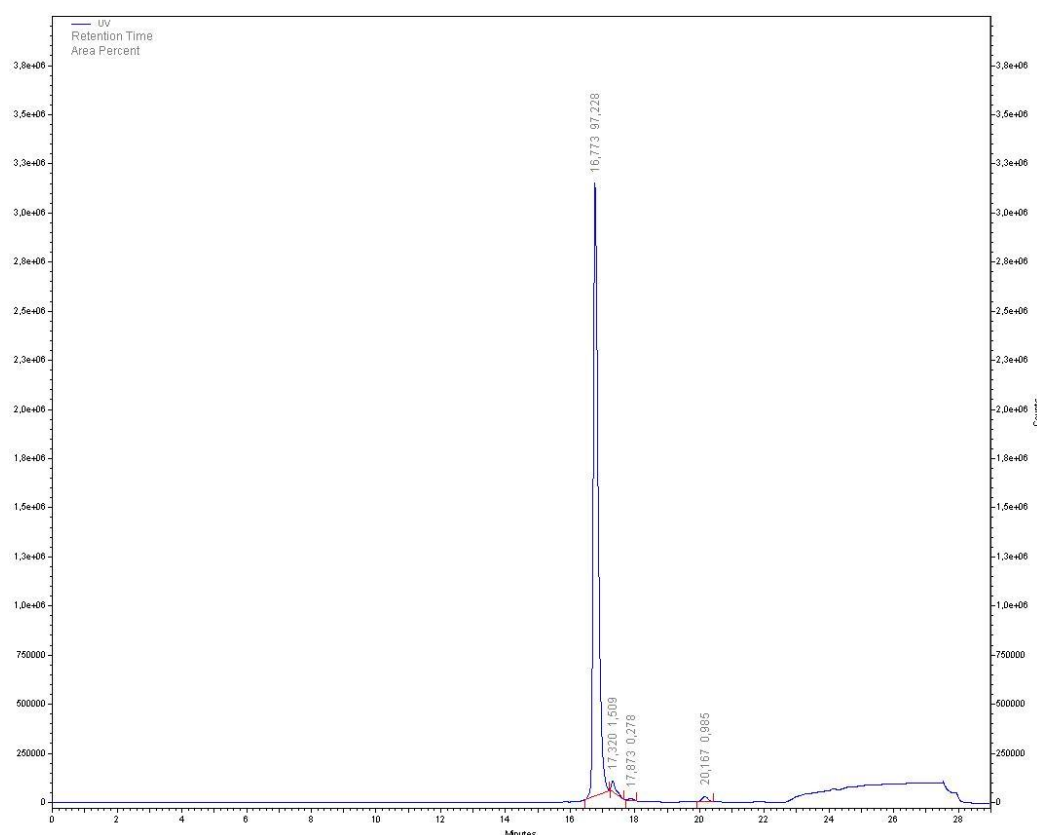


Figure S5: Analytical RP-HPLC of DOTAGA.PEG₂.Glu.(FAPi)₂ **25** (t_R = 16.8 min), 25-35% MeCN + 0.1% TFA (linear gradient) in 20 min, 5 mL/min: purity = 97.2%.

Radiosynthesis - Complex Stabilities

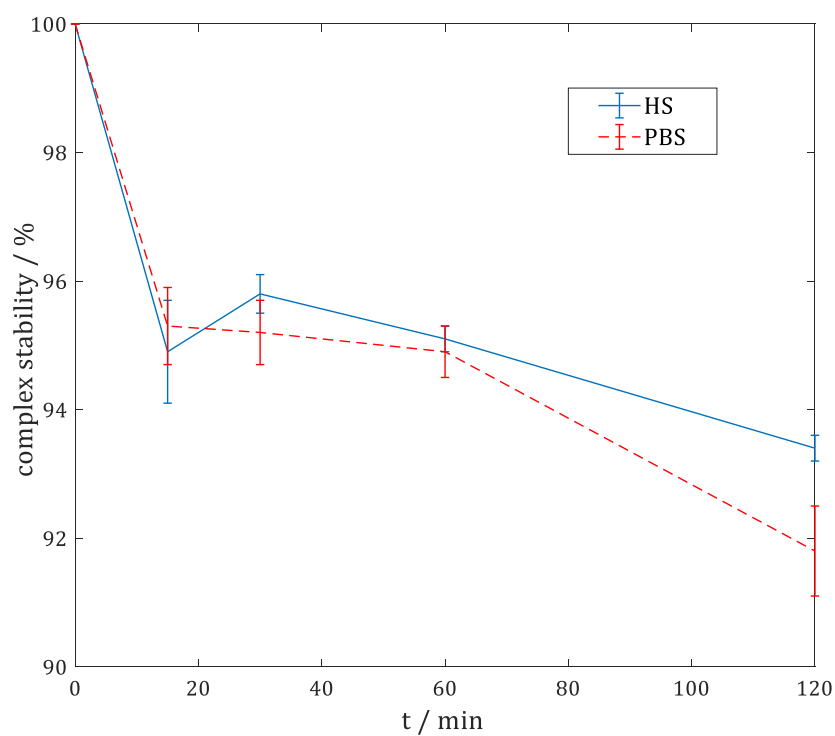


Figure S6: Complex stability of [⁶⁸Ga]Ga-DOTAGA.NPyr.(FAPi)₂ **68Ga-8** (20 MBq/mL) in human serum (HS) and phosphate-buffered saline (PBS) over 120 minutes (n = 3).

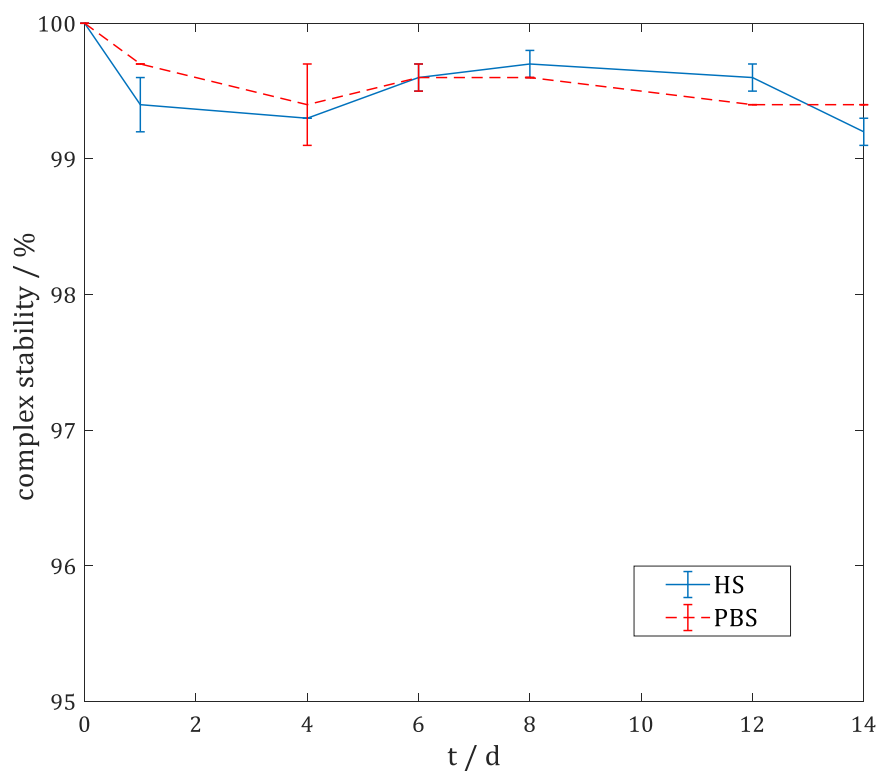


Figure S7: Complex stability of $[^{177}\text{Lu}]\text{Lu-DOTAGA.NPyr.}(\text{FAPi})_2$ $^{177}\text{Lu-8}$ (20 MBq/mL) in human serum (HS) and phosphate-buffered saline (PBS) over 14 days ($n = 3$).

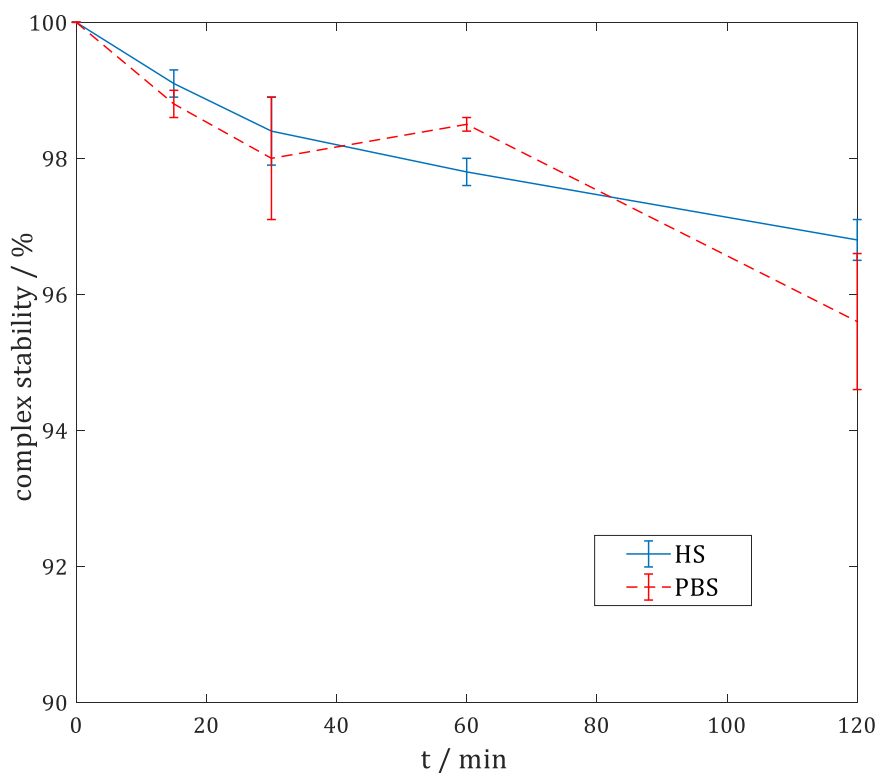


Figure S8: Complex stability of $[^{68}\text{Ga}]\text{Ga-DO3A.NPyr.}(\text{FAPi})_2$ $^{68}\text{Ga-11}$ (20 MBq/mL) in human serum (HS) and phosphate-buffered saline (PBS) over 120 minutes ($n = 3$).

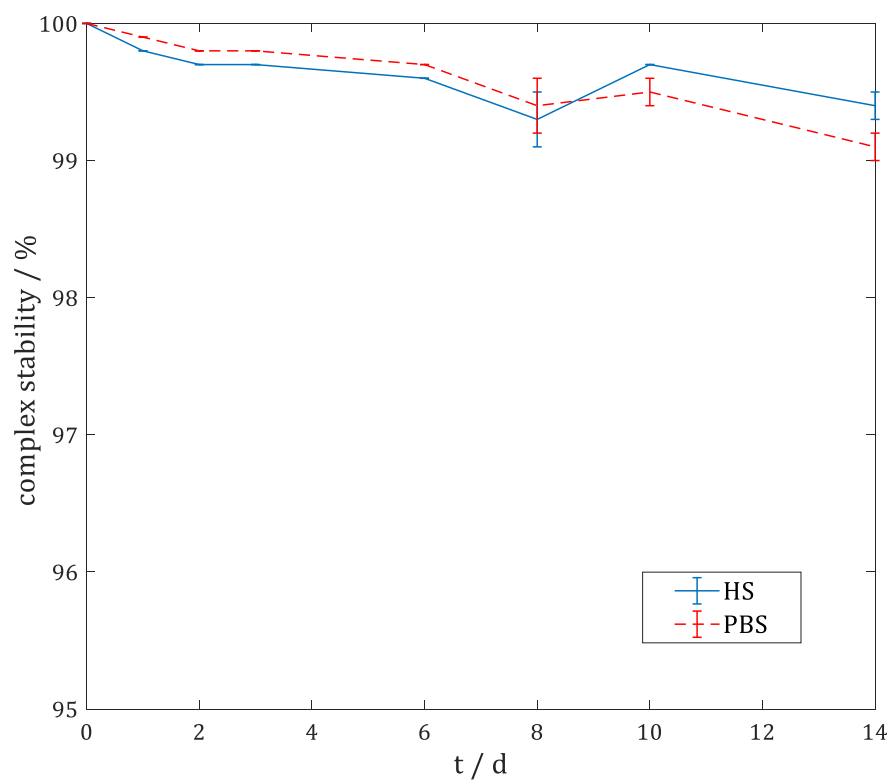


Figure S9: Complex stability of $[^{177}\text{Lu}]\text{Lu-DO3A.NPyr.}(\text{FAPi})_2$ $^{177}\text{Lu-11}$ (20 MBq/mL) in human serum (HS) and phosphate-buffered saline (PBS) over 14 days ($n = 3$).

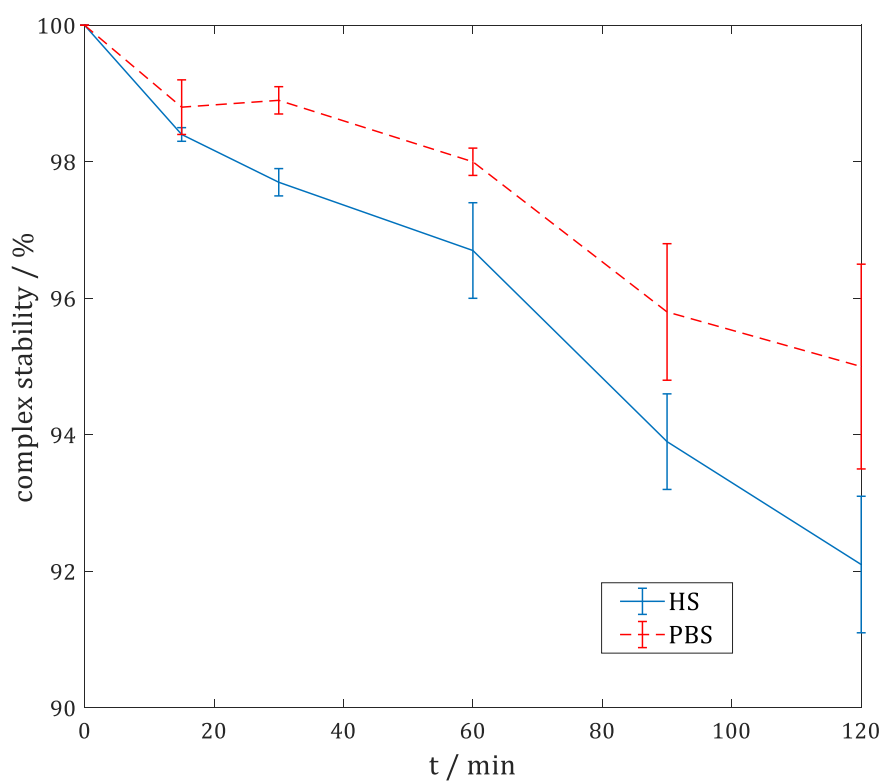


Figure S10: Complex stability of $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu}_2.(\text{FAPi})_2$ $^{68}\text{Ga-18}$ (20 MBq/mL) in human serum (HS) and phosphate-buffered saline (PBS) over 120 minutes ($n = 3$).

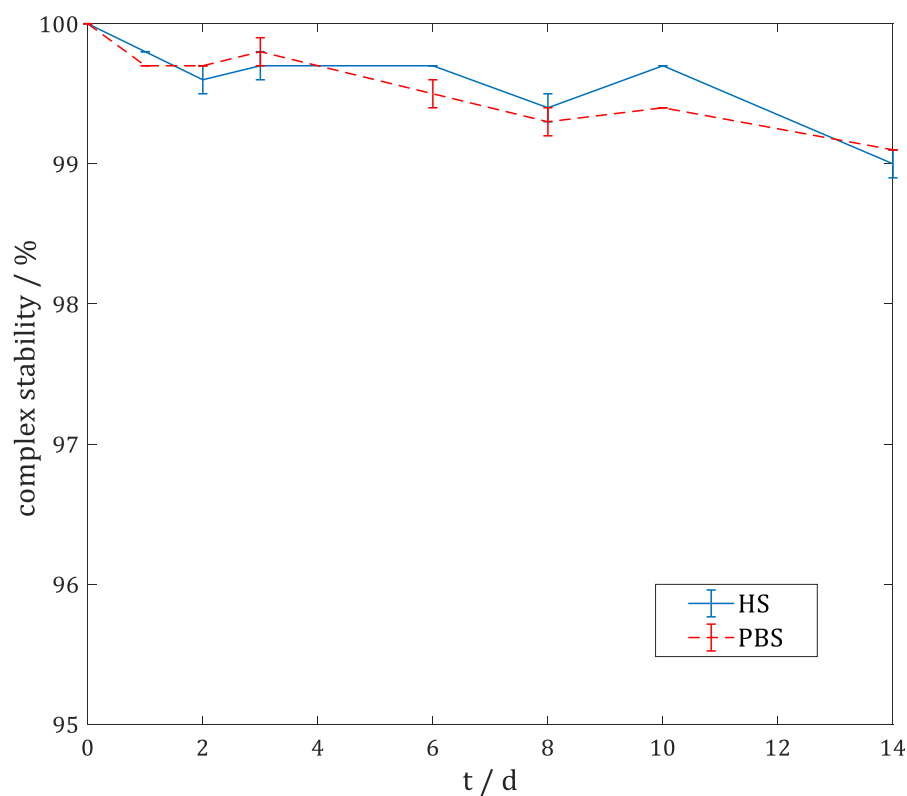


Figure S11: Complex stability of $[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu}_2(\text{FAPi})_2$ $^{177}\text{Lu-18}$ (20 MBq/mL) in human serum (HS) and phosphate-buffered saline (PBS) over 14 days ($n = 3$).

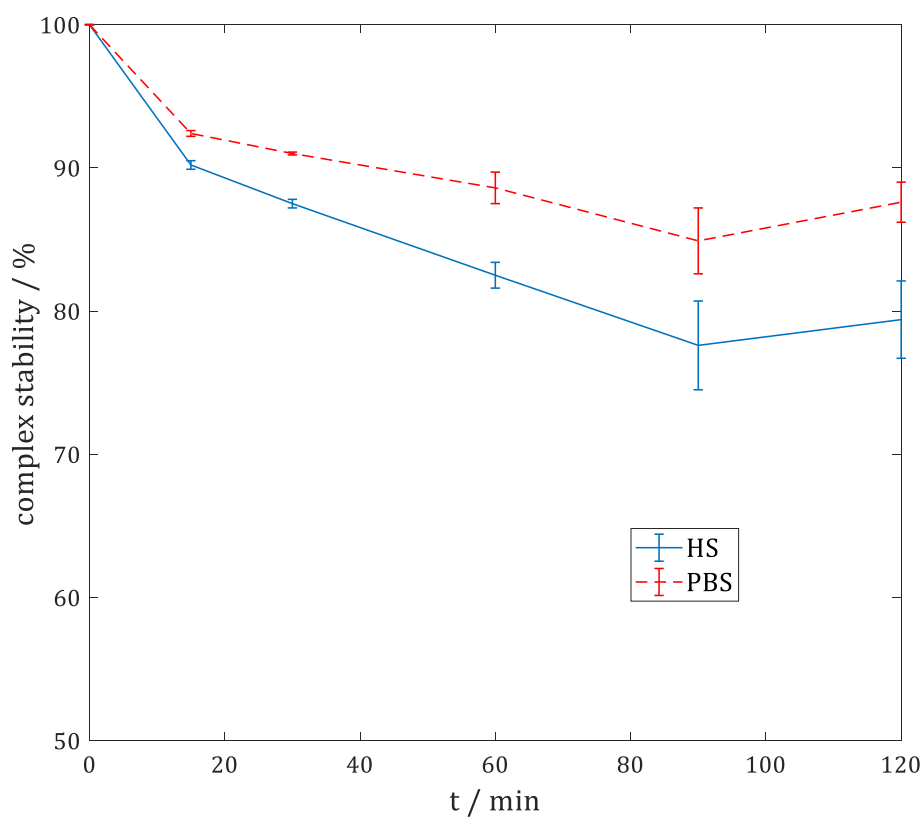


Figure S12: Complex stability of $[^{68}\text{Ga}]\text{Ga-DO3A.Glu}_2(\text{FAPi})_2$ $^{68}\text{Ga-18}$ (20 MBq/mL) in human serum (HS) and phosphate-buffered saline (PBS) over 120 minutes ($n = 3$).

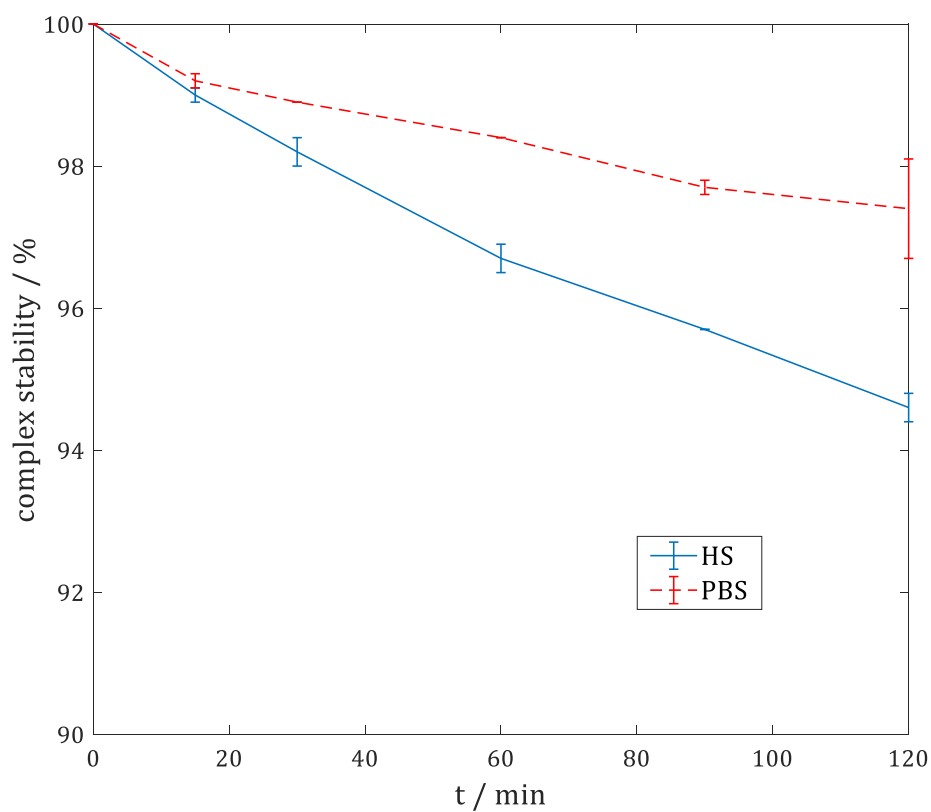


Figure S13: Complex stability of $[^{68}\text{Ga}]\text{Ga-DOTAGA.PEG}_2\text{Glu.(FAPi)}_2$ $^{68}\text{Ga-25}$ (20 MBq/mL) in human serum (HS) and phosphate-buffered saline (PBS) over 120 minutes ($n = 3$).

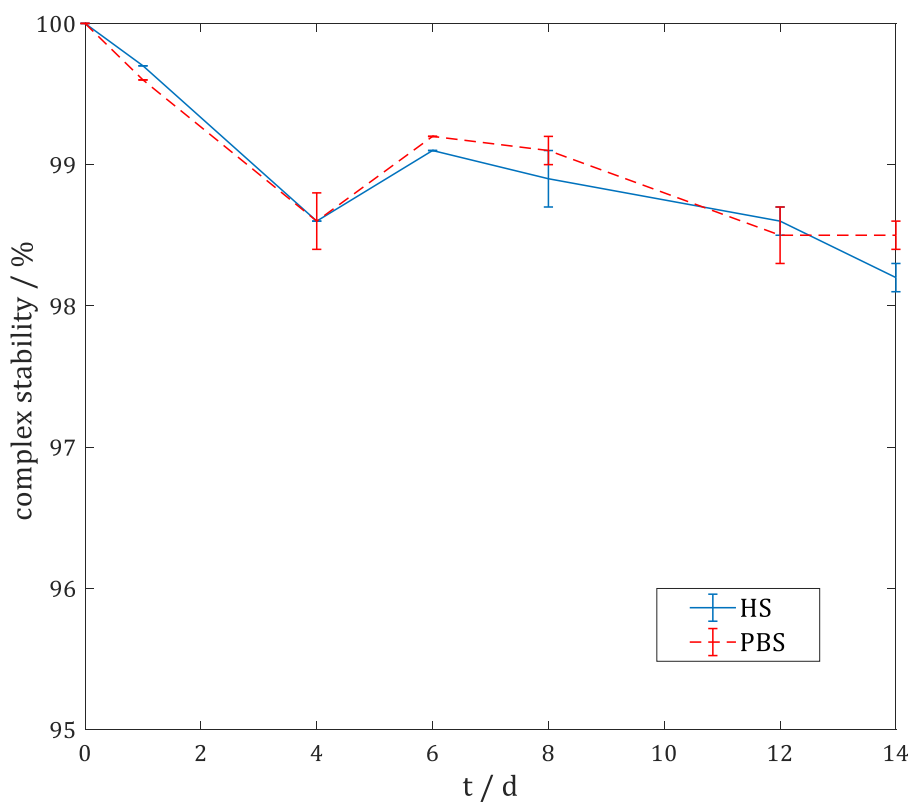


Figure S14: Complex stability of $[^{177}\text{Lu}]\text{Lu-DOTAGA.PEG}_2\text{Glu.(FAPi)}_2$ $^{177}\text{Lu-25}$ (20 MBq/mL) in human serum (HS) and phosphate-buffered saline (PBS) over 14 days ($n = 3$).

Radiosynthesis - Radio-HPLCs

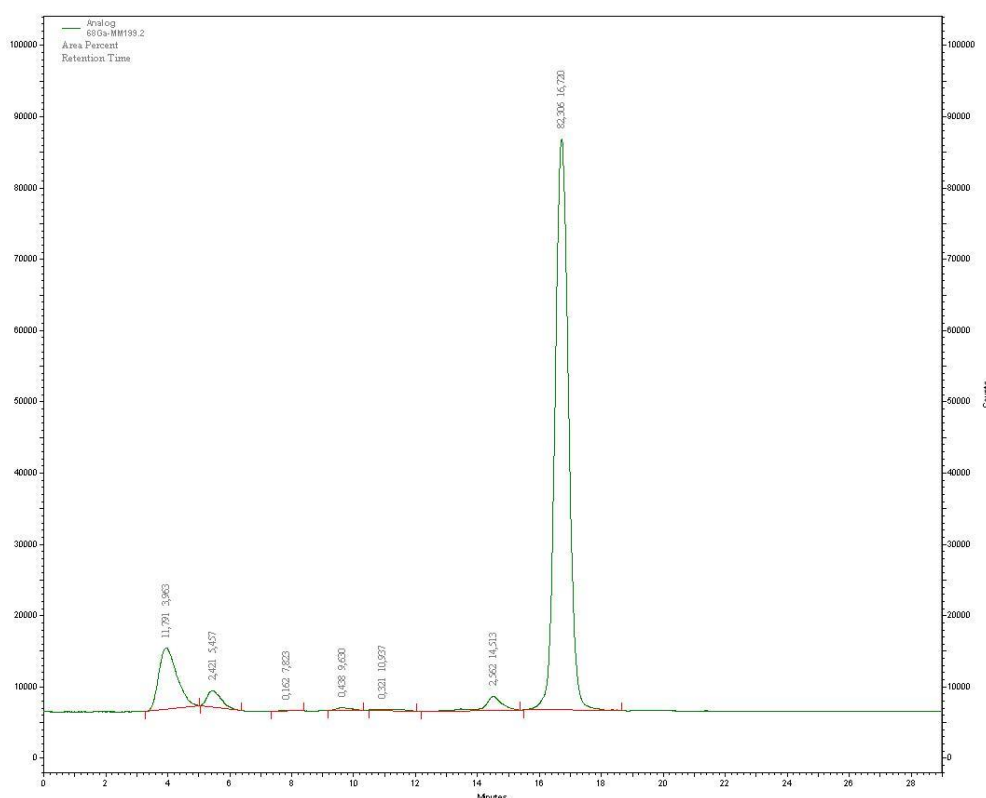


Figure S15: Analytical Radio-HPLC of $[^{68}\text{Ga}]\text{Ga-DOTAGA.NPyr.}(\text{FAPi})_2$ $^{68}\text{Ga-8}$ ($t_R = 16.7$ min) after radiolabeling with 10 nmol **8**, and 50 MBq ^{68}Ga in 1 M HEPES (pH = 5.5), 20-40% MeCN + 0.1% TFA (linear gradient) in 20 min: RCP = 82.3%.

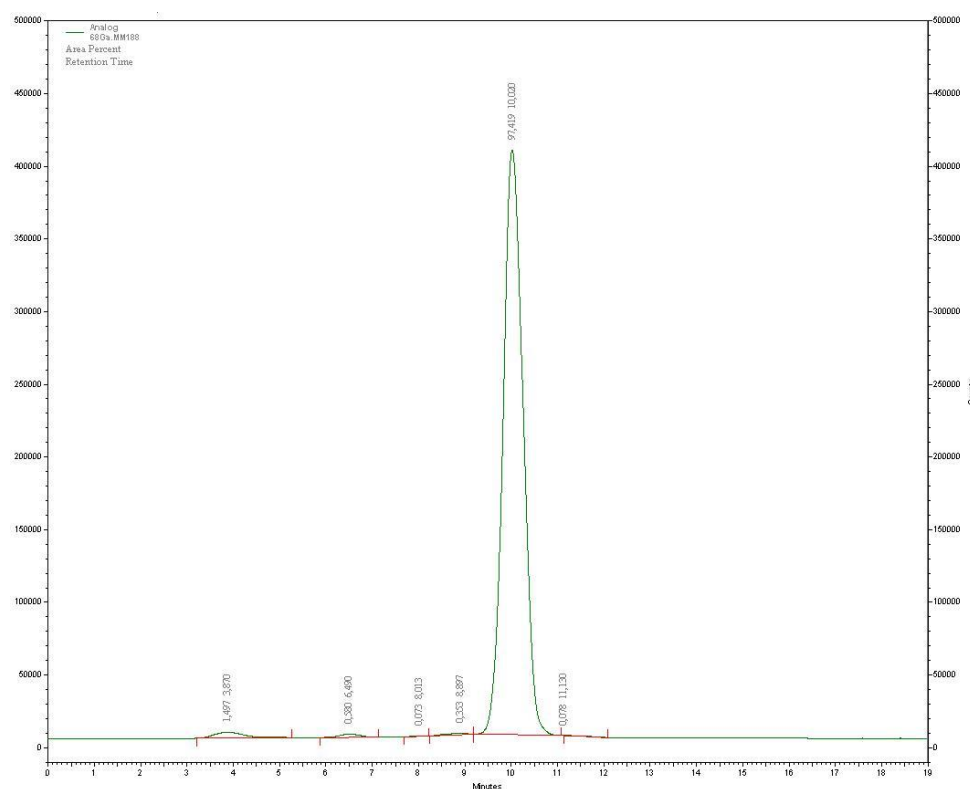


Figure S16: Analytical Radio-HPLC of $[^{68}\text{Ga}]\text{Ga-DOTAGA.NPyr.}(\text{FAPi})_2$ $^{68}\text{Ga-8}$ ($t_R = 10.0$ min) after C18 cartridge purification, 20-50% MeCN + 0.1% TFA (linear gradient) in 10 min: RCP = 97.4%.

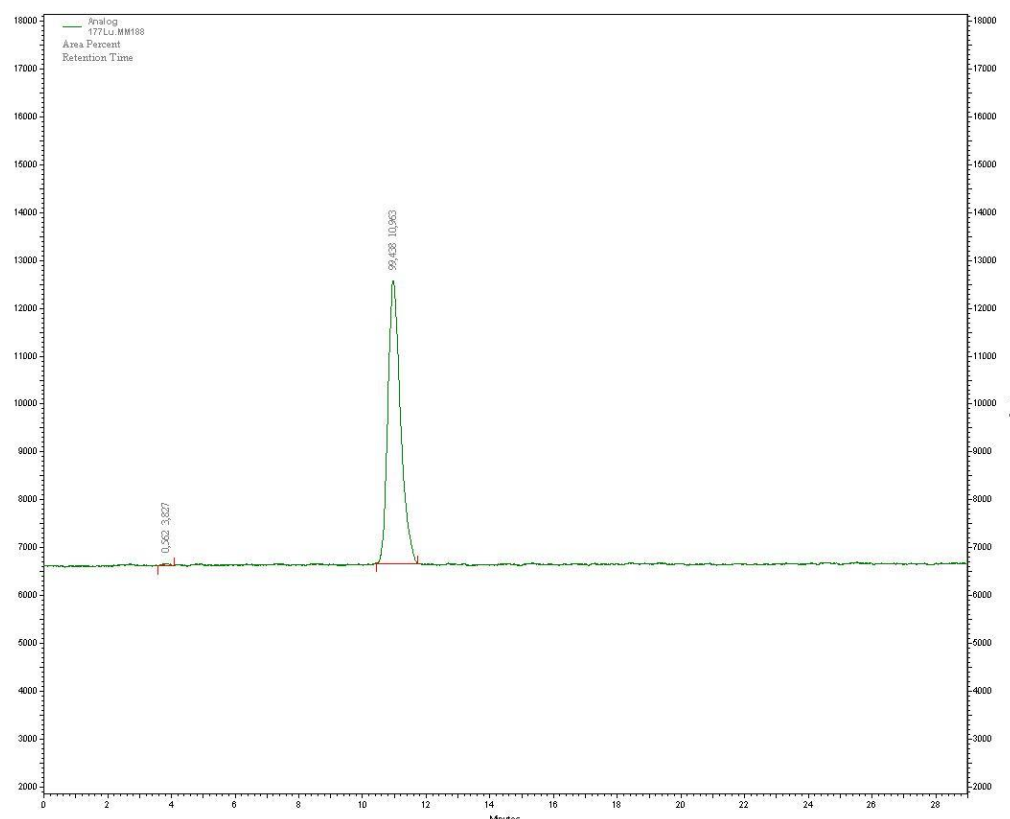


Figure S17: Analytical Radio-HPLC of [^{177}Lu]Lu-DOTAGA.NPyr.(FAPi) $_2$ ^{177}Lu -**8** ($t_R = 11.0$ min) after radiolabeling with 5 nmol **8**, and 50 MBq ^{177}Lu in 1 M HEPES (pH = 5.5), 20-50% MeCN + 0.1% TFA (linear gradient) in 20 min: RCP = 99.4%.

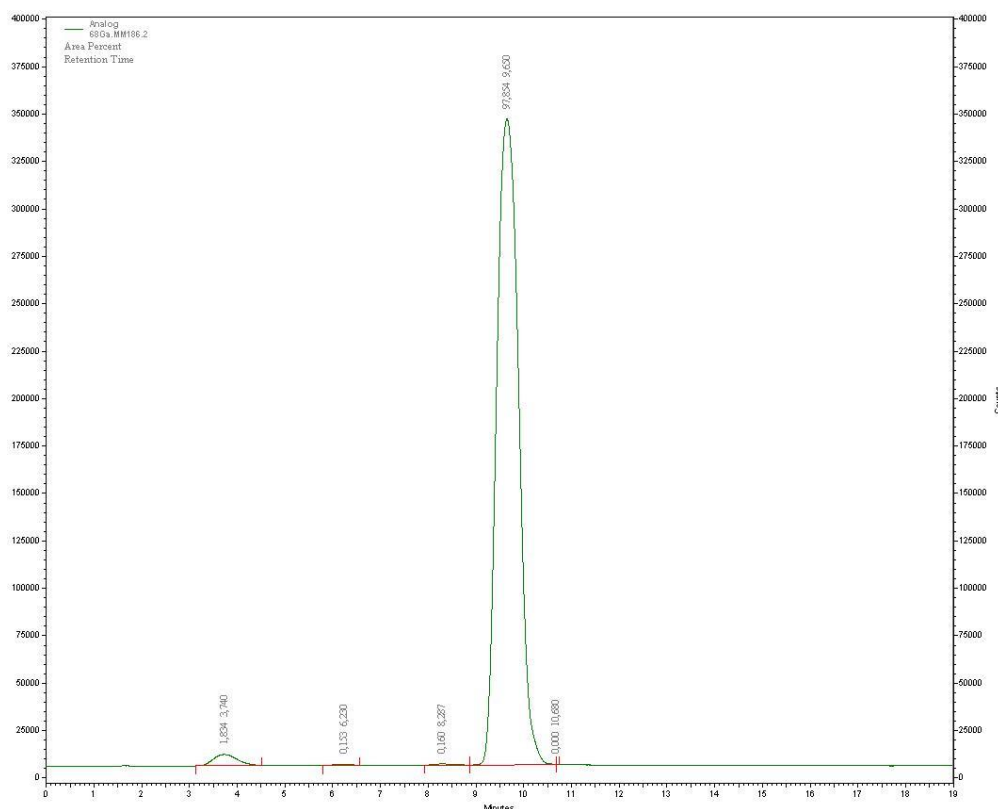


Figure S18: Analytical Radio-HPLC of [^{68}Ga]Ga-DO3A.NPyr.(FAPi) $_2$ ^{68}Ga -**11** ($t_R = 9.7$ min) after radiolabeling with 20 nmol **11**, and 100 MBq ^{68}Ga in 1 M HEPES (pH = 4.5), 20-50% MeCN + 0.1% TFA (linear gradient) in 10 min: RCP = 97.9%.

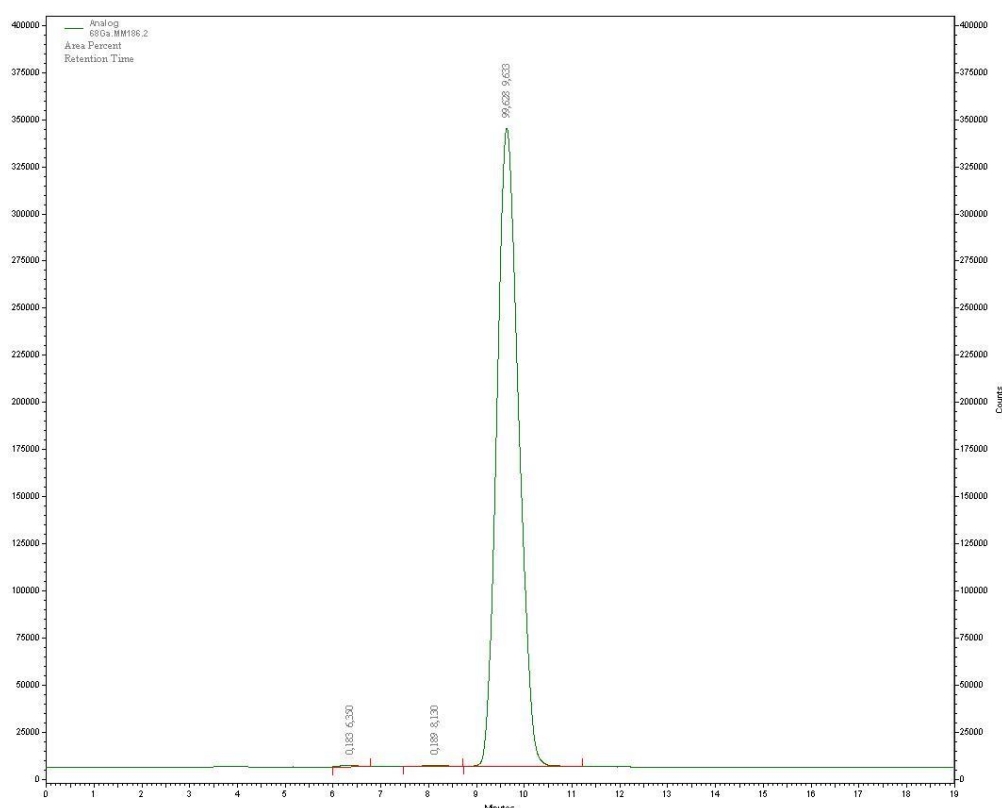


Figure S19: Analytical Radio-HPLC of $[^{68}\text{Ga}]\text{Ga-DO3A.NPyr.}(\text{FAPi})_2$ **68Ga-11** ($t_R = 9.7$ min) after C18 cartridge purification, 20-50% MeCN + 0.1% TFA (linear gradient) in 10 min: RCP = 99.6%.

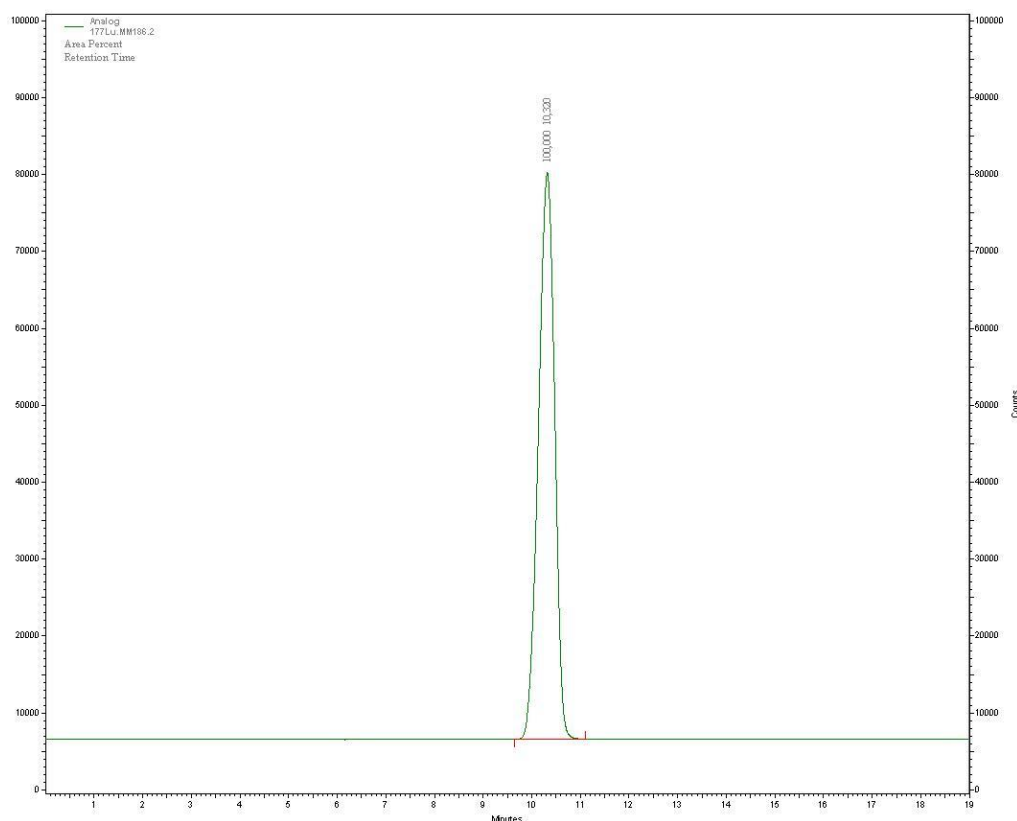


Figure S20: Analytical Radio-HPLC of $[^{177}\text{Lu}]\text{Lu-DO3A.NPyr.}(\text{FAPi})_2$ **177Lu-11** ($t_R = 10.3$ min) after radiolabeling with 2 nmol **11**, and 50 MBq ^{177}Lu in 1 M HEPES (pH = 5.5), 20-50% MeCN + 0.1% TFA (linear gradient) in 10 min: RCP = 100.0%.

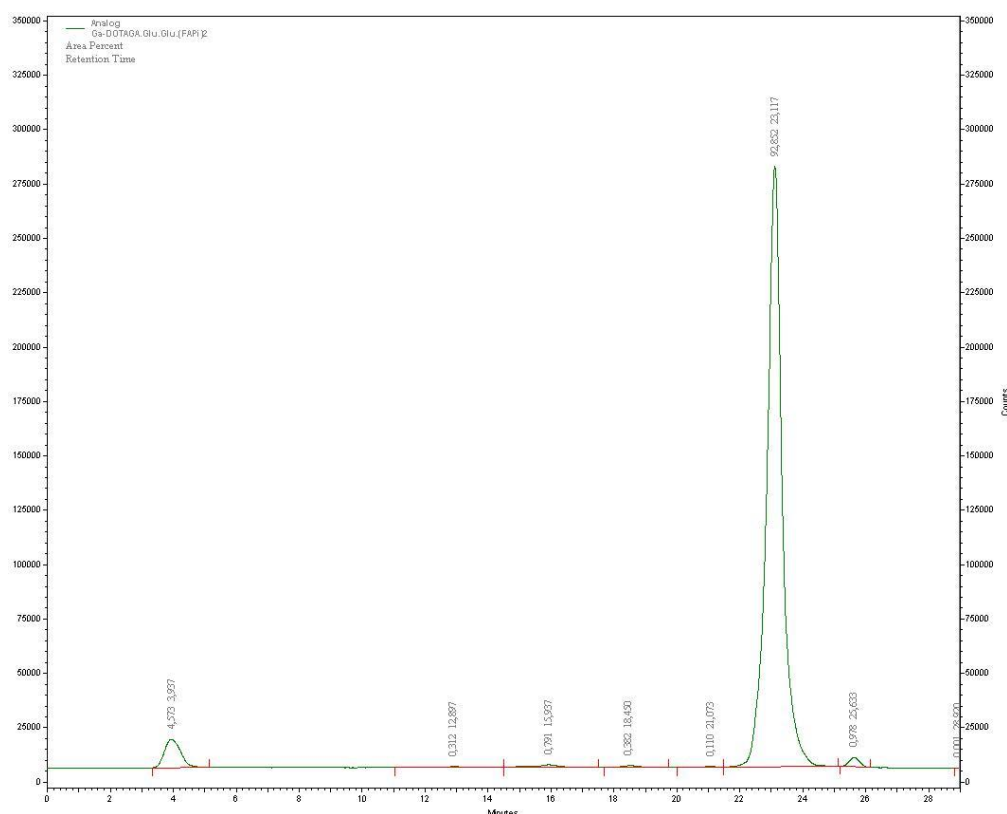


Figure S21: Analytical Radio-HPLC of $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu}_2(\text{FAPi})_2$ $^{68}\text{Ga-18}$ ($t_R = 23.1$ min) after radiolabeling with 10 nmol **18**, and 50 MBq ^{68}Ga in 1 M HEPES (pH = 5.5), 20-30% MeCN + 0.1% TFA (linear gradient) in 20 min: RCP = 92.9%.

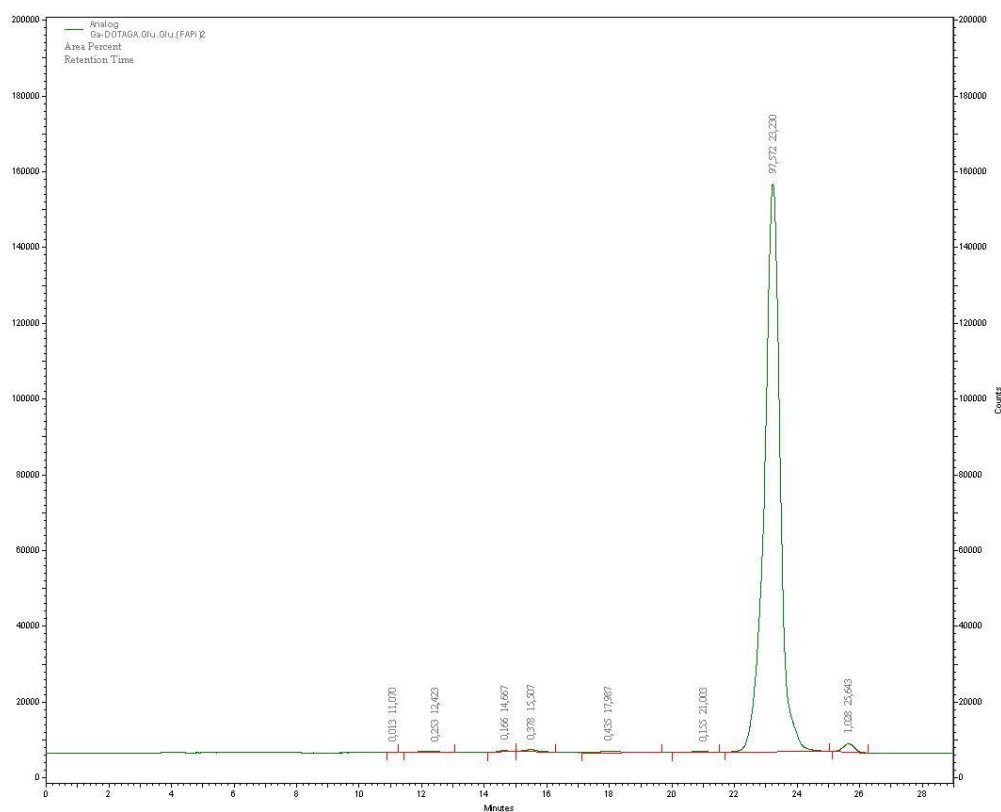


Figure S22: Analytical Radio-HPLC of $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu}_2(\text{FAPi})_2$ $^{68}\text{Ga-18}$ ($t_R = 23.2$ min) after C18 cartridge purification, 20-30% MeCN + 0.1% TFA (linear gradient) in 20 min: RCP = 97.6%.

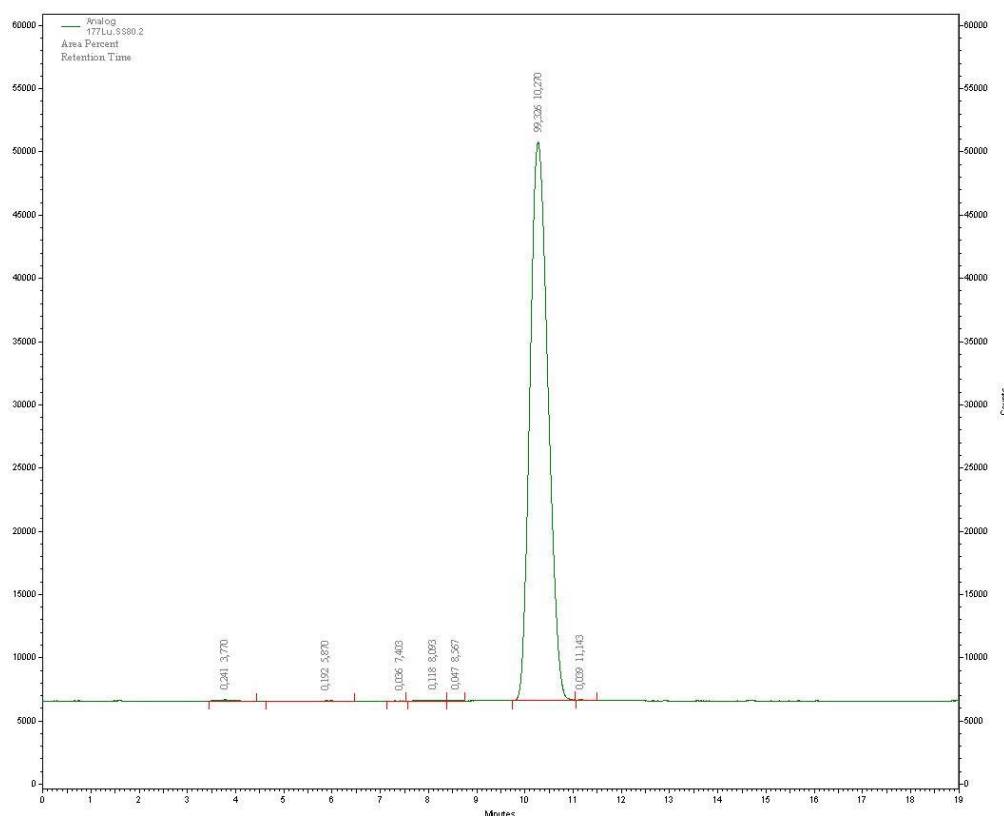


Figure S23: Analytical Radio-HPLC of [^{177}Lu]Lu-DOTAGA.Glu₂.(FAPi)₂ ^{177}Lu -**18** (t_R = 10.3 min) after radiolabeling with 2 nmol **18**, and 50 MBq ^{177}Lu in 1 M HEPES (pH = 5.5), 20-50% MeCN + 0.1% TFA (linear gradient) in 10 min: RCP = 99.3%.

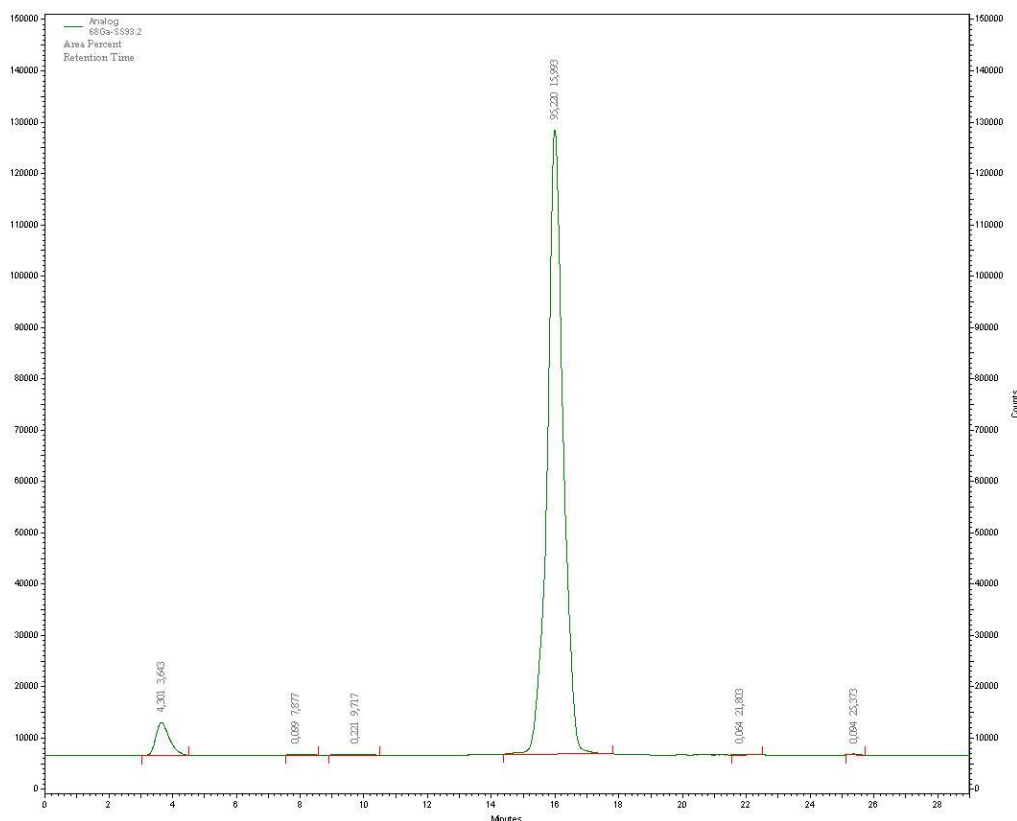


Figure S24: Analytical Radio-HPLC of [^{68}Ga]Ga-DO3A.Glu₂.(FAPi)₂ ^{68}Ga -**19** (t_R = 16.0 min) after radiolabeling with 20 nmol **19**, and 100 MBq ^{68}Ga in 1 M HEPES (pH = 4.5), 20-50% MeCN + 0.1% TFA (linear gradient) in 20 min: RCP = 95.2%.

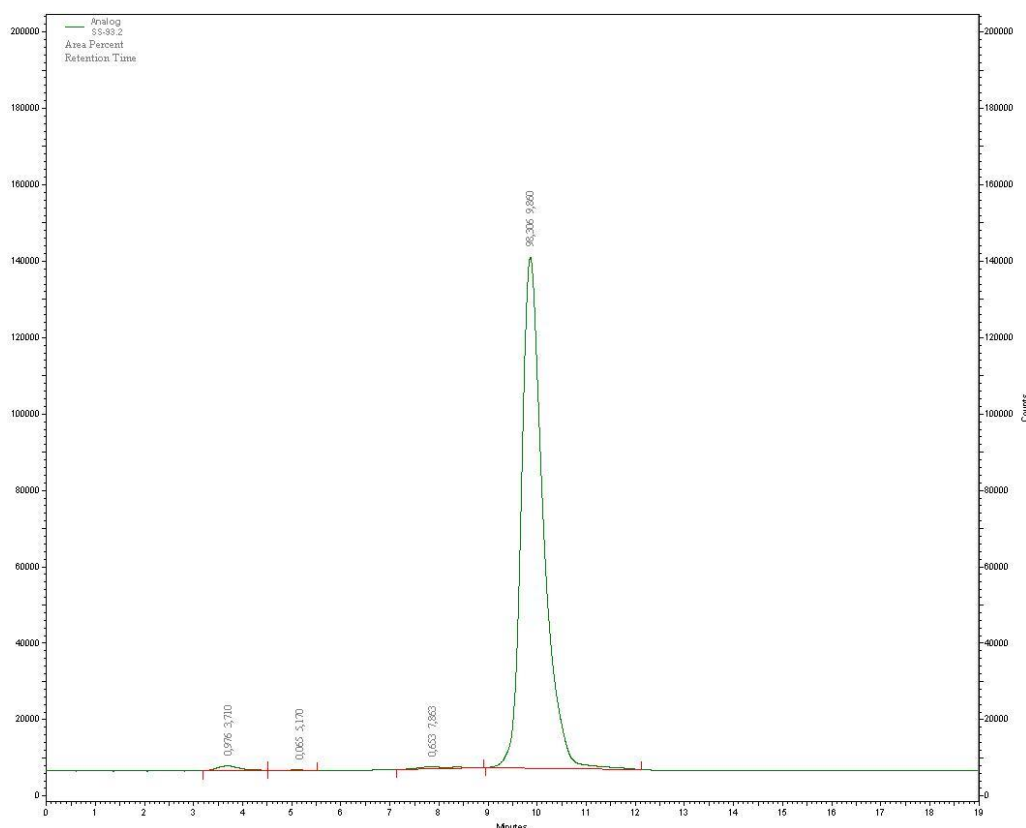


Figure S25: Analytical Radio-HPLC of [^{68}Ga]Ga-DO3A.Glu₂.(FAPi)₂ **$^{68}\text{Ga-19}$** ($t_R = 9.9$ min) after C18 cartridge purification, 20-50% MeCN + 0.1% TFA (linear gradient) in 10 min: RCP = 98.3%.

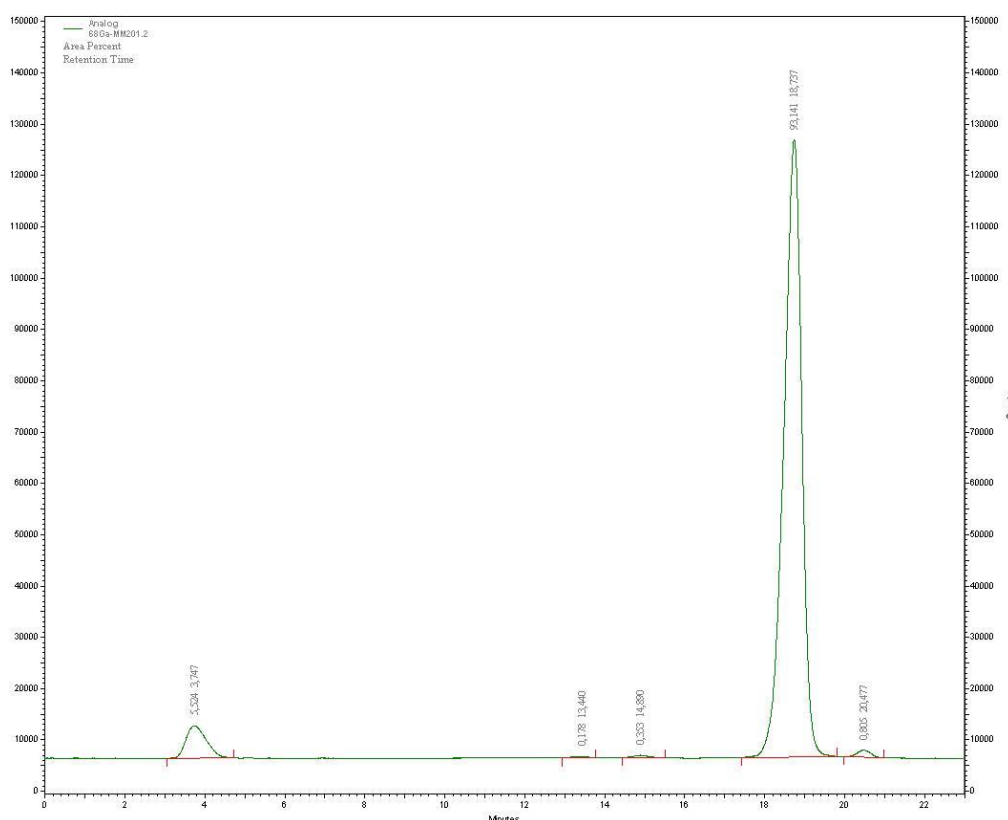


Figure S26: Analytical Radio-HPLC of [^{68}Ga]Ga-DOTAGA.PEG₂.Glu.(FAPi)₂ **$^{68}\text{Ga-25}$** ($t_R = 18.7$ min) after radiolabeling with 10 nmol **25**, and 50 MBq ^{68}Ga in 1 M AmOAc (pH = 4.5), 20-30% MeCN + 0.1% TFA (linear gradient) in 15 min: RCP = 93.1%.

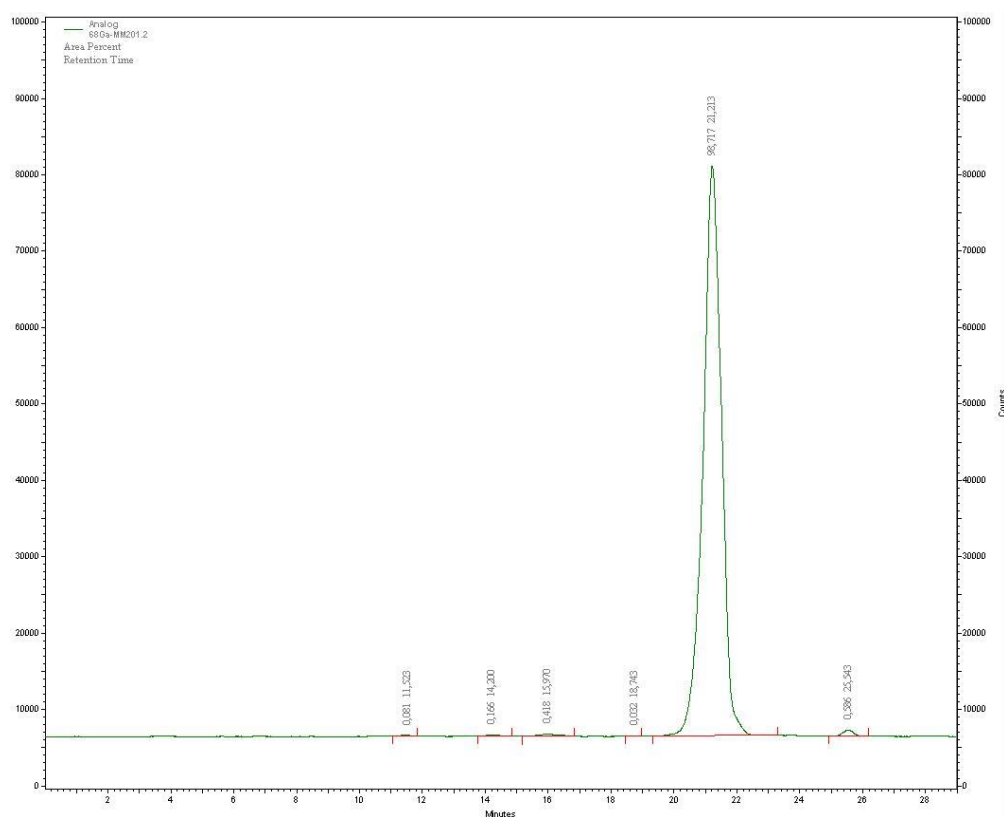


Figure S27: Analytical Radio-HPLC of $[^{68}\text{Ga}]\text{Ga-DOTAGA.PEG}_2.\text{Glu.}(\text{FAPi})_2$ $^{68}\text{Ga-25}$ ($t_R = 21.2$ min) after C18 cartridge purification, 20-30% MeCN + 0.1% TFA (linear gradient) in 20 min: RCP = 98.7%.

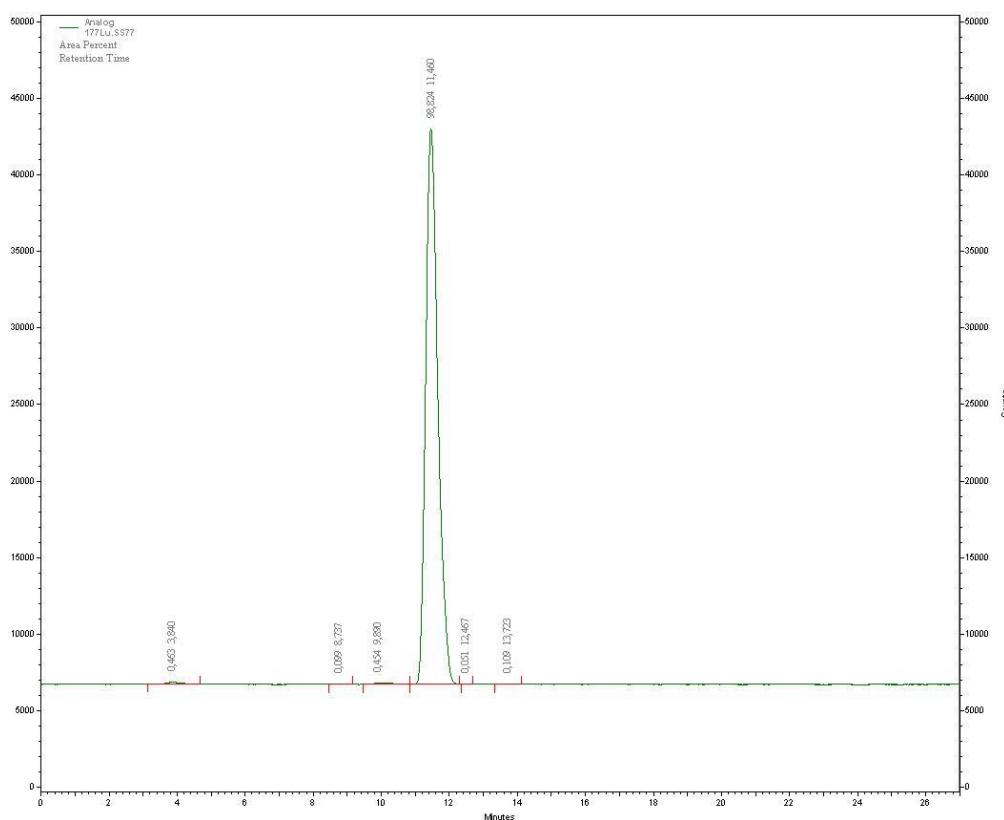


Figure S28: Analytical Radio-HPLC of $[^{177}\text{Lu}]\text{Lu-DOTAGA.PEG}_2.\text{Glu.}(\text{FAPi})_2$ $^{177}\text{Lu-25}$ ($t_R = 11.5$ min) after radiolabeling with 2 nmol **25**, and 50 MBq ^{177}Lu in 1 M HEPES (pH = 5.5), 20-50% MeCN + 0.1% TFA (linear gradient) in 20 min: RCP = 98.8%.

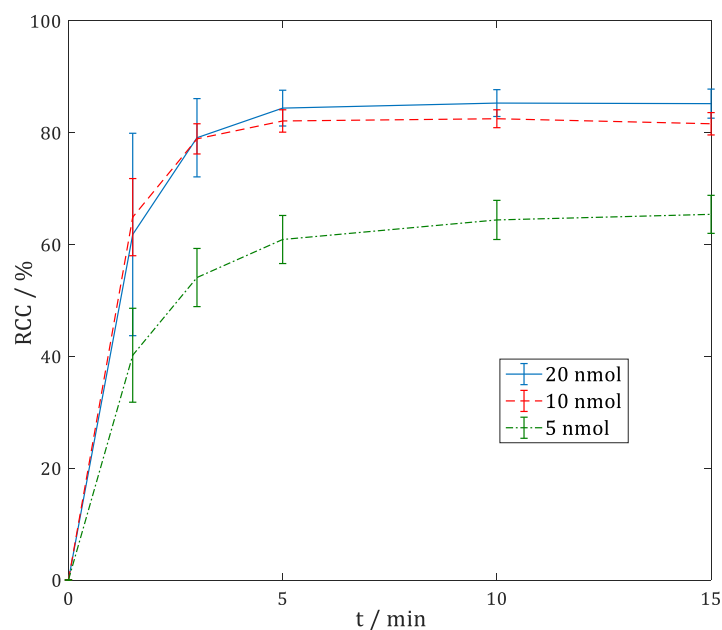


Figure S29: Reaction kinetics (radiochemical conversion (RCC) in %) of ^{68}Ga]-Ga-DOTAGA.NPyr.(FAPi)₂ ^{68}Ga -8 at 95 °C with 20, 10 or 5 nmol DOTAGA.NPyr.(FAPi)₂ 8 and 100 MBq ^{68}Ga in 400 µL 1 M HEPES (pH = 4.5), (n = 3).

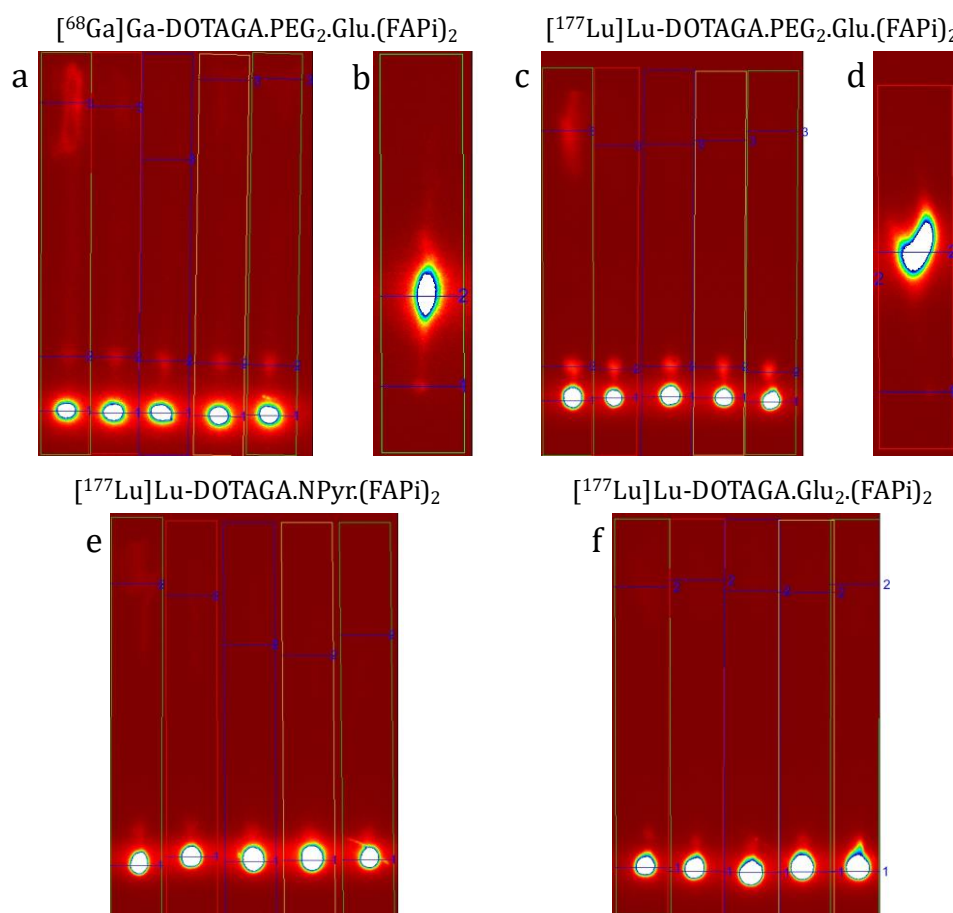


Figure S30: Exemplary reaction kinetic radio-TLCs. a), c), e) and f): 1.5 min on the left to 15 min on the right of respective radiolabeling (^{68}Ga : 5 nmol precursor; ^{177}Lu : 2 nmol precursor) and radio-TLC developed with 0.1 M citrate (pH = 4.0) as mobile phase. b) and d): radio-TLC after respective radiolabeling (15 min) with 1 M ammonium acetate (pH = 4.0)/methanol (1:1) as mobile phase.

In Vitro-Inhibition Assays

Table S1: IC_{50} values of the different inhibitors for the proteases PREP, DPP4, DPP8 and DPP9. The data for $[^{nat}Lu]Lu$ - and DOTAGA.Glu.(FAPi)₂ are taken from Martin et al. [58], for $[^{nat}Lu]Lu$ - and DOTAGA.(SA.FAPi)₂ from Moon et al. [54] and for the initial FAP inhibitor UAMC-1110 from Jansen et al. [35] and Moon et al. [54].

Compound	$IC_{50} / \mu M$			
	PREP	DPP4	DPP8	DPP9
DOTAGA.NPyr.(FAPi) ₂ (8)	0.73 ± 0.16	2.68 ± 0.10	0.106 ± 0.002	0.35 ± 0.02
DO3A.NPyr.(FAPi) ₂ (11)	1.86 ± 0.16	3.90 ± 0.22	0.208 ± 0.004	0.20 ± 0.01
DOTAGA.Glu ₂ .(FAPi) ₂ (18)	0.63 ± 0.05	1.37 ± 0.14	0.041 ± 0.003	0.23 ± 0.05
DO3A.Glu ₂ .(FAPi) ₂ (19)	0.41 ± 0.06	1.03 ± 0.03	0.074 ± 0.001	0.130 ± 0.005
DOTAGA.PEG ₂ .(FAPi) ₂ (25)	0.50 ± 0.08	2.44 ± 0.19	0.023 ± 0.007	0.059 ± 0.002
DOTAGA.Glu.(FAPi) ₂	0.59 ± 0.10	1.19 ± 0.08	0.029 ± 0.004	0.083 ± 0.002
$[^{nat}Lu]Lu$ -DOTAGA.Glu.(FAPi) ₂	0.43 ± 0.16	0.65 ± 0.04	0.22 ± 0.02	0.19 ± 0.01
DOTAGA.(SA.FAPi) ₂	0.39 ± 0.02	0.40 ± 0.07	0.42 ± 0.04	0.16 ± 0.02
$[^{nat}Lu]Lu$ -DOTAGA.(SA.FAPi) ₂	0.56 ± 0.04	0.63 ± 0.07	0.41 ± 0.03	0.18 ± 0.02
UAMC-1110	1.80 ± 0.01	> 10	> 10	4.70 ± 0.40

3.5. Novel FAP Inhibitor-Based Monomers as Diagnostic PET/CT Counterpart for FAPi Dimer Radioligand Therapy

In preparation for submission

Summary

The previous study by MARTIN et al. [51] improved radioligand therapy (RLT) with the FAPi homodimer [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ targeting fibroblast activation protein alpha (FAP). In the clinical setting, ^{177}Lu -FAP-RLT is guided by diagnostic ^{68}Ga -PET imaging to check biodistribution and tumor accumulation of the radiotracer in the patient and estimate radiation dose that will be administered for the following treatment. Until now, ^{68}Ga -PET was carried out using the squaric acid-based [^{68}Ga]Ga-DOTA.SA.FAPi developed by MOON et al. [48].

This study aimed to improve diagnostic ^{68}Ga -PET imaging that guides FAP-RLT with [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$. The monomer DOTA.SA.FAPi is structurally quite different than the new dimer DOTAGA.Glu.(FAPi) $_2$ that does not incorporate squaric acid (SA) linkers, for example. Therefore, new monomeric radiotracers were developed that are structurally closely related to DOTAGA.Glu.(FAPi) $_2$ but one of the two FAPi targeting vectors was substituted by a pyrrolidine heterocyclic group that is supposed to be “inert” in the sense that it should not interfere with the radiolabeling and in particular the binding properties to FAP and the other proteases. Pyrrolidine was chosen because similar nitrogen heterocyclics like piperazine have been used in successful FAPi radiotracers like FAPI-04 or FAPI-46 already. The chelators DO3A and DOTAGA were used in analogy to DO3A- and DOTAGA.Glu.(FAPi) $_2$. Additionally, DATA ^{5m} was used as a hybrid bifunctional chelator that enables radiolabeling with gallium-68 at room temperature.

At first, the intermediate Glu(Pyr).FAPi **6** was synthesized with an high yield of 86% over 4 steps. From this platform, the three different chelators were coupled to it and finally gave the three precursors DO3A.Glu(Pyr).FAPi **9**, DOTAGA.Glu(Pyr).FAPi **11** and DATA ^{5m} .Glu(Pyr).FAPi **13** with 31–33% overall yield over 6 steps, respectively. Subsequently, the ^{nat}Ga -complexes were synthesized with a yield of 63–67%.

The radiolabeling with gallium-68 was at first tested in three different buffer systems: 1 M HEPES (pH = 5.5), 1 M AmOAc (pH = 4.5) and 1 M NaOAc (pH = 4.5). HEPES showed slightly faster reaction kinetics and AmOAc seemed to be slightly superior to NaOAc. Nevertheless, almost quantitative radiochemical conversion (RCC) > 97% could be achieved in all three buffer systems with ^{68}Ga -**9** and ^{68}Ga -**11**. Slight formation of colloidal [^{68}Ga]Ga(OH) $_3$ was observed with ^{68}Ga -**13** but the RCC was still over 90% in all buffer systems. Radiolabeled products with high radiochemical purity (RCP) > 98% were achieved after subsequent purification via C18 cartridge. ^{68}Ga -**9** and ^{68}Ga -**11** were ca. 95% stable in human serum and phosphate-buffered saline for two hours whereas ^{68}Ga -**13** was slightly less stable (85 – 90%). The $\log D_{7.4}$ were determined via shake-flask method and revealed high hydrophilicity for all three compounds. ^{68}Ga -**9** and ^{68}Ga -**11** ($-\log D_{7.4}$ = 2.4 – 2.5) were very similar to [^{68}Ga]Ga-DOTAGA.Glu.(FAPi) $_2$ ($-\log D_{7.4}$ = 2.48 ± 0.05) [51].

Affinity studies *in vitro* resulted in subnanomolar IC_{50} values for FAP and very high selectivity over the related proteases. The selectivity over the important competitive protease PREP in particular,

was even higher than for the initial FAP inhibitor UAMC-1110, especially of **9**, **11** and the respective ^{nat}Ga-complexes. The DPP4 selectivity was comparable to UAMC-1110 for all three compounds. Affinity and selectivity were substantially improved compared to DOTA- and DATA^{5m}.SA.FAPi. The radiolabeling and *in vitro* properties indicated that **⁶⁸Ga-9** and **⁶⁸Ga-11**, in particular, are very promising candidates for ⁶⁸Ga-FAPi-PET imaging *in vivo*.

DO3A.Glu(Pyr).FAPi (**⁶⁸Ga-9**) and DOTAGA.Glu(Pyr).FAPi (**⁶⁸Ga-11**) were investigated in the first patients to demonstrate proof-of-concept. PET/CT scans were taken of two patients, one breast cancer patient and one thyroid cancer. Both radiotracers showed significant uptake in the tumor lesions. Both also showed some uptake in the pancreas similar to [⁶⁸Ga]Ga-DOTA.SA.FAPi where it is unclear if this is due to unspecific binding or physiological FAP expression [35, 68–70]. Physiological FAP expression in the pancreas was reported in several studies and publications [71–74]. **⁶⁸Ga-9** demonstrated fast clearance from the blood pool but some liver uptake was observed at 60 min post-injection. In contrast, **⁶⁸Ga-11** showed almost no liver uptake but the blood pool activity was higher at the same time point.

Overall, the radiotracers seem suitable in detection of tumor lesions of epithelial cancers with good contrast PET/CT images, with limitations probably only in the detection of pancreatic tumor lesions. It is worthwhile to further investigate these new radiotracers, e.g. in comparison to [⁶⁸Ga]Ga-DOTA.SA.FAPi, which is currently used for PET/CT. Maybe the new tracers can optimize the ⁶⁸Ga-PET diagnosis guiding RLT with the FAPi homodimer [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂.

Author Contributions

Frank Roesch supervised this study and conceptualized it with the support of MARCEL MARTIN. Ingrid De Meester and Pieter Van Der Veken supervised the *in vitro* studies whereas Chandrasekhar Bal supervised the *in vivo* studies.

MARCEL MARTIN planned and carried out the organic synthesis and did all the radiochemical investigation, evaluation and validation including all synthesis, analysis and interpretation of the results. He additionally helped to translate the radiolabeling protocol into the clinical routine together with Sanjana Ballal and Madhav Prasad Yadav. *In vitro* investigations were carried out and analyzed by Yentl Van Rymenant, Emile Verhulst and Joni De Loose who further interpreted the *in vitro* results together with MARCEL MARTIN.

Clinical studies were carried out and the clinical data were analyzed by Sanjana Ballal and Madhav Prasad Yadav under the supervision of Chandrasekhar Bal. They were supported by MARCEL MARTIN in the interpretation of the results. MARCEL MARTIN wrote this first draft of the manuscript.

Novel FAP Inhibitor-Based Monomers as Diagnostic PET/CT Counterpart for FAPi Dimer Radioligand Therapy

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Abstract

Targeting fibroblast activation protein alpha (FAP) with FAP inhibitor-based radiotracers has shown great potential in molecular imaging. FAP is overexpressed by cancer-associated fibroblasts in the tumor microenvironment (TME) of most epithelial tumors. While FAP-imaging has already proven its great potential, the search for an optimal therapeutic radiopharmaceutical is still ongoing. The new FAPi dimer [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ is a very promising candidate with selective, high and prolonged tumor uptake. New FAPi monomers with structures closely related to DOTAGA.Glu.(FAPi) $_2$ were synthesized and radiolabeled with gallium-68 to have a complementary diagnostic counterpart with optimized *in vivo* properties. Fast and quantitative radiochemical conversion was achieved in the ^{68}Ga -radiolabeling of DO3A.Glu(Pyr).FAPi and DOTAGA.Glu(Pyr).FAPi. These complexes were also very stable in human serum (HS) and phosphate-buffered saline (PBS) over two hours and showed high hydrophilicity. Very high affinity for FAP (subnanomolar IC_{50} values) and very high selectivity over the related proteases PREP and DPPs was found for all precursors as well as the gallium complexes, especially the DO3A and DOTAGA derivatives.

First proof-of-concept patient studies of one breast cancer and one thyroid cancer patient with the [^{68}Ga]Ga-DO3A.Glu(Pyr).FAPi and [^{68}Ga]Ga-DOTAGA.Glu(Pyr).FAPi did show significant uptake in the tumor lesions via PET/CT 60 min post-injection accompanied by some physiological uptake in the pancreas. DO3A additionally showed fast clearance from blood, yet slight liver uptake was also observed, whereas DOTAGA showed almost no liver uptake but was not completely cleared from the blood. Further studies are needed to evaluate biodistribution in comparison to [^{68}Ga]Ga-DOTA.SA.FAPi and [^{68}Ga]Ga- and [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ to compare these different radiotracers with regard to optimizing PET/CT-guided ^{177}Lu -FAPi dimer radioligand therapy.

Keywords

Positron emission tomography (PET); fibroblast activation protein alpha (FAP); cancer associated fibroblasts (CAF); FAP inhibitors (FAPi); FAPi-based radiotracers; FAPi-PET imaging; gallium-68; ^{68}Ga ; tumor microenvironment (TME); radionuclide therapy (RNT); radioligand therapy (RLT); theranostics

1. Introduction

The tumor microenvironment (TME), or also called tumor stroma, and its different components like activated fibroblasts, inflammatory and immune cells as well as vascular cells and extracellular matrix (ECM) can make up for the majority of the total tumor mass (up to 90%) [1,2]. The TME is formed by every epithelial lesion of a certain size (≥ 1 mm). It is essential for tumor supply and contributes to tumor growth, invasion, metastasis and angiogenesis [1–3]. Cancer-associated fibroblasts (CAFs) are an important component of the TME and are activated mainly by the tumor growth factor TGF- β 1 through mesenchymal-mesenchymal transition (MMT) [4–6]. In turn, CAFs promote epithelial-mesenchymal transition (EMT) and also produce a variety of cytokines, growth factors and enzymes like matrix metalloproteinases (MMPs) that ultimately lead to tumor cell proliferation, angiogenesis, strengthening of apoptosis resistance of tumor cells during invasion into new tissues (metastasis) and ECM remodeling [5–7]. In total, the two-way interaction between tumor cells and CAFs enhances tumor growth and increases therapy resistance [5].

Fibroblast activation protein alpha (FAP, Seprase) is a type II integral membrane glycoprotein that belongs to the S9b peptidase family like the closely related dipeptidyl peptidase 4 (DPP4). In its active form FAP is a serine protease and 170 kDa homodimer. It cleaves peptides after proline units and has dipeptidyl peptidase activity and is a more efficient endopeptidase on top of that [3,8,9]. In contrast, the related DPPs and prolyl endopeptidase (PREP), which is the only other proline-selective endopeptidase, only have one of these enzymatic activities. FAP has the highest endopeptidase efficiency for cleaving peptides after a Gly-Pro units [8]. The role of FAP *in vivo* is not fully understood and the research on its functionalities is still ongoing. Several natural substrates have been identified (neuropeptides, α_2 -antiplasmin and type I collagen) [3,10–13]. Type I collagen, a major component of the ECM, is especially interesting as FAP seems to play a role in ECM remodeling that is also known from the CAFs that are overexpressing FAP [3,14–16]. The influence of FAP expression on tumor growth (tumorigenesis, cell proliferation, metastasis, angiogenesis etc.) was investigated in numerous studies regarding different cancer types [17–22]. High FAP expression is found in around 90% of epithelial tumors such as lung, breast, colon, gastric, ovarian, prostate cancer and others [1,23]. It is also expressed by activated fibroblasts at sites of inflammation, during wound healing and in fibrosis (liver cirrhosis, arthritis etc.) but is absent in most adult, healthy tissue [3,11,24,25].

The selective expression pattern makes FAP an interesting pan-tumor target for potentially broad application in nuclear medicine. The foundation was laid with the works from JANSEN et al. including UAMC-1110 (see Figure 1a), the first FAP inhibitor that showed high affinity for FAP and simultaneously high selectivity over all the other competitive proteases DPP4, DPP8, DPP9 and PREP [26,27].

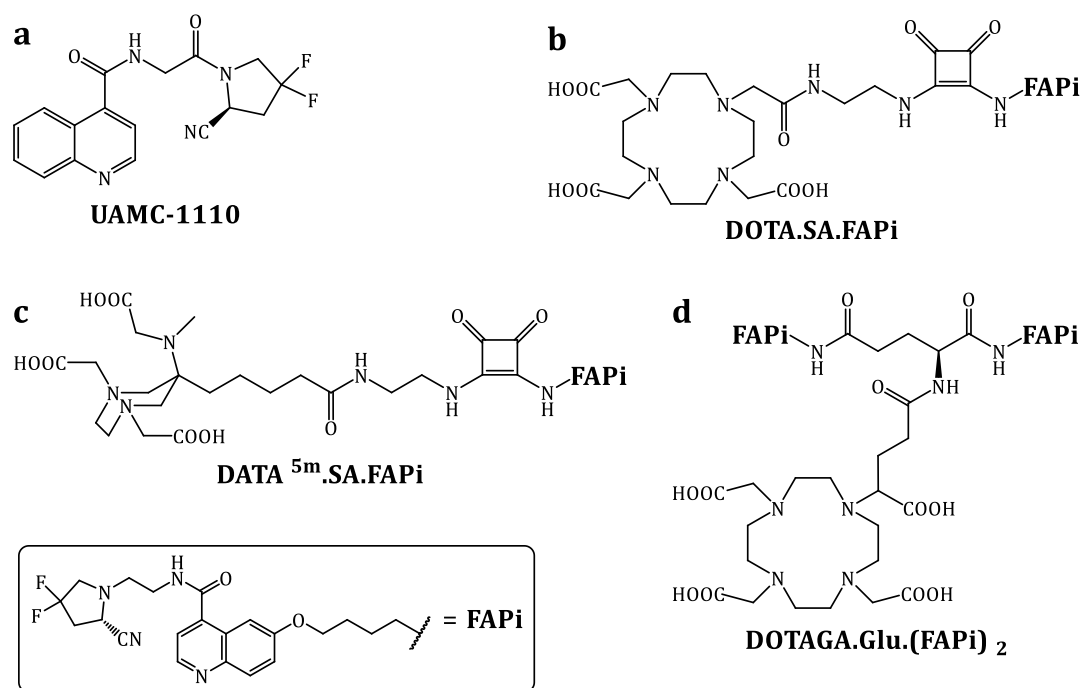


Figure 1: Structures of the FAP inhibitors a) UAMC-1110, b) DOTA.SA.FAPi, c) DATA^{5m}.SA.FAPi and d) DOTAGA.Glu.(FAPi)₂.

The clinical application of FAPi-based radiotracers was pioneered by the group of HABERKORN from the German Cancer Research Centre in Heidelberg with FAPI-04 and FAPI-46 being leading compounds among a series of nitrogen-containing spacer/linker motifs for radiolabeling with the positron emitter gallium-68 [28,29]. DOTA.SA.FAPi and DATA^{5m}.SA.FAPi (see Figure 1b and c) by MOON et al. [30] introduced squaramide as a linker unit. All these compounds and several others (QCP02 [31] or OncoFAP-DOTAGA [32]) containing macrocyclic or hybrid chelators and designed to be radiolabeled with trivalent radiometals. They are particularly suitable for positron emission tomography (PET) with gallium-68 as they showed fast accumulation in various tumor types and renal clearance which resulted in high-contrast PET images at early time points, e.g. 10 minutes post-injection (p.i.) [28,29,33–36]. Extensive patient studies have been carried out over the last years, demonstrating that FAP imaging using FAPi-based radiotracers represent extra value in addition to other standard of care diagnostics such as the most used PET radiotracer [¹⁸F]F-FDG, and may be even superior to [¹⁸F]F-FDG in selected cancer types [35,37–42]. FAPi-PET/CT imaging might even be beneficial compared to MRI or CT for certain indications, but much more comparative clinical data are needed. More FAPi-based tracers have also been synthesized like [¹⁸F]AlF-FAPi-74 [43,44] and [¹⁸F]FGlc-FAPi [45] for PET or [^{99m}Tc]Tc-FAPi-34 [46] and [^{99m}Tc]Tc-HFAPi [47] for SPECT.

While the fast pharmacokinetics are desirable for PET imaging since it allows scanning the patient shortly after administration of the radiotracer, the FAPi monomers all suffer from short tumor residence time. Therapeutic trivalent radiometals all have physical half-lives of several days (¹⁷⁷Lu, ²²⁵Ac, ⁹⁰Y) and the biological half-life of the radiopharmaceutical has to match this for an

optimized treatment response. The tumor retention could be significantly improved with FAPi (homo)dimers. The molecular design of these dimers includes two identical FAPi-targeting vectors that are based on UAMC-1110. The original linear FAPi dimer DOTAGA.(SA.FAPi)₂ showed substantially longer tumor retention of at least one week when labeled with lutetium-177 [48–50] the pharmacologic properties could be improved with DOTAGA.Glu.(FAPi)₂ (see Figure 1d) [51]. The faster pharmacokinetic profile of [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ compared to the rather slow hepatobiliary excretion of [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂ led to a reduced radiation dose to the critical organs (colon) [51]. [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂ based radioligand therapy (RLT) has already shown to be well tolerated by patients [50] but this might be even improved with [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂.

While the promising pharmacokinetic profile of FAPi homodimers such as DOTAGA.(SA.FAPi)₂ and DOTAGA.Glu.(FAPi)₂ when labeled with ¹⁷⁷Lu or ²²⁵Ac appears to be extremely promising in the context of RLT, the homodimers are not optimal for ⁶⁸Ga-PET imaging at early time points. There are several FAPi monomers available which perform well in ⁶⁸Ga-PET/CT, including DOTA.SA.FAPi or DATA^{5m}.SA.FAPi. But [⁶⁸Ga]Ga-DOTA.SA.FAPi or [⁶⁸Ga]Ga-DATA^{5m}.SA.FAPi do not appear optimal as diagnostic counterparts for [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ because they have significant structural differences. Thus, new monomeric FAP ligands are supposed to be as similar as possible structurally to the therapeutic [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂.

The new FAPi monomers share the same branched structural design of the homodimer DOTAGA.Glu.(FAPi)₂ with glutamic acid (Glu) as a central linker unit. One of the two FAPi targeting vectors of the homodimer is replaced with a pyrrolidine moiety. This was chosen to affect the properties of the molecule as little as possible. On the other hand, other FAPi-based radiotracers with similar nitrogen-containing heterocyclic groups have already been used and showed promising properties [28,29,52]. Three different chelators DOTAGA, DO3A and DATA^{5m} were chosen and the respective precursor are shown in Figure 2.

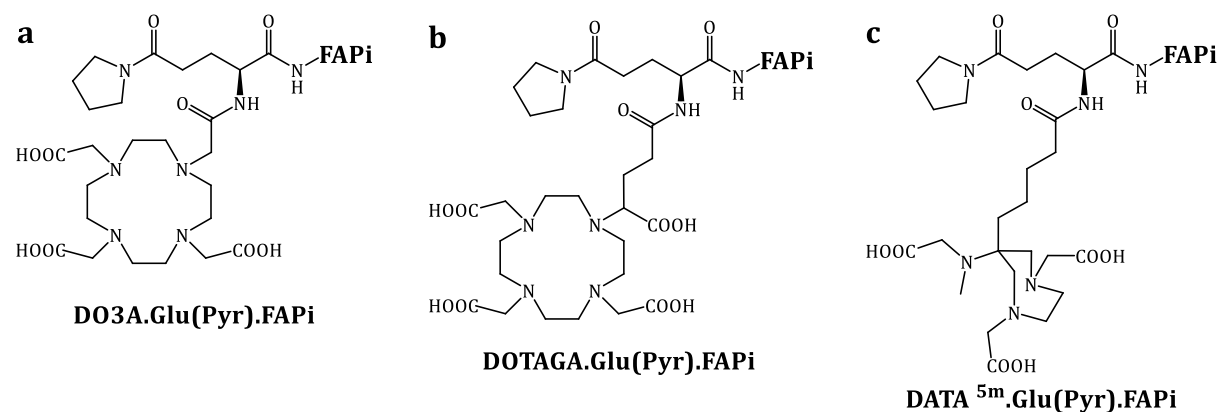


Figure 2: Structures of the new branched FAPi monomers a) DO3A.Glu(Pyr).FAPi, b) DOTAGA.Glu(Pyr).FAPi and c) DATA^{5m}.Glu(Pyr).FAPi.

The new radiotracers like [^{68}Ga]Ga-DOTAGA.Glu(Pyr).FAPi are supposed to enable a more accurate therapy control of the homodimeric FAPi-based RLT using [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ via FAPi monomer-guided ^{68}Ga -PET/CT pre- and post-treatment. After organic synthesis the radiolabeling properties (reaction kinetics) with gallium-68, the complex stability and lipophilicity of the radiolabeled complex were investigated for each compound. Further, IC_{50} values were determined for FAP and the related proteases DPP4, 8, 9 and PREP to quantify *in vitro* affinity and selectivity.

Furthermore, first patients that are considered for treatment with [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ underwent proof-of concept PET/CT scans with the two of the new ^{68}Ga -labeled Glu-monomers [^{68}Ga]Ga-DO3A- and [^{68}Ga]Ga-DOTAGA.Glu(Pyr).FAPi.

2. Materials and Methods

2.1 Chemicals and Equipment

All basic chemicals were purchased from Sigma-Aldrich and Merck (Darmstadt, Germany), TCI (Eschborn, Germany), ABCR (Karlsruhe, Germany), Alfa Aesar by Thermo Fisher Scientific (Kandel, Germany), Acros Organics (Schwerte, Germany) and VWR (Bruchsal, Germany). Dry solvents were bought from Acros Organics, Alfa Aesar by Thermo Fischer Scientific and Merck and deuterated solvents from Deutero (Kastellaun, Germany). The chelators DOTA-tri(^tBu)ester (2-(4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)acetic acid) and DOTA-GA(^tBu) $_4$ (5-(*tert*-butoxy)-5-oxo-4-(4,7,10-tris(2-(*tert*-butoxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecan-1-yl)pentanoic acid) were obtained from CheMatech (Dijon, France) and DATA ^{5m} (^tBu) $_3$ -COOH (5-(1,4-bis(2-(*tert*-butoxy)-2-oxoethyl)-6-((2-(*tert*-butoxy)-2-oxoethyl)(methyl)amino)-1,4-diazepan-6-yl)pentanoic acid) from ChiroBlock GmbH (Wolfen, Germany). (*S*)-6-(4-Aminobutoxy)-*N*-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide (FAPi-NH $_2$ *TFA) was purchased from KE Biochem Co. (Shanghai, China).

Thin-layer chromatography (TLC) plates coated with silica gel 60 F $_{254}$ from Merck were used. For analysis they were investigated via UV lamp (λ = 254 and 366 nm) as well as staining with potassium permanganate. Silica 60 (0.040-0.063 mm particle size) was used for column chromatography.

Nuclear magnetic resonance (NMR) spectra were recorded in deuterated solvents on an Avance II 400 spectrometer (400 MHz, 5 mm BBFO sample head with z-gradient and ATM, SampleXPress 60 sample changer) from Bruker (Rheinstätten, Germany). MestReNova 14.2.0 software from Mestrelab Research (Santiago de Compostela, Spain) was used to analyze the spectra.

Liquid chromatography coupled with electrospray ionization mass spectrometry (ESI-LC/MS) was performed using a LC/MS system from Shimadzu (Japan) with CBM-20A system controller, SIL-20A HT autosampler, two LC-20AD pumps, CTO-20 AC column oven, SPD-M20A photodiode array detector and a LCMS-2020 mass spectrometer. The separation was realized with a Eurosphere II 100-5 C18 column (150x4 mm) from Knauer (Berlin, Germany) and a linear gradient of acetonitrile (MeCN)/H₂O (HPLC grade) + 0.1% formic acid.

Semi-preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) was performed using LaChrom HPLC (7000 series) from Merck Hitachi with L-7100 pump, L-7400 UV detector (λ = 254 nm), a D-7000 interface and autosampler. Separation was performed using a Phenomenex (Aschaffenburg, Germany) Synergi Max-RP C18 column (250x10 mm, 4 μ m) and linear gradient of MeCN (HPLC)/H₂O (Milli Q®) + 0.1% trifluoacetic acid (TFA) and a flow rate of 5 mL/min.

For radiolabeling only chemicals of highest purity (TraceSelect if available) were used. Radio-TLCs were developed using 0.25 M citrate buffer (pH = 4.0). Additional TLCs were developed using 1 M ammonium acetate (AmOAc, pH = 4.0)/methanol (MeOH) (1:1). All radio-TLCs were evaluated using a CR-35 Bio Test-Imager from Duerr-ndt (Bietigheim-Bissingen, Germany) and AIDA software from Elysia-Raytest (Straubenhardt, Germany). Analytical radio-HPLC was performed also using a Merck Hitachi LaChrom HPLC (7000 series). Separation was performed using Phenomenex Luna C18 column (250x4.6 mm, 5 μ m) and linear gradient of MeCN/H₂O + 0.1% TFA and flow rate of 1 mL/min. The radio-HPLC is equipped with a Ramona analog radio detector from Elysia-Raytest with its energy window being set to 100-1200 keV for gallium-68.

2.2 Organic Synthesis

2.2.1. Synthesis of Precursors

Benzyl (S)-2-((tert-butoxycarbonyl)amino)-5-oxo-5-(pyrrolidin-1-yl)pentanoate (3, Boc-Glu(Pyr)-OBzl)

1-Benzyl *N*-Boc-L-glutamate (Boc-Glu-OBzl **1**, 1.00 g, 2.96 mmol, 1.00 eq) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 1.24 g, 3.26 mmol, 1.10 eq) were dissolved in dry dichloromethane (DCM, 15 mL). *N,N*-diisopropylethylamine (DIPEA, 1.51 mL, 8.88 mmol, 3.00 eq) was added and stirred under argon atmosphere at room temperature (RT) for 30 minutes. Then, pyrrolidine **2** (267 μ L, 3.26 mmol, 1.10 eq) was added and stirring at RT

continued overnight. 25 mL of water was added and the organic phase was separated. The aqueous phase was extracted with DCM (5x 25 mL), dried over MgSO₄ and concentrated *in vacuo*. Boc-Glu(Pyr)-OBzl **3** (1.04 g, 2.67 mmol, 90%) was obtained as a colorless solid after column chromatography (cyclohexane/ethyl acetate (CH/EA), 1:1-1:4). ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 7.40 – 7.28 (m, 5H), 5.52 (s, 1H), 5.26 – 5.07 (m, 2H), 4.33 (s, 1H), 3.56 – 3.16 (m, 4H), 2.39 – 1.76 (m, 8H), 1.42 (s, 9H). ¹³C-NMR (400 MHz, CDCl₃): δ [ppm] = 172.3, 170.5, 155.6, 135.5, 128.6, 128.4, 79.8, 67.0, 53.5, 46.5, 45.9, 30.6, 28.3, 27.2, 26.0, 24.4. LC-MS (ESI⁺): m/z (%) = 290.9 (28, [M–Boc+H]⁺), 391.1 (100, [M+H]⁺), 392.1 (23, [M+H]⁺), calculated for C₂₁H₃₀N₂O₅: 390.22 [M].

(S)-2-((*tert*-Butoxycarbonyl)amino)-5-oxo-5-(pyrrolidin-1-yl)pentanoic acid (**4**, Boc-Glu(Pyr)-OH)

A solution of Boc-Glu(Pyr)-OBzl **3** (1.03 g, 2.64 mmol, 1.00 eq) and palladium on carbon (Pd/C, 10%wt, 56.8 mg, 53.0 μmol, 0.02 eq) in dry tetrahydrofuran (THF, 10 mL) was stirred under hydrogen atmosphere for three days. It was then filtered through Celite® and washed with THF and methanol (MeOH). The solvents were completely removed *in vacuo*. A colorless oil (787 mg, 2.62 mmol, 99%) was obtained. ¹H-NMR (400 MHz, MeOD): δ [ppm] = 4.12 (dd, *J* = 9.2, 4.8 Hz, 1H), 3.48 (t, *J* = 6.8 Hz, 2H), 3.41 (td, *J* = 6.8, 1.8 Hz, 2H), 2.56 – 2.34 (m, 2H), 2.31 – 2.11 (m, 1H), 2.02 – 1.84 (m, 5H), 1.44 (s, 9H). LC-MS (ESI⁺): m/z (%) = 300.9 (100, [M+H]⁺), 302.0 (14, [M+H]⁺), 623.35 (14, [2M+Na]⁺), calculated for C₁₄H₂₄N₂O₅: 300.17 [M].

tert-Butyl ((*S*)-1-((4-((4-((2-((*S*)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-1,5-dioxo-5-(pyrrolidin-1-yl)pentan-2-yl)carbamate (**5**, Boc-Glu(Pyr).FAPi)

Boc-Glu(Pyr)-OH **4** (270 mg, 900 μmol, 1.52 eq) and 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU, 371 mg, 975 μmol, 1.64 eq) was dissolved in dry *N,N*-dimethylformamide (DMF, 4 mL) DIPEA (127.5 μL, 750 μmol, 1.89 eq) was added. After stirring for 45 minutes at 30 °C under argon atmosphere, a solution of FAPi-NH₂*TFA (324 mg, 594 μmol, 1.00 eq) and DIPEA (127.5 μL, 750 μmol, 1.89 eq) in dry DMF (5 mL) was added and stirring was continued at 30 °C for one day. The solvent was removed *in vacuo* and subsequent column chromatography (CHCl₃/MeOH, 100:4) gave Boc-Glu(Pyr).FAPi **5** (406 mg, 569 μmol, 96%) as a yellow solid. ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 8.76 (d, *J* = 4.6 Hz, 1H), 8.68 (dd, *J* = 4.4, 1.4 Hz, 1H), 8.15 (d, *J* = 9.3 Hz, 1H), 7.79 (s, 1H), 7.52 (d, *J* = 4.6 Hz, 1H), 7.42 (dd, *J* = 9.2, 2.6 Hz, 1H), 7.08 (s, 1H), 5.67 (d, *J* = 8.1 Hz, 1H), 5.12 (d, *J* = 8.0 Hz, 1H), 4.59 – 3.97 (m, 7H), 3.50 – 3.14 (m, 6H), 2.96 – 2.76 (m, 2H), 2.32 – 1.62 (m, 12H), 1.13 (s, 9H). LC-MS (ESI⁺): m/z (%) = 357.6 (97, [M+H]²⁺), 614.3 (23, [M–Boc+H]⁺), 714.3 (100, [M+H]⁺), 715.4 (35, [M+H]⁺), calculated for C₃₅H₄₅F₂N₇O₇: 713.33 [M].

6-(4-((*S*)-2-Amino-5-oxo-5-(pyrrolidin-1-yl)pentanamido)butoxy)-*N*-(2-((*S*)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide (**6**, Glu(Pyr).FAPi)

Boc-Glu(Pyr).FAPi **5** (393 mg, 550 μ mol) were dissolved in dry MeCN (3 mL) and TFA (1 mL). The reaction solution was stirred at RT for 6 hours. MeOH (10 mL) was added and the solvents were removed *in vacuo*. This step was repeated two more times. A yellow oil was obtained which was used without any further purification. $^1\text{H-NMR}$ (400 MHz, MeOD): δ [ppm] = 8.96 (d, J = 5.1 Hz, 1H), 8.17 (d, J = 2.7 Hz, 1H), 8.12 (d, J = 9.3 Hz, 1H), 7.88 (d, J = 5.1 Hz, 1H), 7.69 (dd, J = 9.3, 2.7 Hz, 1H), 5.17 (dd, J = 9.4, 3.1 Hz, 1H), 4.41 – 4.08 (m, 6H), 3.90 (t, J = 6.1 Hz, 1H), 3.47 – 3.34 (m, 6H), 2.52 (t, J = 7.0 Hz, 2H), 2.16 – 1.78 (m, 12H). LC-MS (ESI⁺): m/z (%) = 307.5 (100, $[\text{M}+\text{H}]^{2+}$), 614.4 (11, $[\text{M}+\text{H}]^+$), calculated for $\text{C}_{30}\text{H}_{37}\text{F}_2\text{N}_7\text{O}_5$: 613.28 [M].

tri-*tert*-Butyl 2,2',2''-(10-(2-((2,5-dioxopyrrolidin-1-yl)oxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetate (**8**, DOTA(*t*Bu)₃-NHS)

DOTA-tris(*tert*-butyl ester) **7** (573 mg, 1.00 mmol, 1.00 eq) and HBTU (417 mg, 1.10 mmol, 1.10 eq) were dissolved in dry acetonitrile (12 mL) and dry DMF (5 mL) and then *N*-hydroxysuccinimide (NHS, 127 mg, 1.10 mmol, 1.10 eq) was added under argon atmosphere. After stirring at 30 °C for 6 hours, HBTU (190 mg, 501 μ mol, 0.50 eq) and NHS (57.5 mg, 500 μ mol, 0.50 eq) was added again and stirring continued overnight. All solvents were removed *in vacuo* and subsequent purification by column chromatography (dichloromethane (DCM)/MeOH, 10:1) gave **8** as a colorless solid (643 mg, 961 μ mol, 96%). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ [ppm] = 2.97 – 2.77 (m, 12H), 2.66 – 2.02 (m, 16H), 1.44 (s, 27H). MS (ESI⁺): m/z (%) = 335.7 (100, $[\text{M}+\text{H}]^{2+}$), 670.4 (50, $[\text{M}+\text{H}]^+$), 671.4 (18, $[\text{M}+\text{H}]^+$), calculated for $\text{C}_{32}\text{H}_{55}\text{N}_5\text{O}_{10}$: 669.39 [M].

2,2',2''-(10-(2-(((*S*)-1-((4-((2-((*S*)-2-Cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-1,5-dioxo-5-(pyrrolidin-1-yl)pentan-2-yl)amino)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (**9**, DO3A.Glu(Pyr).FAPi)

DOTA(*t*Bu)₃-NHS **8** (100 mg, 149 μ mol, 1.49 eq) was dissolved together with Glu(Pyr).FAPi **6** (61.3 mg, 100 μ mol, 1.00 eq) in dry DMF (1 mL) and DIPEA (50 μ L) was added. It was stirred at 40 °C under argon atmosphere for one day and then the solvent was completely removed.

A yellow oil was obtained to which 25 μ L of Milli-Q® water, 50 μ L of triisopropylsilane (TIPS), 100 μ L of acetonitrile and 1 mL of TFA was added (TFA/MeCN/TIPS/H₂O, 100:10:5:2.5) and the solution was stirred at RT for 14 hours. MeOH (10 mL) was added and the solvents were removed again *in vacuo*. This step was repeated two more times. The crude product was purified by semi-preparative RP-HPLC (16.5–18.0% MeCN in 20 min, t_R = 16–17 min). DO3A.Glu(Pyr).FAPi **9** (39. mg, 39.0 μ mol, 39%) was obtained as a yellow solid (99.9% purity: cf. analytical RP-HPLC in Figure S1). LC-MS (ESI⁺): m/z (%) = 334.1 (100, $[\text{M}+\text{H}]^{3+}$), 347.7 (17, $[\text{M}+\text{MeCN}+\text{H}]^{3+}$), 500.8 (45, $[\text{M}+\text{H}]^{2+}$), 1000.5 (6, $[\text{M}+\text{H}]^+$), calculated for $\text{C}_{46}\text{H}_{63}\text{F}_2\text{N}_{11}\text{O}_{12}$: 999.46 [M].

2,2',2''-(10-(1-Carboxy-4-(((S)-1-((4-((4-((2-((S)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl) carbamoyl)quinolin-6-yl)oxy)butyl)amino)-1,5-dioxo-5-(pyrrolidin-1-yl)pentan-2-yl)amino)-4-oxobutyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl)triacetic acid (**11**, DOTAGA.Glu(Pyr).FAPi) DOTAGA(^tBu)₄ **10** (138 mg, 197 μmol, 1.43 eq), HATU (82.5 mg, 217 μmol, 1.57 eq) and DIPEA (31.7 μL, 238 μmol, 1.72 eq) were dissolved in dry DMF (2 mL) under argon atmosphere and stirred at 30 °C for 90 min. A solution of Glu(Pyr).FAPi **6** (84.7 mg, 138 μmol, 1.00 eq) and DIPEA (46.8 μL, 275 μmol, 2.00 eq) in dry DMF (1 mL) was added. It was stirred at 30 °C overnight, then more HATU (52.5 mg, 138 μmol, 1.00 eq) was added. After another 6 hours at 30 °C the solvent was removed *in vacuo* and used without further purification.

25 μL of Milli-Q® water, 50 μL of TIPS, 100 μL of acetonitrile and 1 mL of TFA were added to the yellow oil (TFA/MeCN/TIPS/H₂O, 100:10:5:2.5) and stirred at RT for two days. After co-distillation with MeOH (5x10 mL) and evaporation to dryness the crude product was purified by semipreparative RP-HPLC (18–20% MeCN in 20 min, *t_R* = 12–13 min) and DOTAGA.Glu(Pyr).FAPi **11** (53.0 mg, 49.4 μmol, 36%) was obtained as a yellow solid (99.1% purity: cf. analytical RP-HPLC in Figure S2Figure S). LC-MS (ESI⁺): *m/z* (%) = 358.1 (100, [M+H]³⁺), 371.8 (12, [M+MeCN+H]³⁺), 536.9 (33, [M+H]²⁺), 1072.5 (4, [M+H]⁺), calculated for C₄₉H₆₇F₂N₁₁O₁₄: 1071.48 [M].

2,2'-(6-(((Carboxymethyl)(methyl)amino)-6-(5-(((S)-1-((4-((4-((2-((S)-2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-1,5-dioxo-5-(pyrrolidin-1-yl)pentan-2-yl)amino)-5-oxopentyl)-1,4-diazepane-1,4-diyl)diacetic acid (**13**, DATA^{5m}.Glu(Pyr).FAPi) DATA^{5m}(^tBu)₃-COOH **12** (75.0 mg, 131 μmol, 1.31 eq) and HATU (57.0 mg, 150 μmol, 1.50 eq) were dissolved in dry DMF (1 mL) and DIPEA (29.8 μL, 175 μmol, 1.75 eq) was added. After 45 minutes under argon atmosphere and at 30 °C, a solution of Glu(Pyr).FAPi **6** (61.3 mg, 100 μmol, 1.00 eq) and DIPEA (34.0 μL, 200 μmol, 2.00 eq) in dry DMF (1 mL) was added. After stirring at 30 °C overnight, HATU (19.0 mg, 50.0 μmol, 0.50 eq) was added and after another 4 hours at 30 °C the solvent was removed completely.

A yellow oil was obtained and used directly used in the next step by adding 25 μL of Milli-Q® water, 50 μL of TIPS, 100 μL of ACN, and 1 mL of TFA (TFA/MeCN/TIPS/H₂O, 100:10:5:2.5) and stirring for 14 hours at RT. MeOH (10 mL) was added and the solvents were removed *in vacuo*. This step was repeated two more times. The crude product was purified by semi-preparative RP-HPLC (18–20% MeCN in 20 min, *t_R* = 15–16 min). DATA^{5m}.Glu(Pyr).FAPi **13** (36.7 mg, 36.7 μmol, 37%) was obtained as a yellow solid (98.6% purity: cf. analytical RP-HPLC in Figure S3). LC-MS (ESI⁺): *m/z* (%) = 333.8 (66, [M+H]³⁺), 500.3 (100, [M+H]²⁺), 999.8 (11, [M+H]⁺), calculated for C₄₇H₆₄F₂N₁₀O₁₂: 998.47 [M].

2.2.2. Synthesis of ^{nat}Ga-Complexes

[^{nat}Ga]Ga-DO3A.Glu(Pyr).FAPi (^{nat}Ga-9)

DO3A.Glu(Pyr).FAPi **9** (10.0 mg, 10.0 μmol, 1.00 eq) was dissolved in 500 μL 1 M AmOAc (pH = 5.5). A solution of 200 μL 0.1 M Ga(NO₃)₃ (20.0 μmol, 2.00 eq) in 1 M AmOAc (pH = 5.5) was added and shaken at 90 °C for 16 hours. Subsequent semi-preparative RP-HPLC (20–24% MeCN in 10 min, *t_R* = 8.0–8.5 min) yielded [^{nat}Ga]Ga-DO3A.Glu(Pyr).FAPi as a yellow solid (7.0 mg, 6.6 μmol, 66%). MS (ESI⁺): *m/z* (%) = 534.1 (100, [M+H]²⁺), 534.6 (18, [M+H]²⁺), calculated for C₄₆H₆₀F₂GaN₁₁O₁₂: 1065.37 [M].

[^{nat}Ga]Ga-DOTAGA.Glu(Pyr).FAPi (^{nat}Ga-11)

DOTAGA.Glu(Pyr).FAPi **11** (10.7 mg, 10.0 μmol, 1.00 eq) was dissolved in 500 μL 1 M AmOAc (pH = 5.5), 200 μL Milli-Q® water and 200 μL EtOH. A solution of 200 μL 0.1 M Ga(NO₃)₃ (20.0 μmol, 2.00 eq) in 1 M AmOAc (pH = 5.5) was added and shaken at 90 °C for 6 hours. Subsequent semi-preparative RP-HPLC (20–24% MeCN in 10 min, *t_R* = 8.0–8.5 min) yielded [^{nat}Ga]Ga-DOTAGA.Glu(Pyr).FAPi as a yellow solid (7.2 mg, 6.3 μmol, 63%). MS (ESI⁺): *m/z* (%) = 570.1 (100, [M+H]²⁺), 570.6 (18, [M+H]²⁺), calculated for C₄₉H₆₄F₂GaN₁₁O₁₄: 1137.39 [M].

[^{nat}Ga]Ga-DATA^{5m}.Glu(Pyr).FAPi (^{nat}Ga-13)

DATA^{5m}.Glu(Pyr).FAPi **13** (10.0 mg, 10.0 μmol, 1.00 eq) was dissolved in 500 μL of 1 M AmOAc buffer (pH = 5.5) and 100 μL EtOH. A solution of 200 μL 0.1 M Ga(NO₃)₃ (20.0 μmol, 2.00 eq) in 1 M AmOAc (pH = 5.5) was added and shaken at 90 °C for 6 hours. Subsequent semi-preparative RP-HPLC (20–24% MeCN in 10 min, *t_R* = 9.0–9.5 min) yielded [^{nat}Ga]Ga-DATA^{5m}.Glu(Pyr).FAPi as a yellow solid (7.1 mg, 6.7 μmol, 67%). MS (ESI⁺): *m/z* (%) = 533.6 (100, [M+H]²⁺), 534.1 (18, [M+H]²⁺), 1065.6 (7, [M+H]⁺), 1067.2 (5, [M+H]⁺), calculated for C₄₇H₆₁F₂GaN₁₀O₁₂: 1064.37 [M].

2.3. Radiosynthesis

2.3.1. ⁶⁸Ga-Radiolabeling

Gallium-68 was eluted from a ITG (Isotope Technologies Garching, Germany) ⁶⁸Ge/⁶⁸Ga-generator with 5 mL of 0.05 M HCl (TraceSelect). Cationic post processing was carried out according to the procedure developed by EPPARD et al. [53]. Precursor **9**, **11** or **13** (2.5–10 nmol, 2.5–10 μL 1 mM stock solution) was added to 400 μL buffer. Three different buffer systems were used: 1 M 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES pH = 5.5). 1 M ammonium acetate (AmOAc, pH = 4.5) or 1 M sodium acetate (NaOAc, pH = 4.5). Finally, the generator eluate with ca. 50 MBq (range: 45–55 MBq) ⁶⁸Ga in 100 μL N5 solution (0.9 M HCl in 90% EtOH) was added.

The reaction solutions for $^{68}\text{Ga-9}$ and $^{68}\text{Ga-11}$ were heated at 95 °C whereas the labeling of the DATA^{5m} precursor $^{68}\text{Ga-13}$ was carried out at room temperature (25 °C). Reaction controls were taken at different time points to investigate reaction kinetics analyzed via radio-TLC with 0.25 M citrate buffer (pH = 4.0) as the mobile phase. Additional radio-TLCs (1 M AmOAc (pH = 4.0)/MeOH (1:1)) and radio-HPLC were carried out for the time point to control radiochemical conversion (RCC)/radiochemical purity (RCP).

2.3.2. Complex Stability Measurements

The product was purified via Sep-Pak® Light C18 cartridge after radiolabeling. The cartridge was conditioned with 2 mL ethanol and 5 mL Milli-Q® water. The respective reaction solution was passed through the cartridge and the reaction vial was rinsed with another 200 µL of buffer solution. This was also passed through the cartridge followed by 1 mL of TraceSelect water. The product was eluted with 1 mL ethanol/water (1:1).

The complex stability in human serum (HS) and phosphate-buffered saline (PBS) was investigated in triplicate each (n = 3) by incubating ca. 10 MBq of the labeled tracer in 0.5 mL HS and PBS (≈ 20 MBq/mL) at 37 °C for 120 min. The complex stability was determined via radio-TLC with 0.25 M citrate buffer (pH = 4.0) at different time points.

2.3.3. Determination of $\log D_{7.4}$ (Lipophilicity Measurement)

After radiolabeling was carried out (RCC and RCP > 95%), the $\log D_{7.4}$ value was determined by diluting 5–7 MBq of the respective labeled tracer solution to 700 µL with PBS (n = 4). 700 µL of 1-octanol was added to each sample and shaken vigorously for 1–2 min (1500 rpm) followed by centrifugation for 1–2 min. 400 µL of the organic and aqueous phase were separated into a new Eppendorf tube, respectively. Samples of 3 µL (PBS) or 6 µL (1-octanol) were pipetted onto a TLC plate. Another 300 µL PBS as well as 700 µL 1-octanol were added to the aqueous phase since most of the activity was in PBS. The procedure (shaking, centrifuging, separating, taking samples) was repeated three times in total. The TLC plate was imaged (exposure time: 5 min) and the integral of each spot (octanol phase: I_o , aqueous PBS phase: I_{PBS}) was determined. The $\log D_{7.4}$ value was calculated using equation (1):

$$\log D_{7.4} = \log \left(\frac{I_o}{2 \cdot I_{PBS}} \right) \quad \text{Equation (1)}$$

Only the values of the second and third extraction were considered in the calculation and the different volumes of $V_o = 6$ µL und $V_{PBS} = 3$ µL were taken into account.

2.4. In Vitro-Inhibition Assays (IC_{50} Measurements)

DPP8 (dipeptidyl peptidase VIII), DPP9 (dipeptidyl peptidase IX) and rhFAP (recombinant human fibroblast activation protein) were expressed and purified as described previously [54,55]. DPP4 was purified from human seminal plasma as described previously [56]. Human recombinant PREP (prolyl endopeptidase) was expressed and purified as described before [54].

IC_{50} values for FAP and PREP were determined as described before [55,57]. Z-Gly-Pro-7-amino-4-methylcoumarine (AMC, Bachem) was used as the substrate for IC_{50} (FAP) measurements at a final concentration of 50 μ M at pH = 8.0 in 0.05 M Tris-HCl buffer, 1 mg/mL BSA and 140 mM NaCl. N-succinyl-Gly-Pro-AMC (Bachem, 250 μ M) at pH = 7.4 in 0.1 M K-phosphate, 1 mM EDTA, 1 mM DTT and 1 mg/mL BSA buffer was used for PREP.

IC_{50} measurements for DPP4, DPP8 and DPP9 were performed as published before [57,58]. Ala-Pro-pNA was used as the substrate at the respective concentrations of 25 μ M (DPP4), 300 μ M (DPP8) and 150 μ M (DPP9) at pH = 7.4 in 0.05 M HEPES-NaOH buffer with 0.1% Tween-20, 0.1 mg/mL BSA and 150 mM NaCl.

All enzymatic activities were determined by pre-incubating the inhibitor sample with the enzyme for 15 minutes at 37 °C. Then the substrate was added and velocities of AMC release (λ_{ex} = 380 nm and λ_{em} = 465 nm) or pNA release (405 nm) were measured kinetically for at least 10 minutes. Measurements were performed on the Infinite 200 from Tecan Group Ltd. (Männedorf, Switzerland) and Magellan software was used to process the data. Data fitting was performed using a non-linear fitting model in Grafit 7.4. All IC_{50} measurements were carried out in triplicate with at least eight different inhibitor concentrations.

2.5. Patient Study

2.5.1. Radiosynthesis

The radiosynthesis procedure for the preparation of [^{68}Ga]Ga-DO3A.Glu(Pyr)FAPi and [^{68}Ga]Ga-DOTAGA.Glu(Pyr).FAPi was carried out as follows: a vial containing FAPi precursor (67.5 μ g/GBq) and 1 M ammonium acetate (pH = 5) was added to the gallium generator eluate solution ($\approx$ 925 MBq). The mixture was then heated at 95°C for 10 minutes. The resulting radiolabeled product was loaded onto a Sep-Pak C18 cartridge. 1 mL water was passed through the cartridge as a washing step. Subsequently, the product was eluted with 50% ethanol (1 mL) and diluted with saline (10 mL). To assess the quality of the radiolabeled product, two methods were employed: radio-HPLC and instant TLC using 0.1 M citrate buffer (pH = 4.0) as the mobile phase. RCP of the products was determined to be 95%, respectively, and RCY exceeded 95% (half-life-corrected).

2.5.2. Clinical Image Acquisition and Analysis

PET scans were conducted using a dedicated GE Discovery 710 PET/CT scanner with a 40 mm detector and a rotation speed of 0.35 sec. For each patient, a dose of 180 – 200 MBq of the [⁶⁸Ga]Ga-D03A.Glu(Pyr)FAPi or [⁶⁸Ga]Ga-DOTAGA.Glu(Pyr).FAPi was administered. Whole-body PET/CT studies were performed at one-hour post-injection. The acquisition protocol consisted of a scout image, followed by a CT scan, and PET acquisition with a duration of 2 minutes per bed position.

The CT scanner parameters included 300-350 mAs, 120kVp, a slice thickness of 5 mm, and a pitch of 1. The Carina landmark registration technique was utilized for the alignment and registration of PET/CT scans. Two nuclear medicine specialists independently interpreted the scans, with a third physician involved in case of any disagreement in the case reports. Regions of interest (ROIs) based on the PET response criteria in solid tumors (PERCIST 1.0) were delineated for comparison purposes.

Table 1 provides an overview of the clinical details of the different patients involved in the study.

Table 1: Clinical details of the 3 patients that underwent ⁶⁸Ga-FAPi PET/CT imaging.

Patient No.	Age/ Gender	Diagnosis	Histopathology	Prior treatments	FAPi radiotracer
1	70/F	Invasive ductal carcinoma	ER+ PR+ Her2Neu +	Left breast MRM, hormonal therapy, chemotherapy	[⁶⁸ Ga]Ga-D03A.Glu. (Pyr).FAPi
2	19/F	Thyroid cancer	Medullary thyroid cancer Synaptophysin+, CgA+	Total thyroidectomy	[⁶⁸ Ga]Ga-DOTAGA. Glu.(Pyr).FAPi

ER: estrogen receptor, PR: progesterone receptor, HER2/neu: human epidermal growth factor receptor-2, LAR: long-acting, WHO world health organization, CgA chromogranin A.

The clinical investigations of this study were approved by the Ethics Committee of the All India Institute of Medical Sciences (IECPG-22, 27 February 2020), and written informed consent was obtained from all participating patients.

3. Results and Discussion

3.1. Organic Synthesis

The first four steps of the organic synthesis are identical for all three precursors DO3A.Glu(Pyr).FAPi **9**, DOTAGA.Glu(Pyr).FAPi **11** and DATA^{5m}.Glu(Pyr).FAPi **13** and started from Boc-Glu-OBzl **1** to obtain the intermediate Glu(Pyr).FAPi **6** as shown in Figure 3.

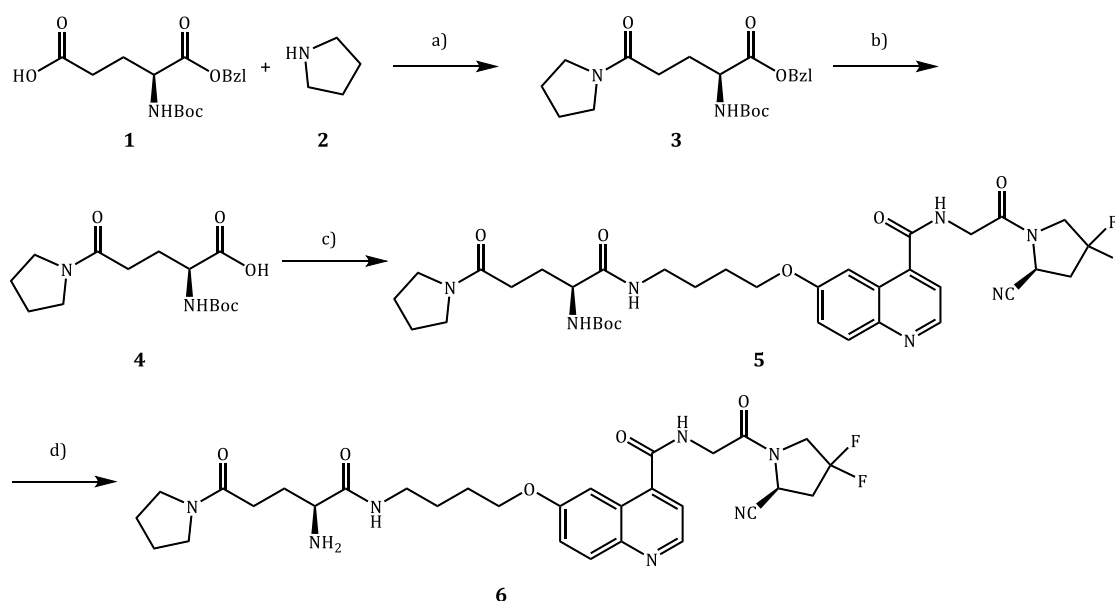


Figure 3: Synthesis scheme of Glu(Pyr).FAPi **6**: a) 1. **1**, HBTU, DIPEA, DMF, RT, 30 min; 2. **2**, DIPEA, DMF, RT, 1 d, 90%; b) H₂, Pd/C, THF, RT, 3 d, 99%; c) 1. HATU, DIPEA, DMF, 30 °C, 45 min; 2. **FAPi-NH₂*TFA**, DIPEA, DMF, 30 °C, 3 d, 96%; d) TFA/MeCN (1:3), RT, 6 h, 100%.

At first, **1** and pyrrolidine **2** were coupled with HBTU in the presence of DIPEA in dry DMF to obtain Boc-Glu(Pyr)-OBzl **3**. Subsequent reductive cleavage of the benzyl ester under hydrogen atmosphere (atmospheric pressure) and with palladium on carbon as catalyst yielded Boc-Glu(Pyr)-OH **4**. This was followed by coupling to FAPi-NH₂ in dry DMF, with HATU as coupling reagent and DIPEA as base to obtain Boc-Glu(Pyr).FAPi **5**. The Boc-protective group was cleaved with a 25 %v/v of trifluoroacetic acid (TFA) in dry acetonitrile. All reactions gave very high to quantitative yields (90–100%) and the overall yield was 86% over 4 steps.

The final precursors **9**, **11** and **13** were obtained from Glu(Pyr).FAPi **6** according to the synthesis scheme in Figure 4.

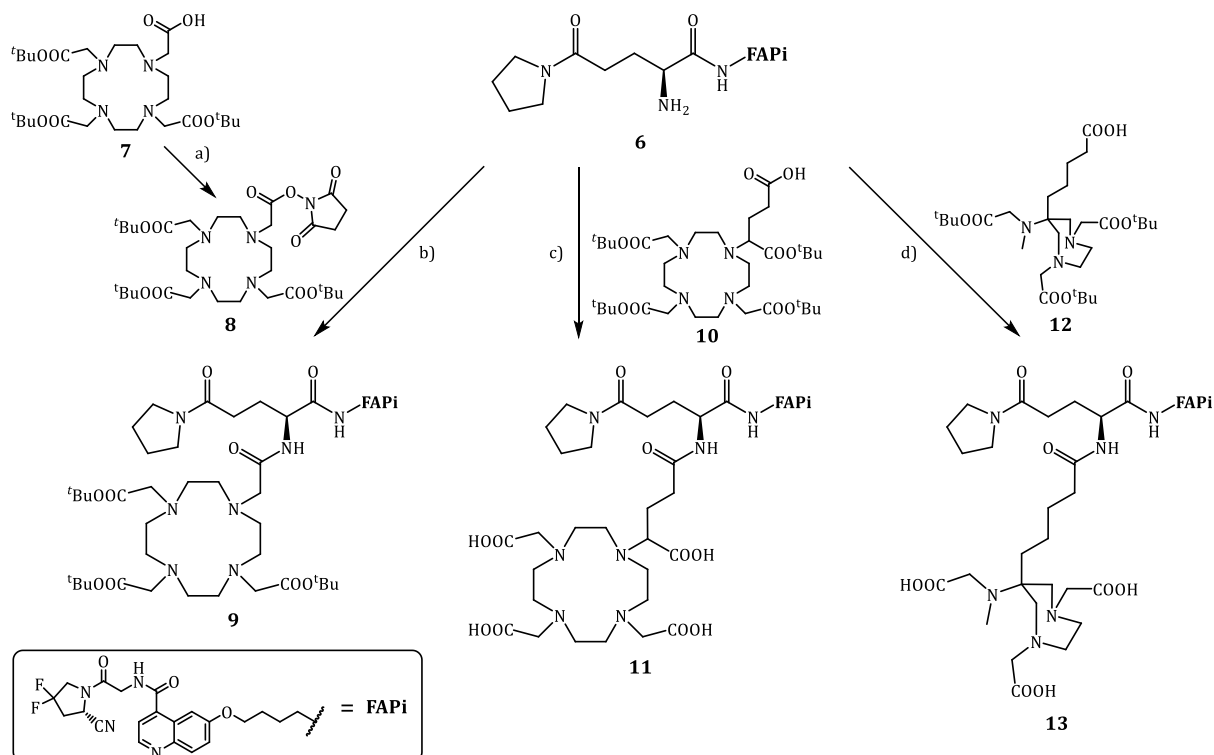


Figure 4: Synthesis scheme to obtain the final precursors DO3A.Glu(Pyr).FAPI **9**, DOTAGA.Glu(Pyr).FAPI **11** and DATA^{5m}.Glu(Pyr).FAPI **13**: a) NHS, HBTU, MeCN, DMF, 30 °C, 1 d, 96%; b) 1. DIPEA, DMF, 40 °C, 1 d; 2. TFA/MeCN/TIPS/H₂O (100:10:5:2.5), RT, 14 h, 39% (over 2 steps); c) 1. HATU, DIPEA, DMF, 30 °C, 90 min, 2. **6**, DIPEA, DMF, 30 °C, 1 d; 3. TFA/MeCN/TIPS/H₂O (100:10:5:2.5), RT, 2 d, 36% (over 3 steps); d) 1. HATU, DIPEA, DMF, 30 °C, 45 min; 2. **6**, DIPEA, DMF, 30 °C, 1 d; 3. TFA/MeCN/TIPS/H₂O (100:10:5:2.5), RT, 14 h, 37% (over 3 steps).

For the DO3A chelator, DOTA-tris(*tert*-butyl ester) **7** was first transferred into the corresponding NHS-ester **8** with *N*-hydroxysuccinimide (NHS) and HBTU because this gave much better conversion rates as direct coupling of **6** and **7** with HATU, HBTU or EDC·HCl and HOBT as could be seen in small test reactions and analysis via TLC and LC/MS. Without any further purification, the crude product was then deprotected with TFA/MeCN/TIPS/H₂O (100:10:5:2.5) to cleave the *tert*-butyl esters and yield the precursor DO3A.Glu(Pyr).FAPI **9** after RP-HPLC purification with 39% over two steps. The overall yield was 33% over 6 steps.

For the other two precursors the coupling was carried out with HATU and DIPEA in dry DMF under the same conditions. Both crude products were also deprotected without any prior purification under the same conditions (TFA/MeCN/TIPS/H₂O) and yielded DOTAGA.Glu(Pyr).FAPI **11** and DATA^{5m}.Glu(Pyr).FAPI **13** after RP-HPLC purification with 36% and 37%, and an overall yield of 32% and 31%, respectively.

The ^{nat}Ga-complexes were obtained by adding as solution of 0.1 M Ga(NO₃)₃ (2.00 eq) in 1 M ammonium acetate (pH = 5.5) to the corresponding precursors **9**, **11** and **13** (10 μmol), respectively. For the DOTAGA and DATA^{5m} derivatives some ethanol was added to improve

solubility. Subsequent purification via RP-HPLC yielded the ^{nat}Ga -complexes in moderate to good yields (63–67%).

3.2. Radiosynthesis

3.2.1. $[^{68}\text{Ga}]\text{Ga-DO3A.Glu(Pyr).FAPi}$ ($^{68}\text{Ga-9}$)

Radiolabeling of DO3A.Glu(Pyr).FAPi **9** with gallium-68 was first investigated in three different buffer systems: 1 M 4-(2-hydroxyethyl)-1-piperazineethansulfonic acid (HEPES pH = 5.5). 1 M ammonium acetate (AmOAc, pH = 4.5) and 1 M sodium acetate (NaOAc, pH = 4.5), as seen in Figure 5a.

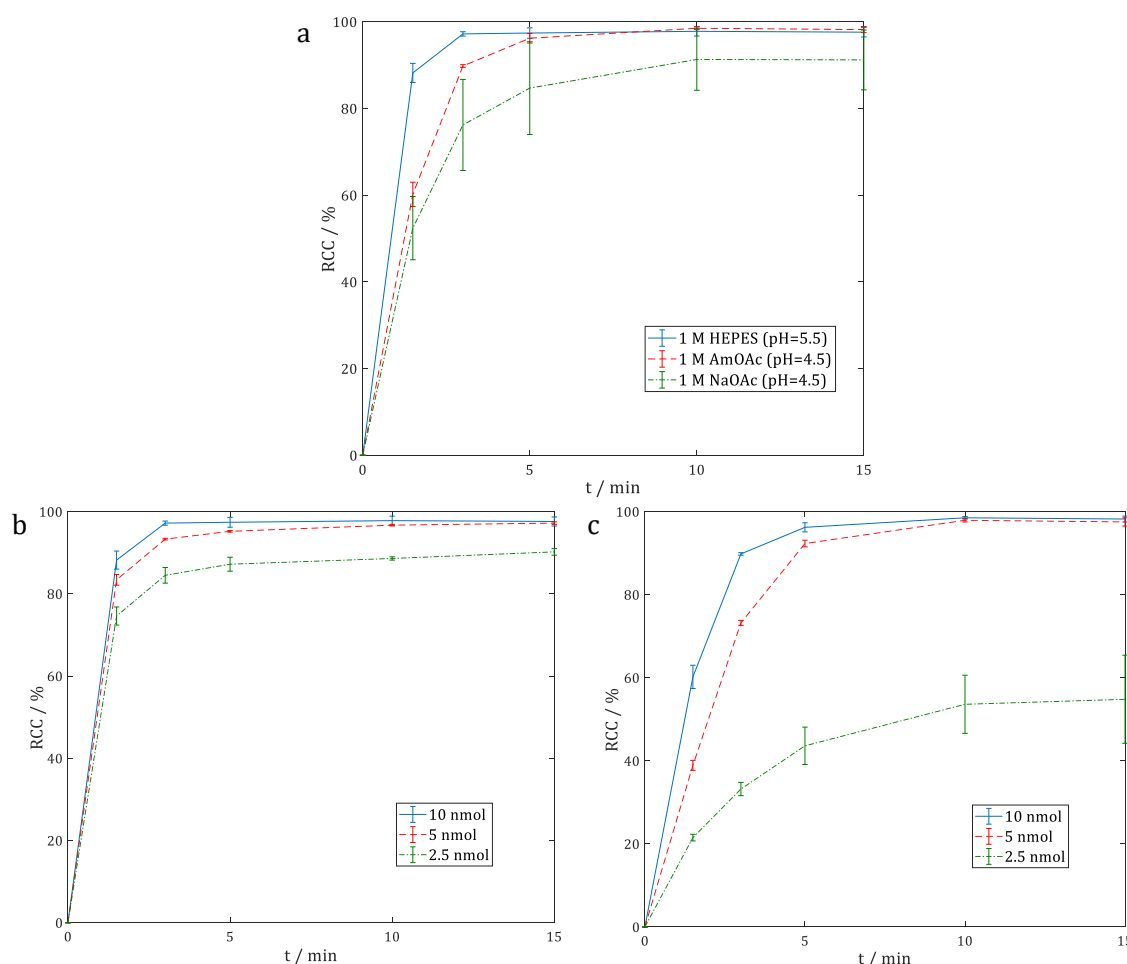


Figure 5: Reaction kinetics (radiochemical conversion (RCC) in %) of $[^{68}\text{Ga}]\text{Ga-DO3A.Glu(Pyr).FAPi}$ $^{68}\text{Ga-9}$ at 95 °C with 50 MBq ^{68}Ga , 400 μL buffer solution and a) 10 nmol DO3A.Glu(Pyr).FAPi **9** in 1 M HEPES (pH = 5.5), 1 M AmOAc (pH = 4.5) or 1 M NaOAc (pH = 4.5); b) 10, 5 or 2.5 nmol precursor **9** in 1 M HEPES (pH = 5.5); c) 10, 5 or 2.5 nmol precursor **9** in 1 M AmOAc (pH = 4.5). All reactions were carried out in triplicate ($n = 3$).

10 nmol precursor **9** was used and each labeling was carried out three times at 95 °C. The radiolabeling in NaOAc seemed to be less robust and reproducible as can be seen in the larger error bars. The labeling in AmOAc resulted in slightly higher radiochemical conversion (RCC) of

> 98% after 10 minutes as confirmed via radio-HPLC (cf. Figure S7 vs. S8: 94.4% vs. 98.7%) whereas the labeling in HEPES was a bit faster (RCC > 97% after 3 minutes). Therefore, different precursor amounts (10, 5 and 2.5 nmol) were tested in HEPES (see Figure 5b) and AmOAc (see Figure 5c). Almost quantitative RCC > 97% could be reached with 5 nmol **9** in both buffer systems. Only with 2.5 nmol **9**, differences were significant. The labeling with AmOAc resulted in insufficient RCC = $54.8 \pm 10.6\%$ after 15 minutes whereas in HEPES RCC = $90.2 \pm 0.8\%$ could be achieved. Radio-TLCs with the second solvent system 1 M AmOAc (pH = 4.0)/MeOH (1:1) gave conforming results for all reactions (data not shown).

The labeling principally works in all three buffer systems, yet the results were slightly better in AmOAc and HEPES. Quantitative RCC and therefore optimal yields can be achieved with 5 nmol **9**. HEPES showed faster reaction kinetics than AmOAc and significant differences were found in the labeling with 2.5 nmol precursor where HEPES is superior to AmOAc. Either HEPES is playing a role in breaking up the hydration shell or is directly involved in complexation and perhaps forms a weak intermediate complex with Ga(III) to accelerate labeling with gallium-68. Acetate can form weak complexes with Ga(III) but only acts as monodentate ligand [59]. The hydroxy group and the neighboring nitrogen atom of HEPES can act as a bidentate ligand than can form weak complexes with different metal ions such as Cu(II) and Eu(III) [60,61]. The trivalent metal Ga(III) might behave similar to Eu(III). An intermediate complex with the weak ligand HEPES might be formed first before the strong ligand DOTA forms a stable complex with Ga(III) comparable to ^{99m}Tc -labeling where a weak intermediate ligand (like tartrate, citrate or EDTA etc.) is used to stabilize the oxidation state Tc(V) of the $[\text{TcO}]^{3+}$ core before a stable complex with a strong N_xS_y -type ligand is formed [62–65].

On the other hand, HEPES has the disadvantage of a very low threshold value ($\leq 200 \mu\text{g}$ per dose) in the European Pharmacopeia for radiopharmaceuticals like ^{68}Ga -DOTATOC and the removal must be verified analytically although the need for the limit is questionable as it was discussed for example by LE ROUX et al. [66].

Purification with Sep-Pak® Light C18 cartridge resulted in product with high radiochemical purity (RCP) as determined via radio-TLCs and radio-HPLC (see Figure S9). The complex stability was investigated in human serum (HS) and phosphate-buffered saline (PBS) in triplicate each ($n = 3$) by incubating ca. 10 MBq ^{68}Ga -**9** in 500 μL HS and PBS at 37 °C for 120 minutes, respectively, which means the activity concentration was around 20 MBq/mL. Only very little radiolysis was observed and the stability was $97.4 \pm 0.7\%$ (PBS) and $95.8 \pm 0.2\%$ (HS) after 120 min as analyzed via radio-TLC (cf. Figure S4).

3.2.2. [^{68}Ga] $\text{Ga-DOTAGA.Glu(Pyr).FAPi}$ ($^{68}\text{Ga-11}$)

Radiolabeling of DOTAGA.Glu(Pyr).FAPi **11** with gallium-68 was investigated in the same three buffer systems 1 M HEPES (pH = 5.5), 1 M (AmOAc, pH = 4.5) and 1 M NaOAc (pH = 4.5) and with 10 nmol precursor **11** at 95 °C (Figure 6a).

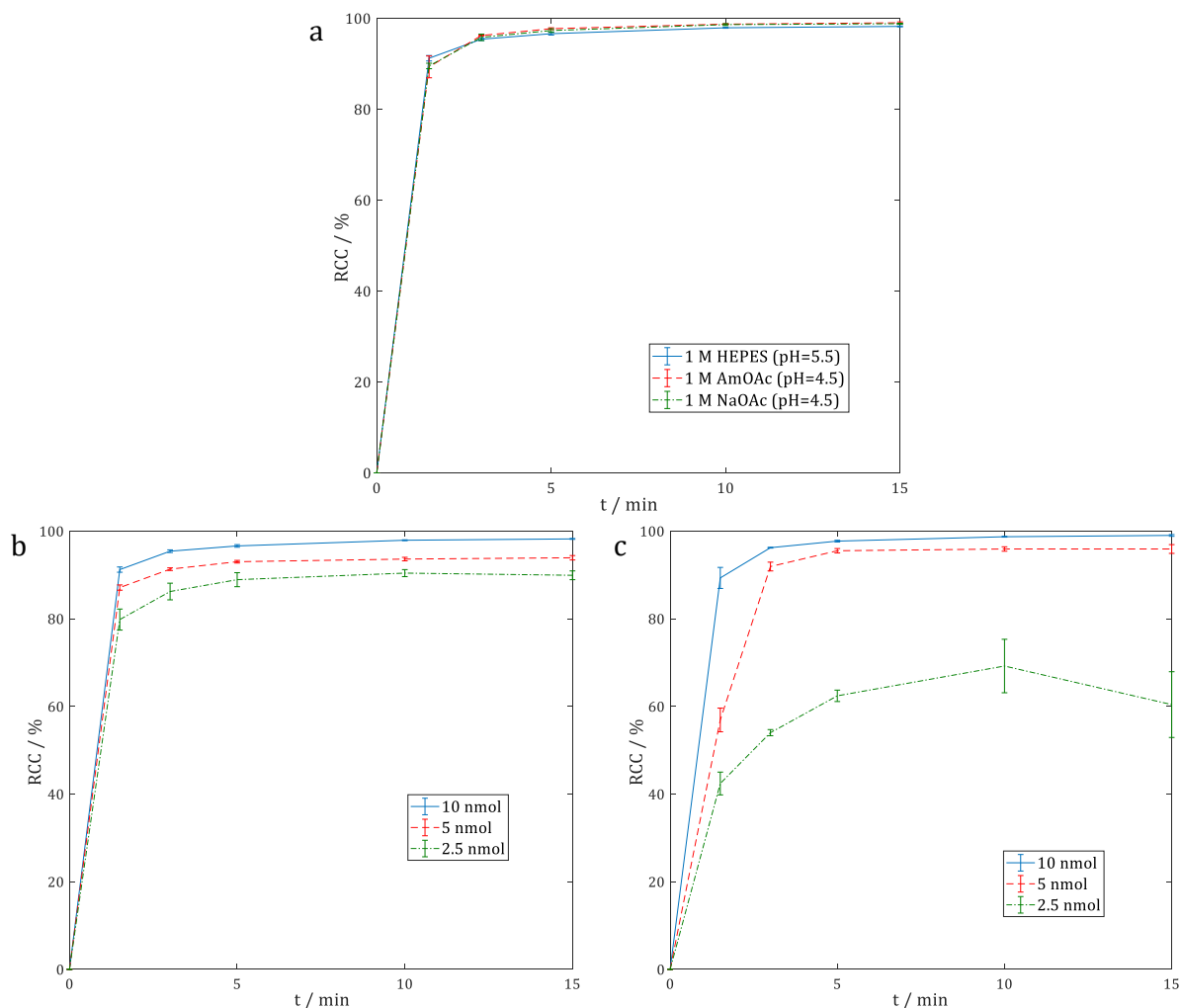


Figure 6: Reaction kinetics (radiochemical conversion (RCC) in %) of [^{68}Ga] $\text{Ga-DOTAGA.Glu(Pyr).FAPi}$ $^{68}\text{Ga-11}$ at 95 °C with 50 MBq ^{68}Ga , 400 μL buffer solution and a) 10 nmol DOTAGA.Glu(Pyr).FAPi **11** in 1 M HEPES (pH = 5.5), 1 M AmOAc (pH = 4.5) or 1 M NaOAc (pH = 4.5); b) 10, 5 or 2.5 nmol precursor **11** in 1 M HEPES (pH = 5.5); c) 10, 5 or 2.5 nmol precursor **11** in 1 M AmOAc (pH = 4.5). All reactions were carried out in triplicate ($n = 3$).

Quantitative RCC of 98–99% and very similar reaction kinetics were achieved with all buffer systems. Figure S10 and S11 show exemplary radio-HPLCs after the radiolabeling in HEPES and AmOAc. Subsequently, the precursor amount was optimized in HEPES and AmOAc (Figure 6b and c). High RCCs were observed with 5 nmol **11** in HEPES (RCC = $93.4 \pm 0.4\%$) and AmOAc (RCC = $95.9 \pm 0.5\%$) after 10 minutes. It dropped to RCC = $90.4 \pm 0.8\%$ (HEPES) and RCC = $69.2 \pm 6.1\%$ (AmOAc) with just 2.5 nmol **11** after 10 minutes. The results were confirmed with 1 M AmOAc (pH = 4)/MeOH (1:1) radio-TLCs.

The experiments proved that DOTAGA.Glu(Pyr).FAPi **11** can be labeled quantitatively in HEPES, AmOAc and NaOAc. 5 nmol **11** is enough to get high RCCs in AmOAc while only 2.5 nmol resulted in insufficient RCC. In contrast, RCC $\approx$ 90% was achieved in HEPES with only 2.5 nmol precursor analogous to the results of ^{68}Ga -**9**.

After purification with C18 cartridge the RCP was determined via radio-TLCs and radio-HPLC (see Figure S12) and ^{68}Ga -**11** was incubated in 500 μL HS and PBS ($\approx$ 20 MBq/mL) in triplicate each ($n = 3$) at 37 $^{\circ}\text{C}$ for 120 min and analyzed via radio-TLC (see Figure S5). Complex stability was $95.7 \pm 1.0\%$ (PBS) and $94.2 \pm 0.7\%$ (HS) after 120 min, respectively, which means that only little radiolysis occurred.

3.2.3. [^{68}Ga]Ga-DATA^{5m}.Glu(Pyr).FAPi (^{68}Ga -**13**)

Labeling of DATA^{5m}.Glu(Pyr).FAPi **13** with gallium-68 in the buffer systems 1 M HEPES (pH = 5.5), 1 M (AmOAc, pH = 4.5) and 1 M NaOAc (pH = 4.5) with 10 nmol precursor at room temperature (25 $^{\circ}\text{C}$) is shown in Figure 7a.

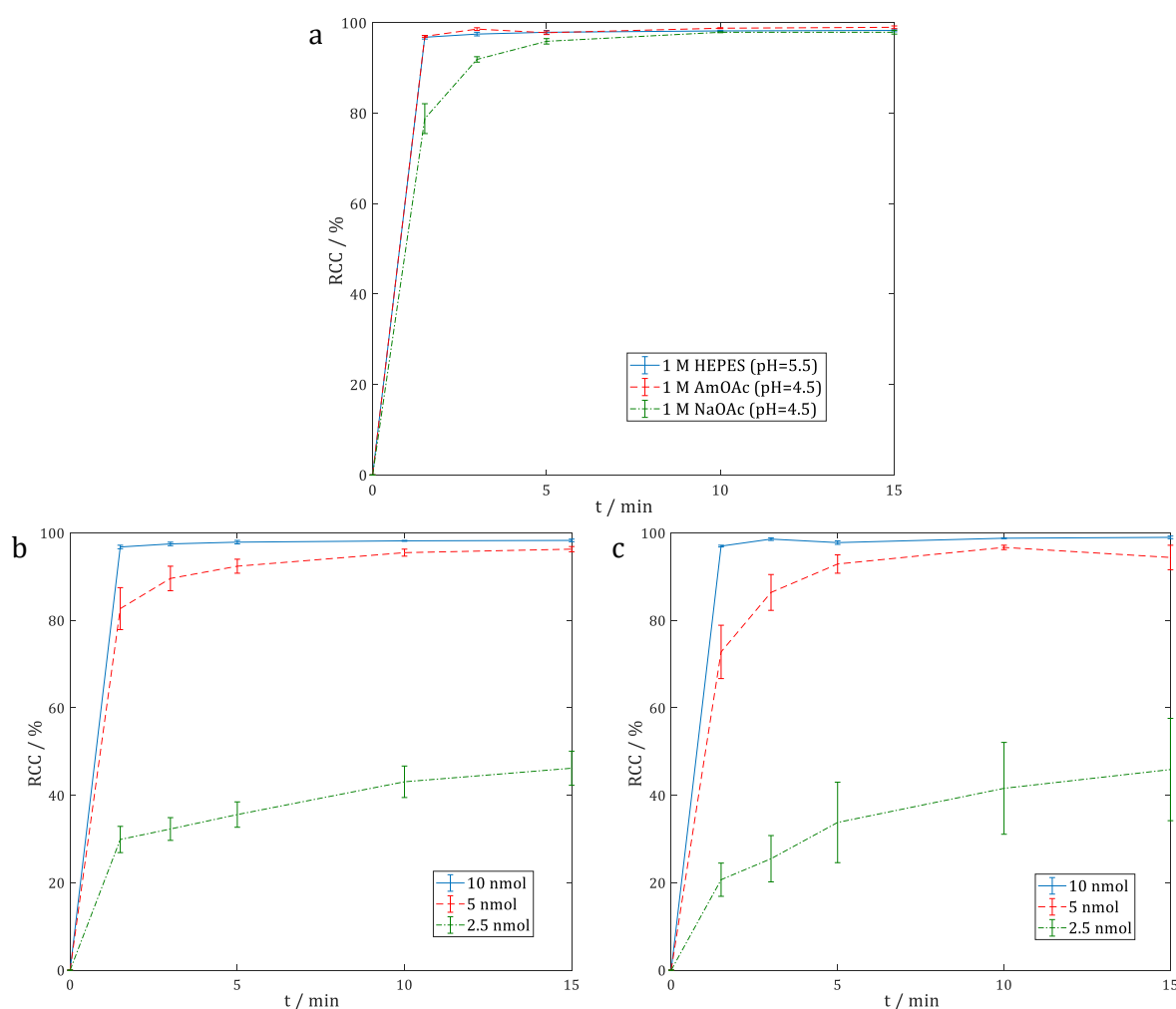


Figure 7: Reaction kinetics (radiochemical conversion (RCC) in %) of [^{68}Ga]Ga-DATA^{5m}.Glu(Pyr).FAPi ^{68}Ga -**13** at 25 $^{\circ}\text{C}$ with 50 MBq ^{68}Ga , 400 μL buffer solution and a) 10 nmol DATA^{5m}.Glu(Pyr).FAPi **13** in 1 M HEPES

(pH = 5.5), 1 M AmOAc (pH = 4.5) or 1 M NaOAc (pH = 4.5); b) 10, 5 or 2.5 nmol precursor **13** in 1 M HEPES (pH = 5.5); c) 10, 5 or 2.5 nmol precursor **13** in 1 M AmOAc (pH = 4.5). All reactions were carried out in triplicate ($n = 3$).

Quantitative RCC = 97–99% was observed with all three buffers after 5–10 minutes when analyzed *via* citrate radio-TLC. The reaction kinetics in NaOAc were slightly slower than in HEPES and AmOAc. Slight formation of colloidal gallium ($[^{68}\text{Ga}]\text{Ga}(\text{OH})_3$ and other hydroxo species) was observed in the second TLC system (1 M AmOAc (pH = 4)/MeOH (1:1)) where the RCC = 92–93% for HEPES and AmOAc was also slightly higher than for NaOAc (RCC = $90.5 \pm 0.5\%$).

By labeling 5 nmol **13** in 1 M AmOAc (pH = 4.0), it was tested if less colloidal gallium-68 ($[^{68}\text{Ga}]\text{Ga}(\text{OH})_3$) was formed as its formation usually increases with rising pH value. This resulted in only 45.5% RCC after 15 minutes (data not shown) and pH value after the reaction was < 2 meaning the buffer capacity was exceeded.

Subsequently, the precursor amount was optimized in HEPES and AmOAc (Figure 6b and c). High RCCs were observed with 5 nmol **11** in HEPES (RCC = $93.4 \pm 0.4\%$) and AmOAc (RCC = $95.9 \pm 0.5\%$) after 10 minutes. Radio-HPLCs after radiolabeling in HEPES and AmOAc are shown in Figure S13 and S14, respectively. The RCC dropped to $90.4 \pm 0.8\%$ (HEPES) and $69.2 \pm 6.1\%$ (AmOAc) with just 2.5 nmol **11** after 10 minutes.

RCP was determined via radio-TLCs and radio-HPLC after purification with C18 cartridge (see Figure S15). $[^{68}\text{Ga}]\text{Ga-DATA}^{5\text{m}}.\text{Glu}(\text{Pyr}).\text{FAPi}$ **$^{68}\text{Ga-13}$** (ca. 10 MBq) was incubated in 500 μL HS and PBS ($\approx 20 \text{ MBq/mL}$, $n=3$) at 37 °C for 120 min and analyzed via radio-TLC (Figure S6). The complex was not completely stable under these conditions and the stability was $90.1 \pm 0.7\%$ (PBS) and $85.0 \pm 2.0\%$ (HS) after 120 min.

For potential kit-type labeling, elevated temperatures (e.g. 50 or 90 °C) should be tried to investigate if the formation of colloidal gallium-68 can be suppressed this way to not need any purification after labeling.

3.2.4. Lipophilicity

The lipophilicity of the ^{68}Ga -labeled compounds **$^{68}\text{Ga-9}$** , **$^{68}\text{Ga-11}$** and **$^{68}\text{Ga-13}$** was investigated experimentally via shake-flask method with ca. 5–7 MBq per vial ($n = 4$) in PBS and 1-octanol. The $\log D_{7.4}$ value was calculated via equation (1). Table 2 summarizes the $\log D_{7.4}$ values and compares them with those of compounds from previous works [30,51].

Table 2: $\log D_{7.4}$ values of different labeled FAPi-based radiopharmaceuticals. The values for $[^{68}\text{Ga}]\text{Ga-DO3A.SA.FAPi}$ is taken from MOON et al. [30] and for $[^{68}\text{Ga}]\text{Ga-}$ and $[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu.(FAPi)}_2$ from MARTIN et al. [51].

Complex	$\log D_{7.4}$
$[^{68}\text{Ga}]\text{Ga-DO3A.SA.FAPi}$	-2.68 ± 0.06
$[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu.(FAPi)}_2$	-2.48 ± 0.05
$[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu.(FAPi)}_2$	-2.77 ± 0.10
$[^{68}\text{Ga}]\text{Ga-DO3A.Glu(Pyr).FAPi}$ ($^{68}\text{Ga-9}$)	-2.40 ± 0.09
$[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu(Pyr).FAPi}$ ($^{68}\text{Ga-11}$)	-2.45 ± 0.07
$[^{68}\text{Ga}]\text{Ga-DOTA}^{5m}\text{.Glu(Pyr).FAPi}$ ($^{68}\text{Ga-13}$)	-2.29 ± 0.07

The new FAPi monomers have high hydrophilicity and, especially the DO3A and DOTAGA derivatives **$^{68}\text{Ga-9}$** and **$^{68}\text{Ga-11}$** , show $\log D_{7.4}$ values comparable to those of $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu.(FAPi)}_2$. This can be seen as a first indicator that these compounds might also behave similarly *in vivo*.

3.3. In Vitro-Inhibition Assays

The precursors and the respective ^{nat}Ga -complexes were tested for their affinity for FAP as well as the related proteases PREP, DPP4, DPP8 and DPP9. The IC_{50} value of FAP and all selectivity indexes SI , with $SI = IC_{50}(\text{respective protease})/IC_{50}(\text{FAP})$, are summarized in Table S1 and compared to compounds from earlier works. Furthermore, the IC_{50} values for PREP, DPP4, DPP8 and DPP9 can be found in Table S2. The $IC_{50}(\text{FAP})$ for each new compound as well as selected literature compounds (including the respective error bar) is plotted against the SI over the two most important competitive proteases DPP4 and PREP in Figure 8a and b, respectively.

The affinity for FAP of all new compounds is very high and the IC_{50} values are in the subnanomolar range. Together with PREP and DPP4 affinities that are all $> 1 \mu\text{M}$, this results in very high selectivity $> 10^4$ for all new DO3A and DOTAGA compounds **9**, **$^{nat}\text{Ga-9}$** , **11** and **$^{nat}\text{Ga-11}$** . Even the PREP selectivity for **13** and **$^{nat}\text{Ga-13}$** ($5 \cdot 10^3 - 1 \cdot 10^4$) is still higher than for the initial FAP inhibitor UAMC-1110 ($SI(\text{PREP}/\text{FAP}) = 4186$). Since DPP4 and PREP are ubiquitously expressed in healthy tissue, the selectivity for these two proteases is crucial. The selectivity for DPP8 and DPP9 is less critical because these proteases are only expressed intracellularly [67]. Nevertheless, as can be derived from the IC_{50} values, the DPP8- and DPP9-selectivity is > 1000 for all new compounds except the DPP8 selectivity of **11** (cf. Table S1).

Overall, the new compounds show excellent results *in vitro* and have comparable DPP4 selectivity and improved PREP selectivity compared to UAMC-1110. As found in earlier works, the IC_{50} and SI values do not differ significantly between precursor and Ga-complex. The results of the DO3A

and DOTAGA precursors and Ga-complexes (**natGa-9**) and (**natGa-11**) are superior to those of the FAPi monomer DOTA.SA.FAPi and [^{natGa}Ga-DOTA.SA.FAPi], respectively. This indicates that the radiotracers [⁶⁸Ga]Ga-DO3A- and [⁶⁸Ga]Ga-DOTAGA.Glu(Pyr).FAPi might be suitable radiotracers for *in vivo* application.

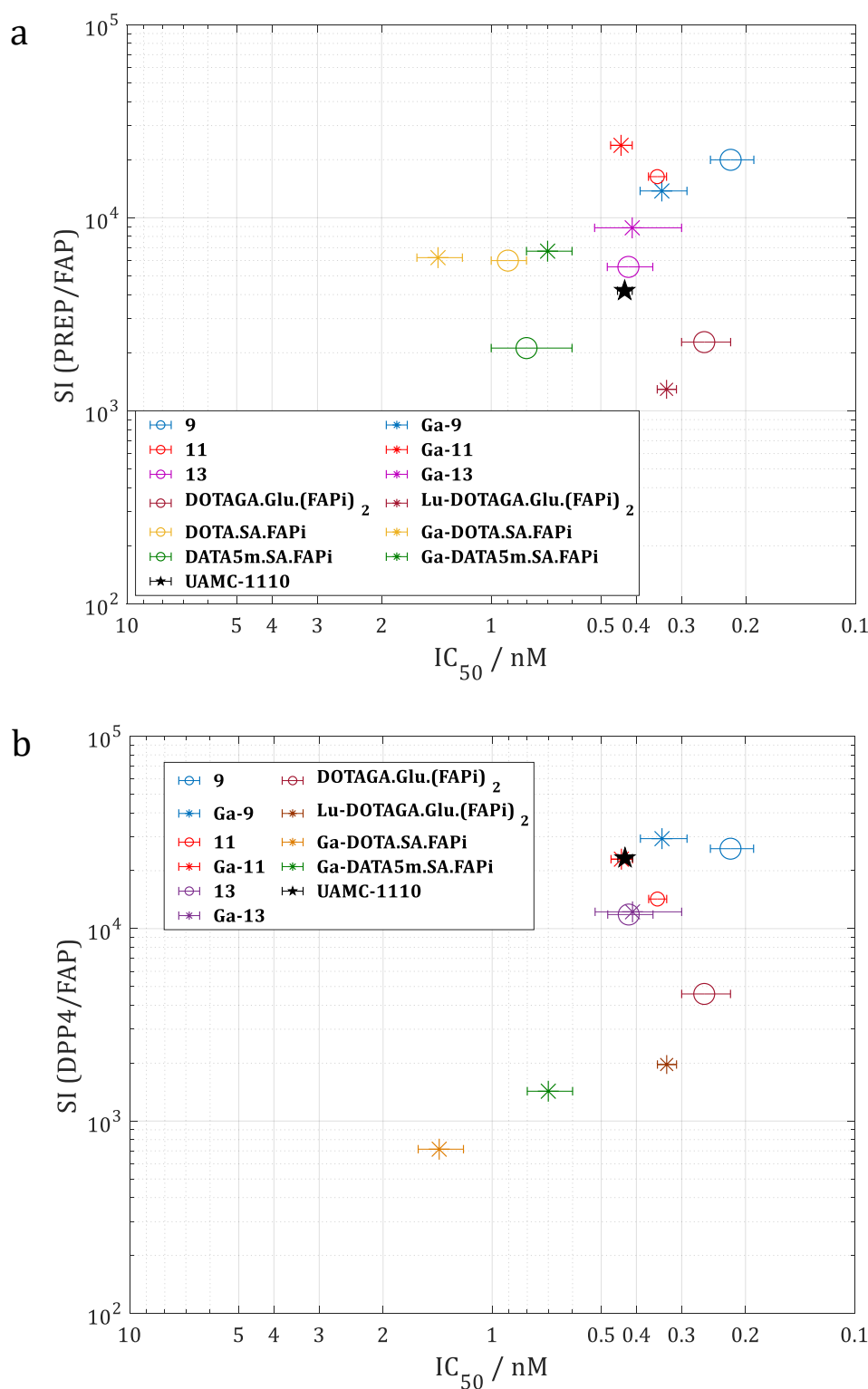


Figure 8: FAP affinity and selectivity. IC₅₀ value for FAP plotted against a) the selectivity index (SI) for FAP over PREP and b) SI for FAP over DPP4 with $SI = IC_{50}(PREP \text{ or } DPP4) / IC_{50}(FAP)$. The symbol "o" stands for the precursor and "*" for the Ga- or Lu-complex.

3.4. Proof-of-Principle Patient Studies

For the initial proof-of-concept PET/CT scan, two radiotracers were evaluated: [^{68}Ga]Ga-DO3A and [^{68}Ga]Ga-DOTAGA.Glu(Pyr).FAPi. These radiotracers were chosen to assess their feasibility and efficacy in the PET/CT imaging procedure.

After successful radiolabeling, [^{68}Ga]Ga-DO3A.Glu(Pyr).FAPi was administered to a patient with invasive ductal carcinoma (cf. Table 1 for patient details). Figure 9 displays the PET/CT scan taken at 60 minutes post-injection, showcasing the imaging results obtained.

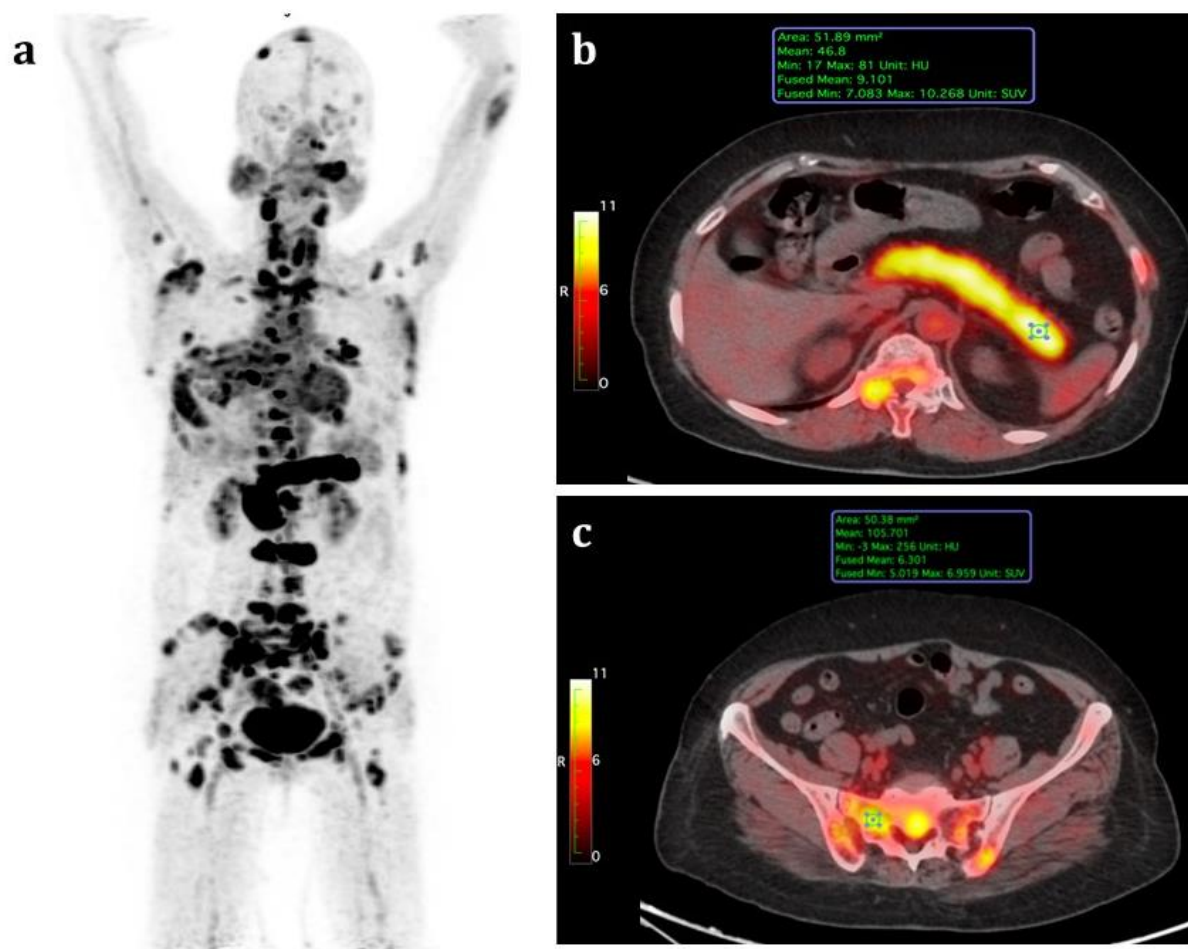


Figure 9: A 70-year-old female was diagnosed with metastatic invasive ductal breast carcinoma (ER+, Pr+, Her2Neu+). The [^{68}Ga]Ga-DO3A.Glu(Pyr).FAPi PET/CT scan 60 min p.i. revealed physiological uptake in pancreas (a and b), and extensive bone metastases with intense uptake (a and c).

The biodistribution analysis, based on lean body mass-corrected standard uptake values (SUL peaks), for relevant organs is summarized in Table 3.

Table 3: SUL values of various organs and tumor lesions of the PET/CT scan with the two FAPi radiotracers [⁶⁸Ga]Ga-DO3A.Glu(Pyr).FAPi and [⁶⁸Ga]Ga-DOTAGA.Glu(Pyr).FAPi at 60 min p.i.

FAPi radiotracer	Liver background (SUL peak)	Blood pool (SUL peak)	Pancreas (SUL peak)	Lesion (SUL peak)
[⁶⁸ Ga]Ga-DO3A.Glu.(Pyr).FAPi	3	2.7	10.3	Mediastinal lymph node: 7.5 Bone: 12.9
[⁶⁸ Ga]Ga-DOTAGA Glu.(Pyr).FAPi	0.8	4.6	8	Liver: 7.4 Lymph node: 3.5 Bone: 8.6

* all values are calculated with ROI volume of 1.26 cm³

[⁶⁸Ga]Ga-DO3A.Glu(Pyr).FAPi exhibited effective clearance from the blood pool and demonstrated significant uptake in tumor lesions and bone metastases. There was also noticeable physiological uptake in the pancreas and some uptake in the liver. The liver uptake suggests a possible, at least partially, hepatobiliary excretion pattern.

Overall, the new radiotracer provided good-contrast images, highlighting its potential for effective imaging.

Figure 10 depicts the PET/CT scan obtained from the investigation of a patient with thyroid cancer using [⁶⁸Ga]Ga-DOTAGA.Glu(Pyr).FAPi. The scan provides visual information on the biodistribution and uptake of the radiotracer within the patient's body.

[⁶⁸Ga]Ga-DOTAGA Glu.(Pyr).FAPi demonstrated favorable tumor uptake in bone lesions and liver metastasis, with lower background activity in the liver compared to [⁶⁸Ga]Ga-DO3A.Glu.(Pyr).FAPi. However, there was still a higher amount of circulating activity in the blood. The uptake in the pancreas was comparable to DO3A.Glu.(Pyr).FAPi, which is consistent with previous studies, e.g. with [⁶⁸Ga]Ga-DOTA.SA.FAPi, reporting physiological uptake in the pancreas [35,68–70]. FAP has been reported to be physiologically expressed in the pancreas in several studies [71–74].

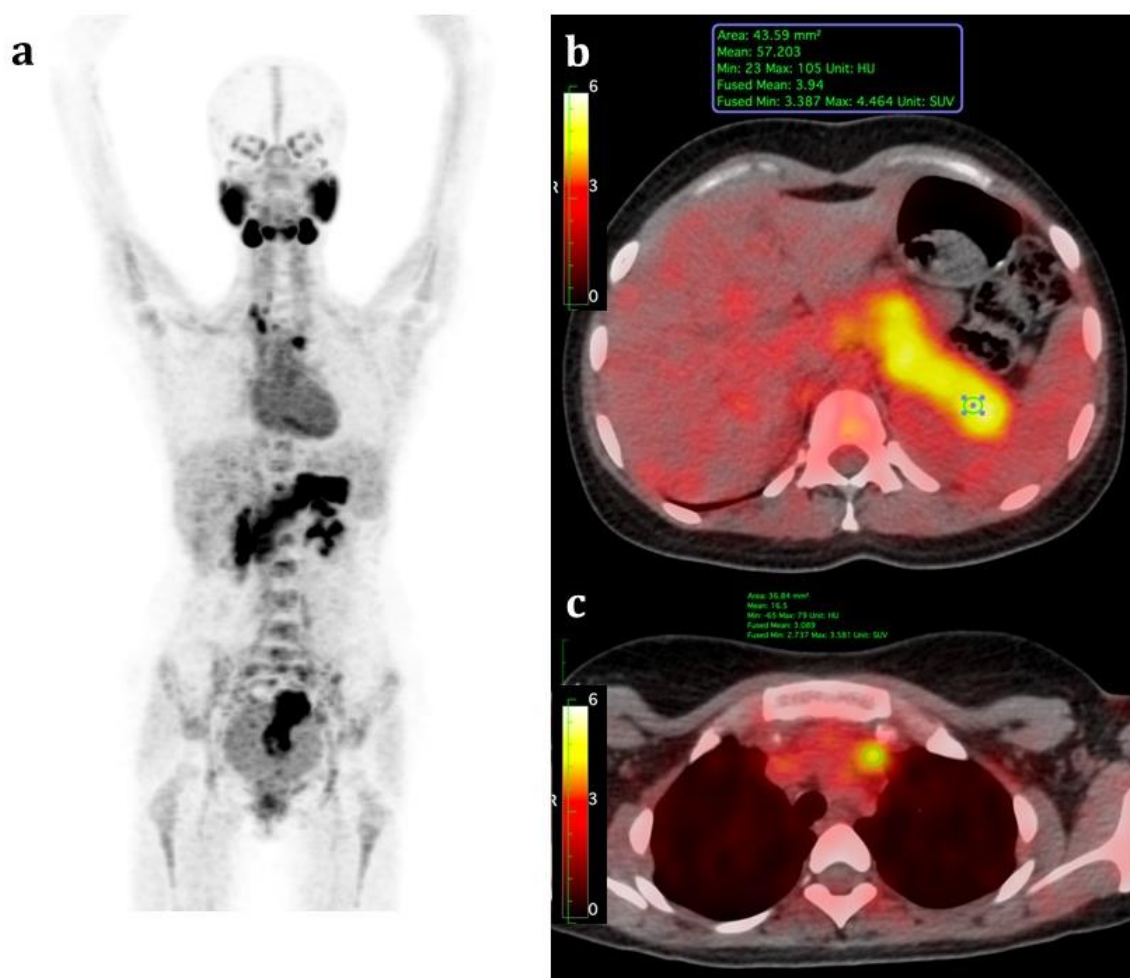


Figure 10: A 19-year-old-female diagnosed with medullary thyroid cancer underwent [⁶⁸Ga]Ga-DOTAGA.Glu(Pyr).FAPi PET/CT. Scan at 60 min p.i. showed uptake in the pancreas (a and b) and mediastinal lymph node metastases (a and c).

To gain a more detailed understanding, further investigations are necessary such as histological examinations of pancreatic tissue to determine FAP expression in cases like the ones observed here. Additionally, conducting blocking studies in murine xenograft models (ideally with FAP-expressing pancreas) could help determine if the observed uptake in the pancreas is indeed non-specific binding or if it is FAP-specific binding and the uptake that results from physiological FAP expression.

It would be valuable to conduct systematic biodistribution studies comparing the new compounds with [⁶⁸Ga]Ga-DOTA.SA.FAPi and [⁶⁸Ga]Ga-DOTAGA.Glu.(FAPi)₂. This would be particularly interesting in patients who are being considered for FAPi-based radioligand therapy using [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂. Given that [¹⁷⁷Lu]Lu-FAPi dimer RLT is a promising therapeutic option for thyroid cancer, breast cancer, lung cancer and other epithelial cancer types, an improved diagnostic counterpart for this theranostic pair would be beneficial for patient care.

4. Conclusion

New monomeric FAPi-based radiotracers were successfully synthesized and radiolabeled with gallium-68. The compounds DO3A.Glu(Pyr).FAPi, DOTAGA.Glu(Pyr).FAPi and DATA^{5m}.Glu(Pyr).FAPi have a branched structure with the central linker unit glutamate (Glu) similar to the dimer DOTAGA.Glu.(FAPi)₂ which can be used for therapeutic applications when radiolabeled with lutetium-177 or actinium-225. The new molecules are intended to act as the diagnostic counterpart for PET and are supposed to form an optimized theranostic pair with [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂. FAPi dimers do not have an optimal pharmacokinetic profile for acquiring high-contrast PET images at early time points like monomers. Despite the different pharmacokinetic profile expected, pharmacodynamic profile of the new FAPi monomers is expected to be more similar to DOTAGA.Glu.(FAPi)₂ than DOTA.SA.FAPi. [⁶⁸Ga]Ga-DOTA.SA.FAPi is currently used for pre- and post-treatment assessment via PET/CT after radioligand therapy with [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂.

The DO3A and DOTAGA derivatives showed excellent ⁶⁸Ga-radiolabeling properties which means quantitative radiochemical conversion that results in high radiochemical yields in several different buffer systems, high complex stability in different media (HS and PBS) and high hydrophilicity.

The respective precursors and Ga-complexes also showed excellent FAP affinity and selectivity over the related proteases being even higher than UAMC-1110. The properties of DATA^{5m}.Glu(Pyr).FAPi were also promising but not as good as the DO3A and DOTAGA compounds although the PREP selectivity was still better than of UAMC-1110. In comparison to DOTA.SA.FAPi, all new compounds have improved *in vitro* properties and have proven to be interesting candidates for translation into *in vivo* PET imaging.

First proof-of-concept PET/CT scans using [⁶⁸Ga]Ga-DO3A.Glu(Pyr).FAPi on two different patients [⁶⁸Ga]Ga-DOTAGA Glu.(Pyr).FAPi demonstrated significant tumor uptake and very good clearance from the blood for the DO3A derivative. Future comparative studies with DOTA.SA.FAPi are interesting to find the optimal ⁶⁸Ga-PET/CT radiotracer guiding ¹⁷⁷Lu-FAPi dimer RLT.

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Conflicts of interest

The authors declare no conflicts of interest.

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Supporting Information

Organic Synthesis - Analytical RP-HPLC

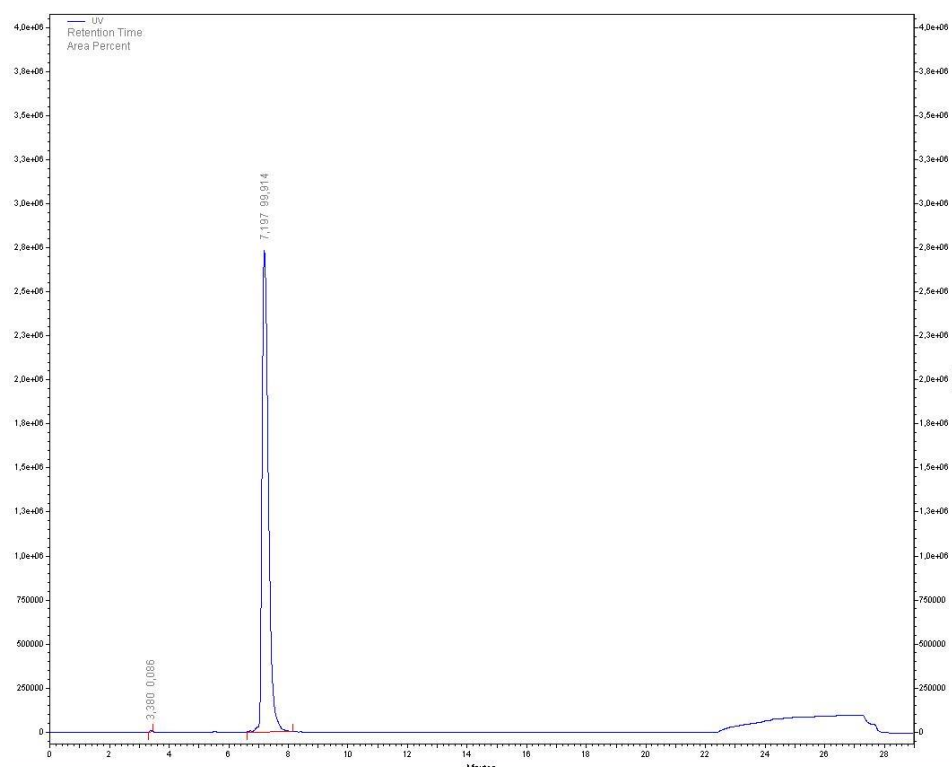


Figure S1: Analytical RP-HPLC of DO3A.Glu(Pyr).FAPi **9** ($t_R = 7.2$ min), 20-30% MeCN + 0.1% TFA (linear gradient) in 20 min, 5 mL/min: purity = 99.9%.

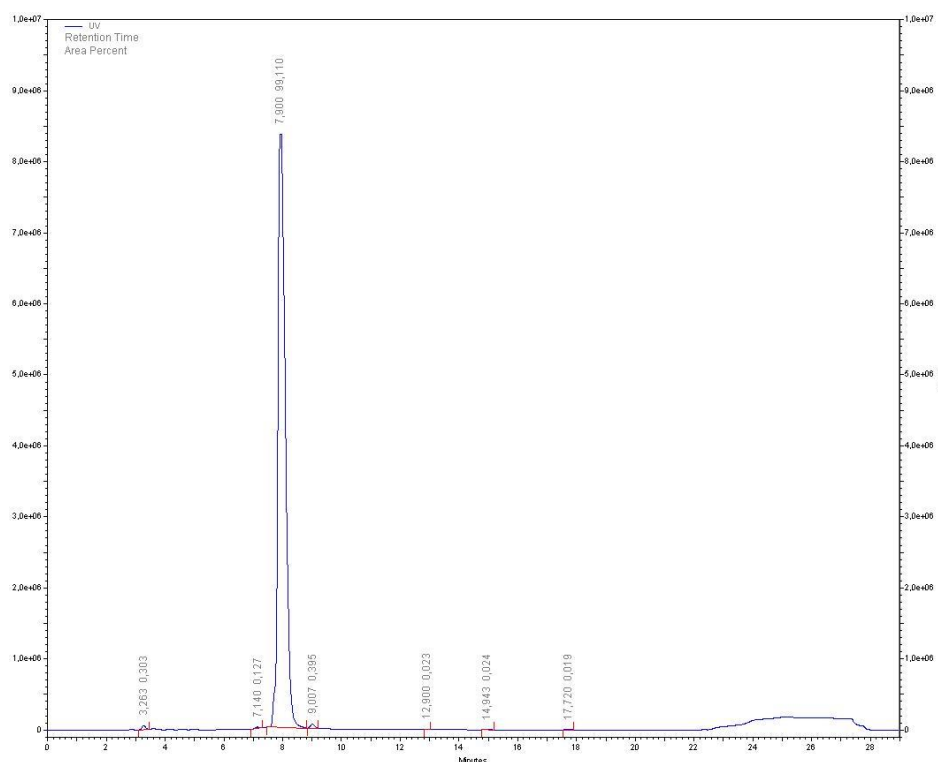


Figure S2: Analytical RP-HPLC of DOTAGA.Glu(Pyr).FAPi **11** ($t_R = 7.9$ min), 20-30% MeCN + 0.1% TFA (linear gradient) in 20 min, 5 mL/min: purity = 99.1%.

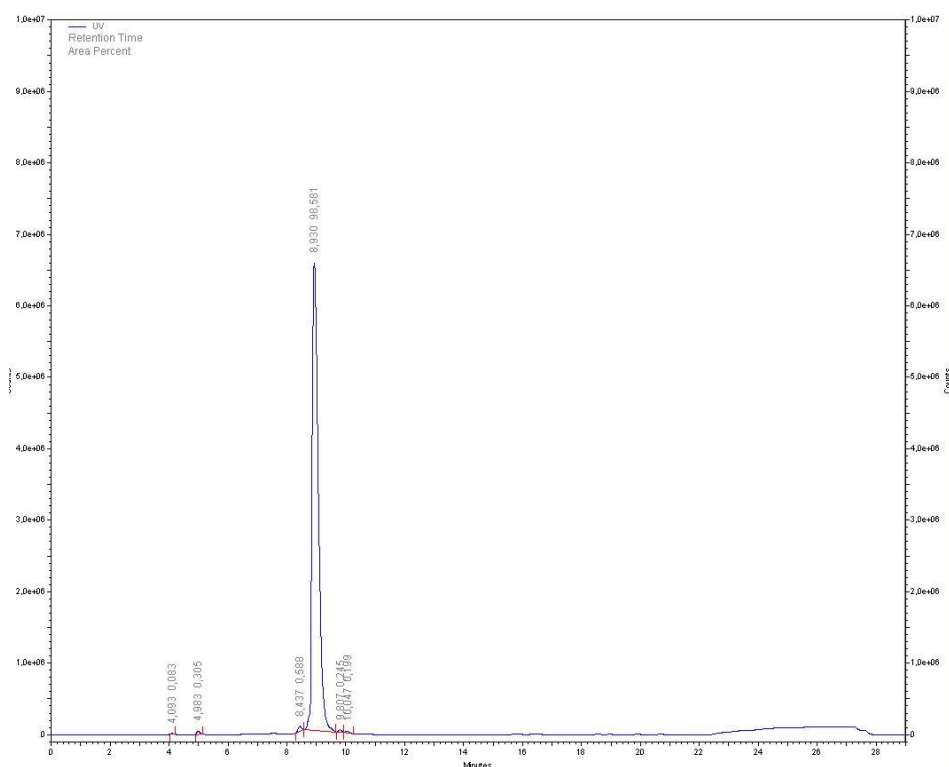


Figure S3: Analytical RP-HPLC of DATA^{5m}.Glu(Pyr).FAPi **13** ($t_R = 8.9$ min), 20-30% MeCN + 0.1% TFA (linear gradient) in 20 min, 5 mL/min: purity = 98.6%.

Radiosynthesis - Complex Stabilities

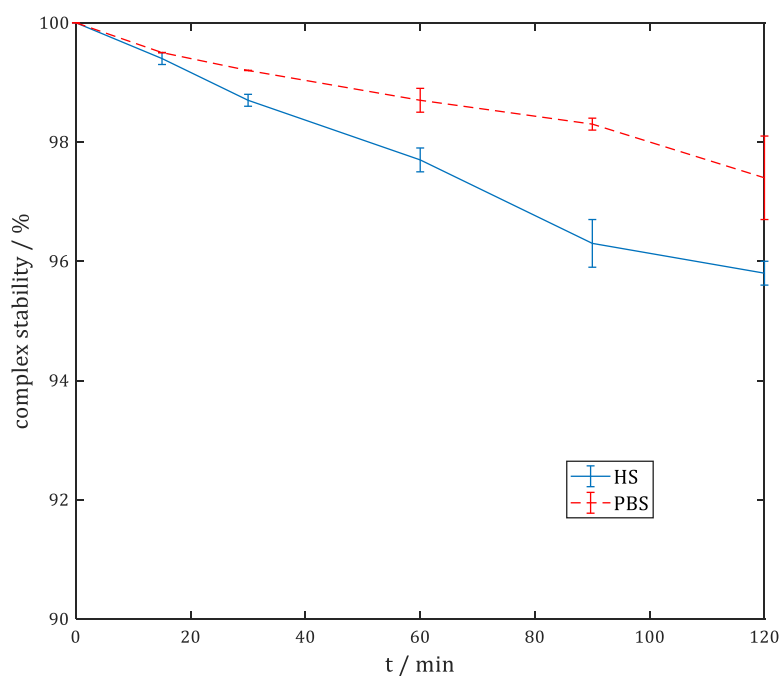


Figure S4: Complex stability of [⁶⁸Ga]Ga-DO3A.Glu(Pyr).FAPi **68Ga-9** (20 MBq/mL) in human serum (HS) and phosphate-buffered saline (PBS) over 120 minutes ($n = 3$).

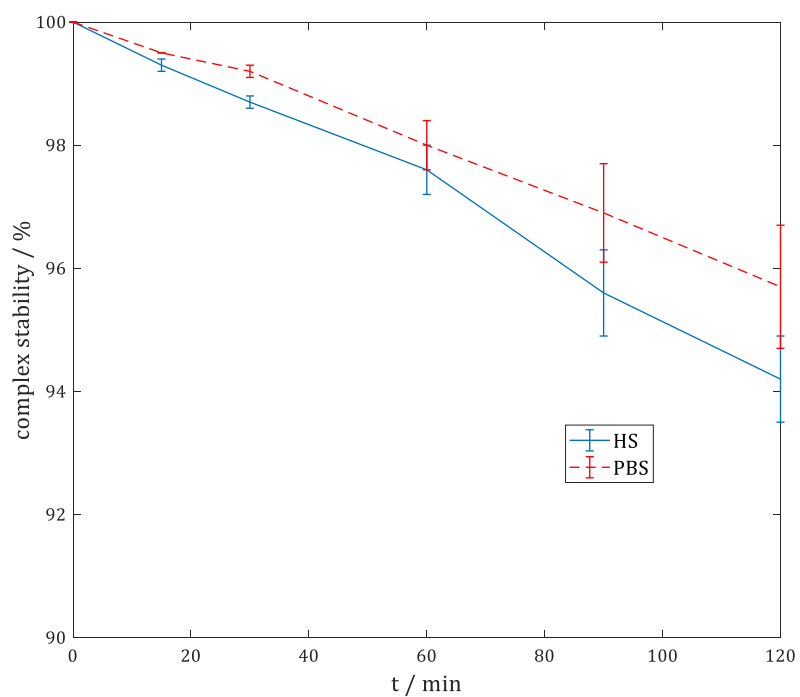


Figure S5: Complex stability of $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu(Pyr).FAPi } ^{68}\text{Ga-11}$ (20 MBq/mL) in human serum (HS) and phosphate-buffered saline (PBS) over 120 minutes ($n = 3$).

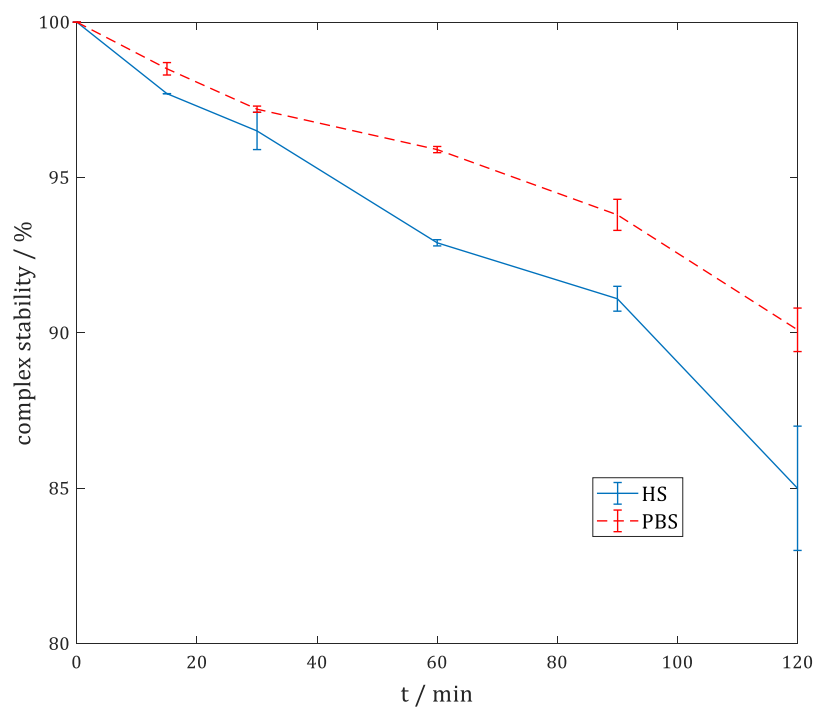


Figure S6: Complex stability of $[^{68}\text{Ga}]\text{Ga-DATA}^{5m}.\text{Glu(Pyr).FAPi } ^{68}\text{Ga-13}$ (20 MBq/mL) in human serum (HS) and phosphate-buffered saline (PBS) over 120 minutes ($n = 3$).

Radiosynthesis - Radio-HPLCs

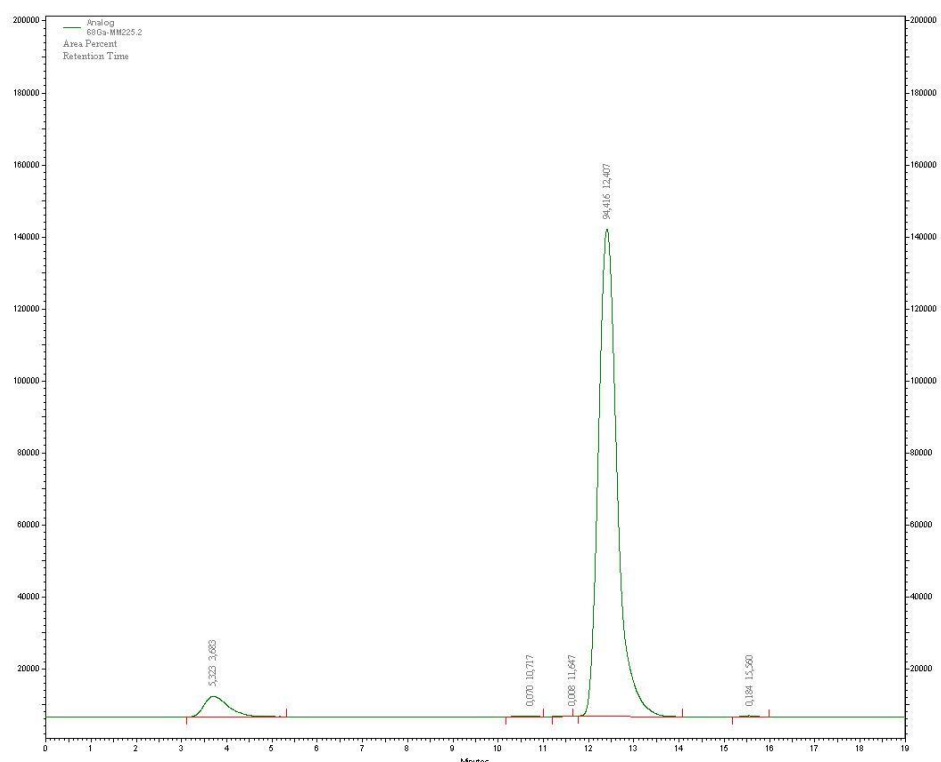


Figure S7: Analytical Radio-HPLC of [^{68}Ga]Ga-DO3A.Glu(Pyr).FAPi **^{68}Ga -9** ($t_R = 12.4$ min) after radiolabeling with 10 nmol **9**, and 50 MBq ^{68}Ga in 1 M HEPES (pH = 5.5), 20-30% MeCN + 0.1% TFA (linear gradient) in 10 min: RCP = 94.4%.

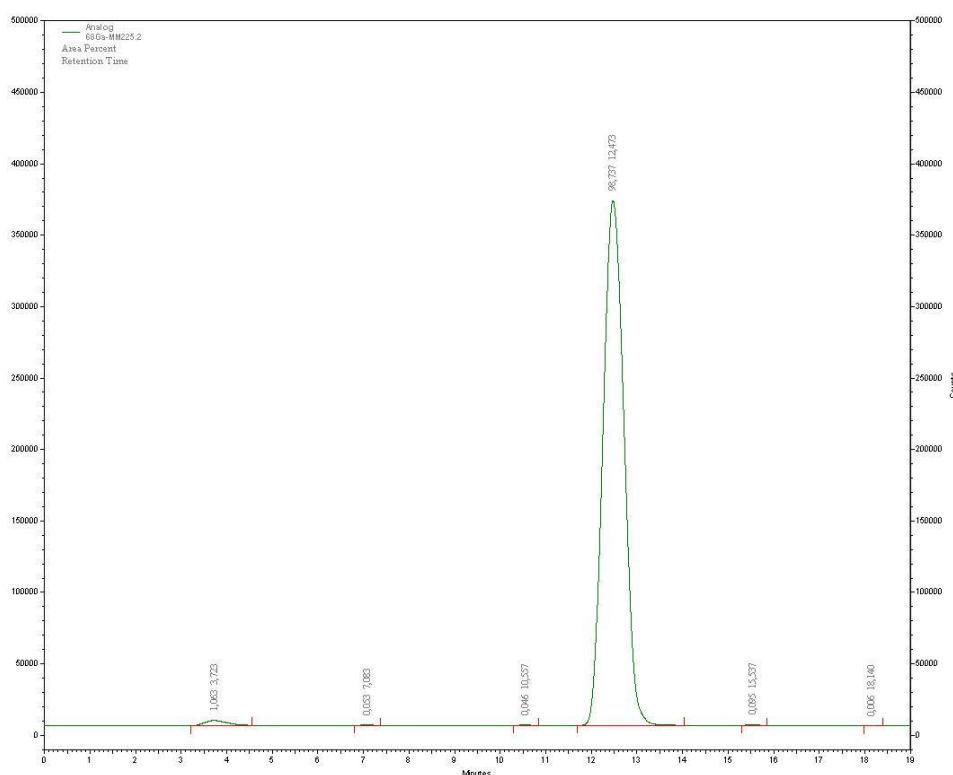


Figure S8: Analytical Radio-HPLC of [^{68}Ga]Ga-DO3A.Glu(Pyr).FAPi **^{68}Ga -9** ($t_R = 12.5$ min) after radiolabeling with 10 nmol **9**, and 50 MBq ^{68}Ga in 1 M AmOAc (pH = 4.5), 20-30% MeCN + 0.1% TFA (linear gradient) in 10 min: RCP = 98.7%.

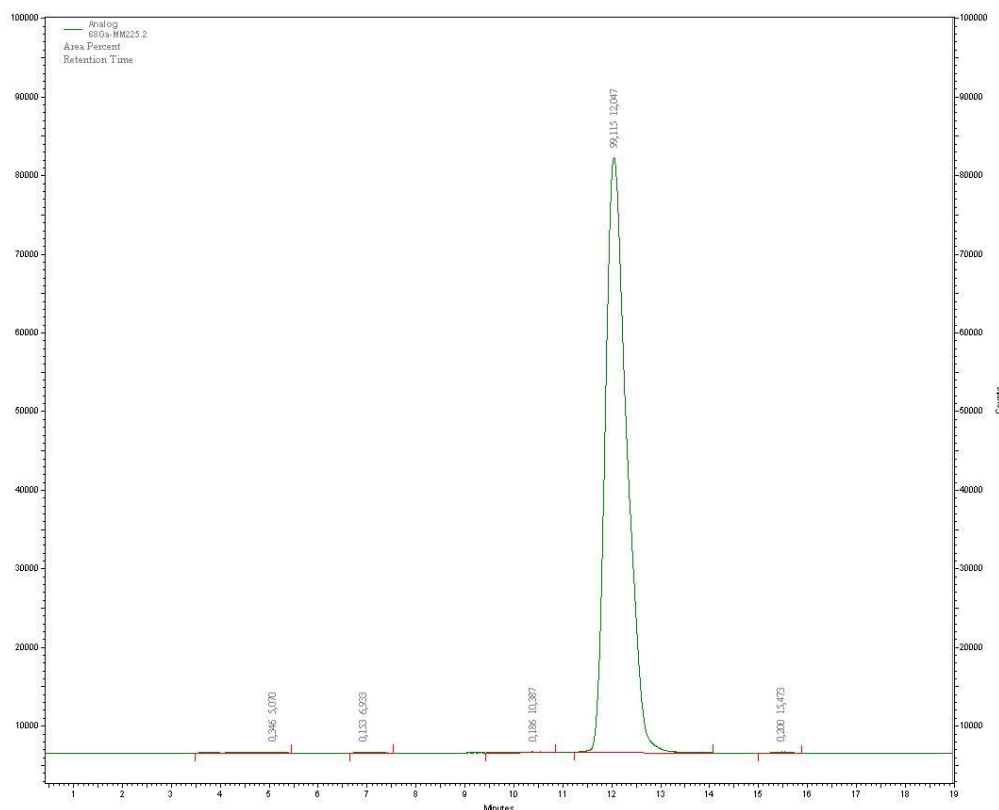


Figure S9: Analytical Radio-HPLC of $[^{68}\text{Ga}]\text{Ga-DO3A.Glu(Pyr).FAPi } ^{68}\text{Ga-9}$ ($t_R = 12.1$ min) after C18 cartridge purification, 20-30% MeCN + 0.1% TFA (linear gradient) in 10 min: RCP = 99.1%.

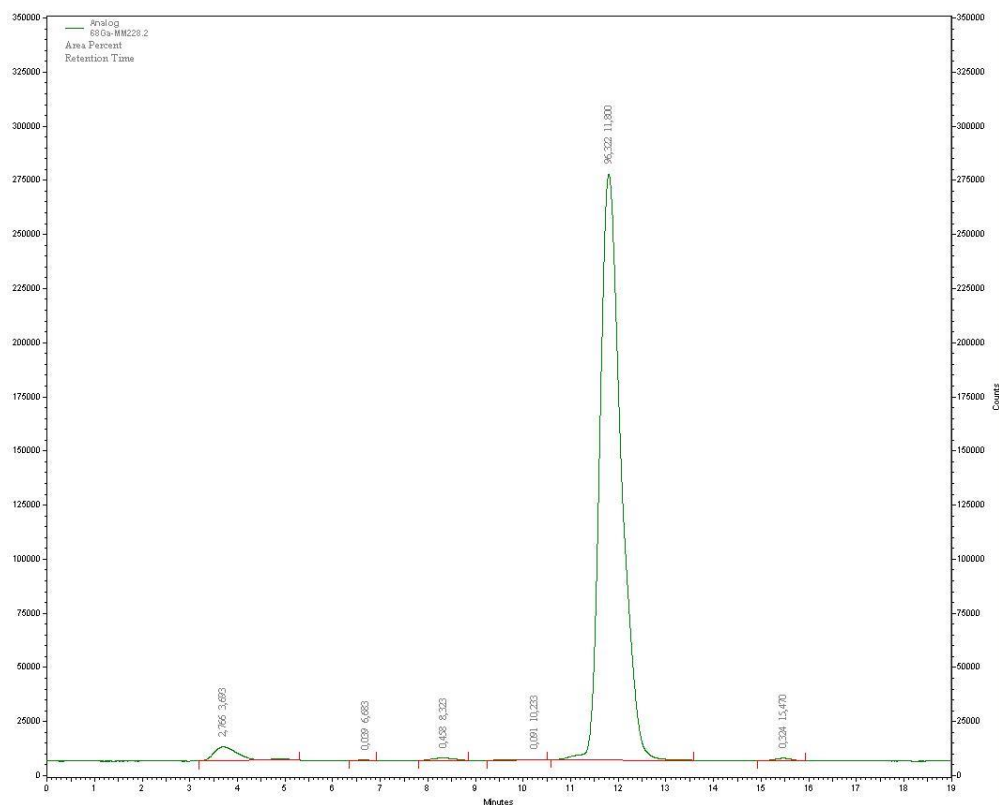


Figure S10: Analytical Radio-HPLC of $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu(Pyr).FAPi } ^{68}\text{Ga-11}$ ($t_R = 11.8$ min) after radiolabeling with 10 nmol **11**, and 50 MBq ^{68}Ga in 1 M HEPES (pH = 5.5), 20-30% MeCN + 0.1% TFA (linear gradient) in 10 min: RCP = 96.3%.

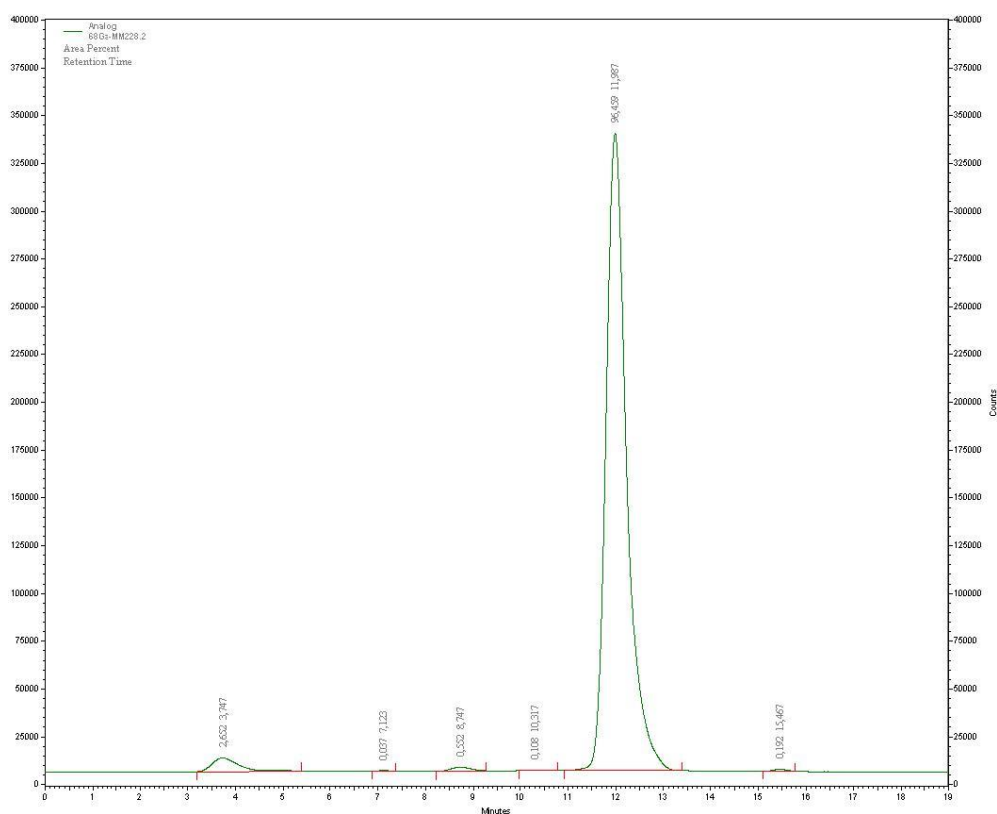


Figure S11: Analytical Radio-HPLC of [^{68}Ga]Ga-DOTAGA.Glu(Pyr).FAPi ^{68}Ga -**11** ($t_R = 12.0$ min) after radiolabeling with 10 nmol **11**, and 50 MBq ^{68}Ga in 1 M AmOAc (pH = 4.5), 20-30% MeCN + 0.1% TFA (linear gradient) in 10 min: RCP = 96.5%.

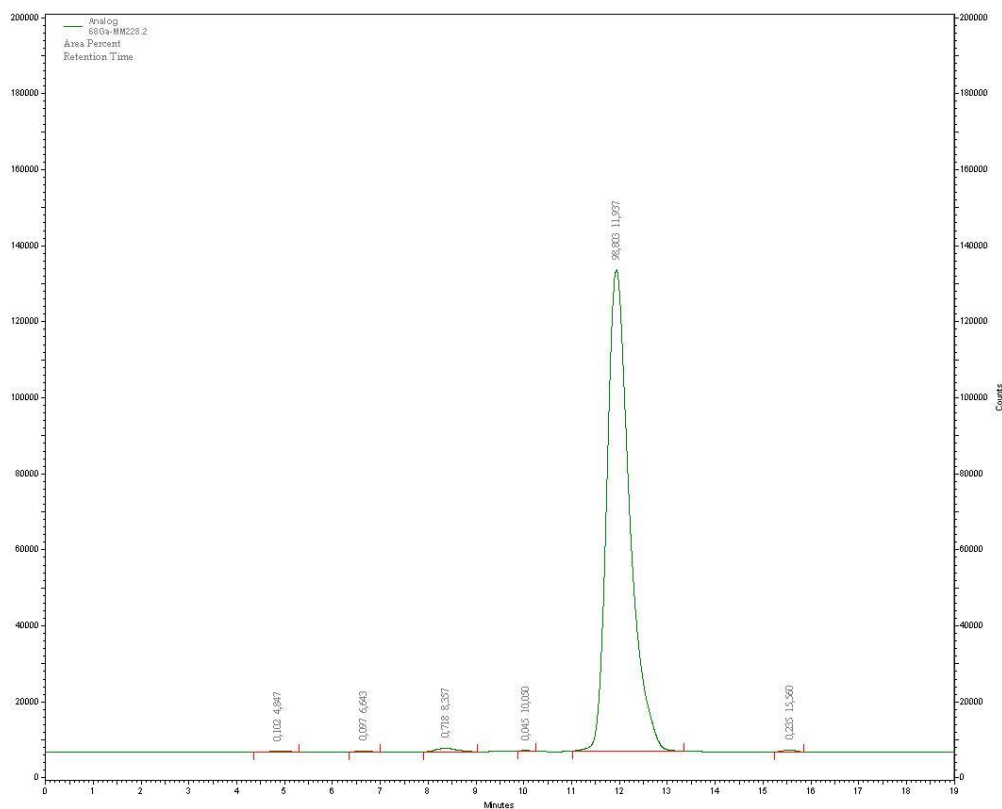


Figure S12: Analytical Radio-HPLC of [^{68}Ga]Ga-DOTAGA.Glu(Pyr).FAPi ^{68}Ga -**11** ($t_R = 11.9$ min) after C18 cartridge purification, 20-30% MeCN + 0.1% TFA (linear gradient) in 10 min: RCP = 98.8%.

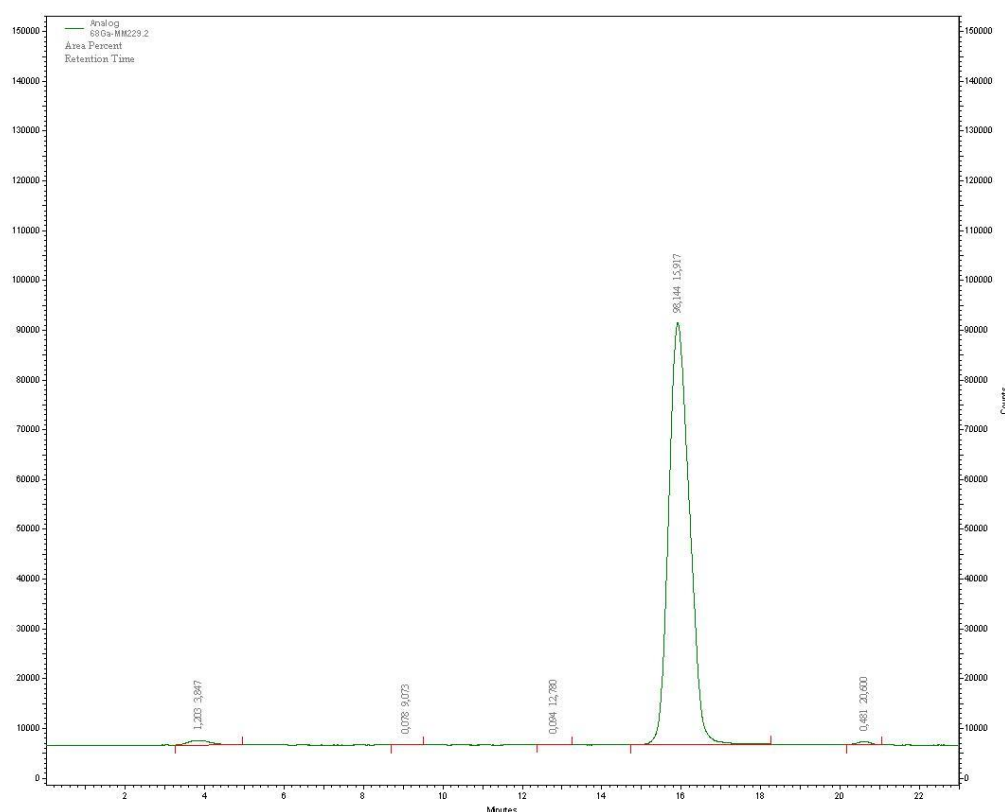


Figure S13: Analytical Radio-HPLC of $[^{68}\text{Ga}]\text{Ga-DATA}^{5\text{m}}.\text{Glu}(\text{Pyr}).\text{FAPi } ^{68}\text{Ga-13}$ ($t_R = 15.9$ min) after radiolabeling with 5 nmol **13**, and 50 MBq ^{68}Ga in 1 M HEPES (pH = 5.5), 20-30% MeCN + 0.1% TFA (linear gradient) in 15 min: RCP = 98.1%.

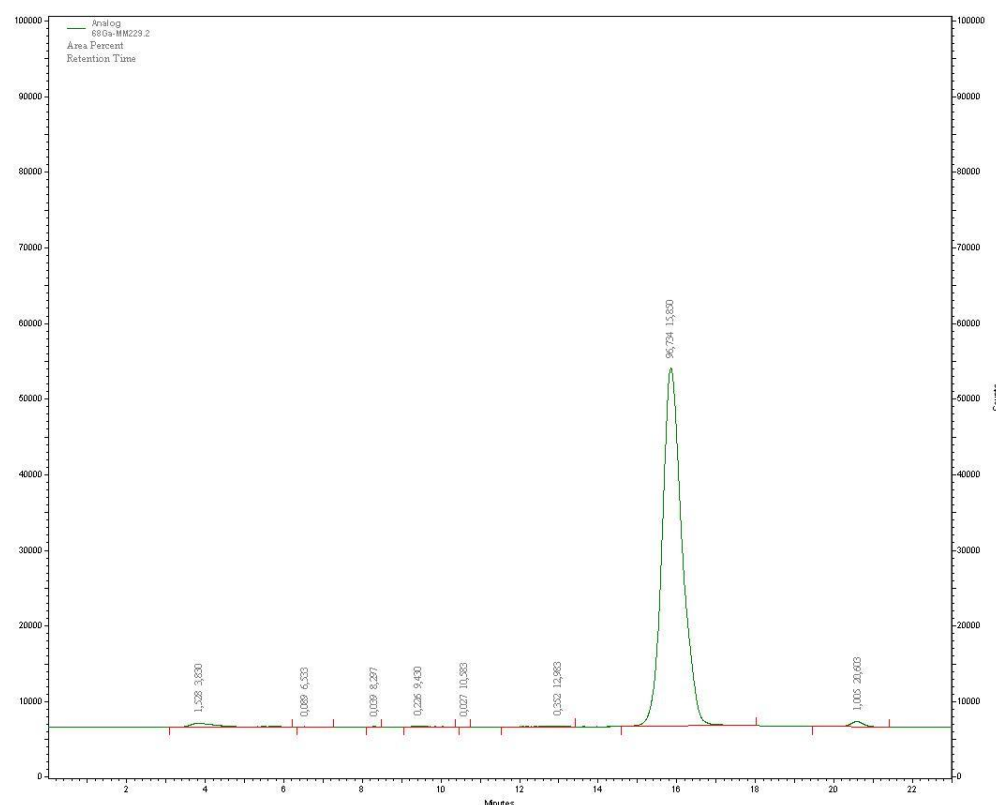


Figure S14: Analytical Radio-HPLC of $[^{68}\text{Ga}]\text{Ga-DATA}^{5\text{m}}.\text{Glu}(\text{Pyr}).\text{FAPi } ^{68}\text{Ga-13}$ ($t_R = 15.9$ min) after radiolabeling with 5 nmol **13**, and 50 MBq ^{68}Ga in 1 M AmOAc (pH = 4.5), 20-30% MeCN + 0.1% TFA (linear gradient) in 15 min: RCP = 96.7%.

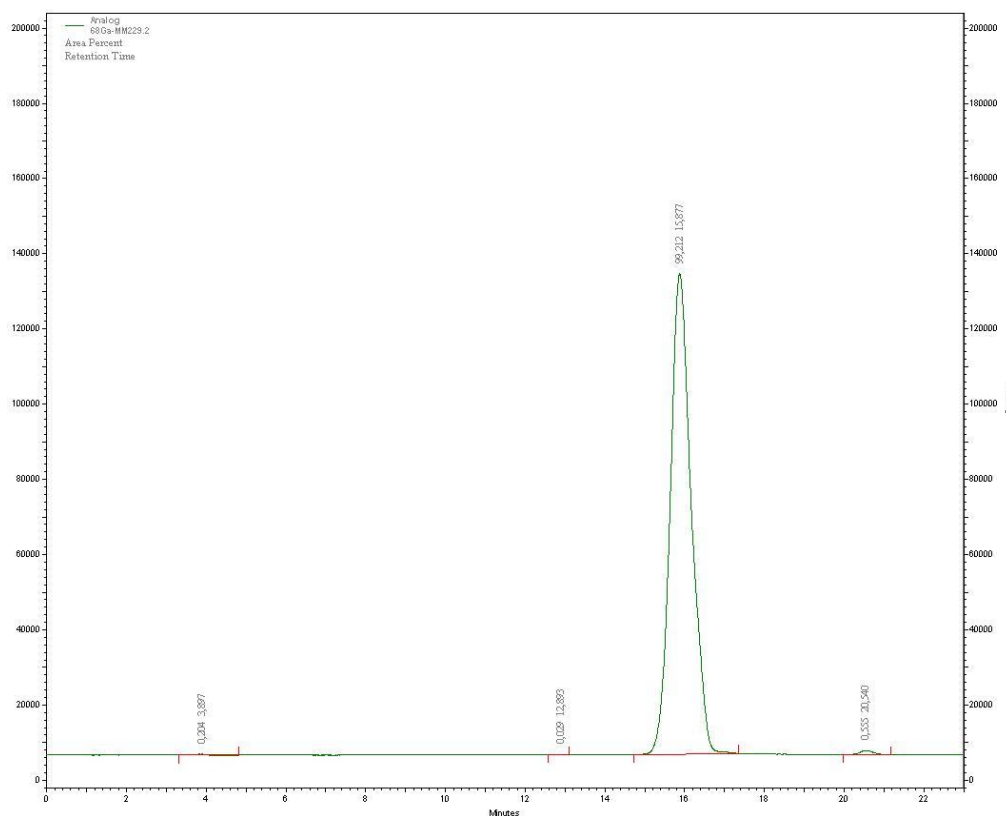


Figure S15: Analytical Radio-HPLC of $[^{68}\text{Ga}]\text{Ga-DATA}^{5\text{m}}.\text{Glu}(\text{Pyr}).\text{FAPi } ^{68}\text{Ga-13}$ ($t_R = 15.9$ min) after C18 cartridge purification, 20-30% MeCN + 0.1% TFA (linear gradient) in 15 min: RCP = 99.2%.

In Vitro-Inhibition Assays

Table S1: IC_{50} values for FAP and the respective selectivity index (SI) of different FAP inhibitors (precursors and metal complexes). The data for $[^{nat}Lu]Lu$ - and DOTAGA.Glu.(FAPi)₂ are taken from MARTIN et al. [51], for $[^{nat}Ga]Ga$ - and DOTA- and DATA^{5m}.SA.FAPi from MOON et al. [30,48] and for the initial FAP inhibitor UAMC-1110 from JANSEN et al. [27] and MOON et al. [48].

Compound	IC_{50} / nM	SI			
	FAP	PREP/FAP	DPP4/FAP	DPP8/FAP	DPP9/FAP
DO3A.Glu.(Pyr).FAPi (9)	0.22 ± 0.03	19955	> 26023	3290	5406
$[^{nat}Ga]Ga$ - DO3A.Glu.(Pyr).FAPi (^{nat} Ga-9)	0.34 ± 0.05	13767	> 29354	> 29354	> 29354
DOTAGA.Glu.(Pyr).FAPi (11)	0.35 ± 0.02	16328	> 14254	330	3648
$[^{nat}Ga]Ga$ - DOTAGA.Glu.(Pyr).FAPi (^{nat} Ga-11)	0.44 ± 0.03	23719	> 22962	3572	5188
DATA ^{5m} .Glu.(Pyr).FAPi (13)	0.42 ± 0.06	5565	> 11838	10581	4669
$[^{nat}Ga]Ga$ - DATA ^{5m} .Glu.(Pyr).FAPi (^{nat} Ga-13)	0.41 ± 0.11	8868	> 12227	> 12227	5590
DOTAGA.Glu.(FAPi) ₂	0.26 ± 0.04	2269	4577	112	319
$[^{nat}Lu]Lu$ - DOTAGA.Glu.(FAPi) ₂	0.33 ± 0.02	1292	1967	661	557
DOTA.SA.FAPi	0.9 ± 0.1	6000	-	-	-
$[^{nat}Ga]Ga$ -DOTA.SA.FAPi	1.4 ± 0.2	6214	> 714	-	-
DATA ^{5m} .SA.FAPi	0.8 ± 0.2	2113	-	-	-
$[^{nat}Ga]Ga$ - DATA ^{5m} .SA.FAPi	0.7 ± 0.1	6714	> 1429	-	-
UAMC-1110	0.43 ± 0.02	4186	23256	> 23256	10930

Table S2: IC_{50} values for FAP and respective selectivity index ($SI = IC_{50}(\text{respective protease})/IC_{50}(\text{FAP})$) of different FAP inhibitors. The data for $[^{nat}\text{Lu}]\text{Lu-}$ and $\text{DOTAGA.Glu.}(\text{FAPi})_2$ are taken from MARTIN et al. [51], for $[^{nat}\text{Ga}]\text{Ga-}$ and DOTA- and $\text{DATA}^{5m}.\text{SA.FAPi}$ from MOON et al. [30,48] and for the initial FAP inhibitor UAMC-1110 from JANSEN et al. [27] and MOON et al. [48].

Compound	$IC_{50} / \mu\text{M}$			
	PREP	DPP4	DPP8	DPP9
DO3A.Glu.(Pyr).FAPi (9)	4.36 ± 1.33	> 5	0.72 ± 0.23	1.18 ± 0.37
$[^{nat}\text{Ga}]\text{Ga-}$ DO3A.Glu.(Pyr).FAPi ($^{nat}\text{Ga-9}$)	4.69 ± 0.19	> 10	> 10	> 10
DOTAGA.Glu.(Pyr).FAPi (11)	5.73 ± 0.68	> 5	0.116 ± 0.004	1.28 ± 0.23
$[^{nat}\text{Ga}]\text{Ga-}$ DOTAGA.Glu.(Pyr).FAPi ($^{nat}\text{Ga-11}$)	10.33 ± 1.06	> 10	1.56 ± 0.26	2.26 ± 0.09
DATA^{5m}.Glu.(Pyr).FAPi (13)	2.35 ± 0.43	> 5	4.45 ± 0.07	1.97 ± 0.14
$[^{nat}\text{Ga}]\text{Ga-}$ DATA^{5m}.Glu.(Pyr).FAPi ($^{nat}\text{Ga-13}$)	3.63 ± 0.72	> 5	> 5	2.29 ± 0.33
DOTAGA.Glu.(FAPi)₂	0.59 ± 0.10	1.19 ± 0.08	0.029 ± 0.004	0.083 ± 0.0015
$[^{nat}\text{Lu}]\text{Lu-}$ DOTAGA.Glu.(FAPi)₂	0.43 ± 0.16	0.65 ± 0.04	0.22 ± 0.02	0.19 ± 0.01
DOTA.SA.FAPi	5.4 ± 0.3	-	-	-
$[^{nat}\text{Ga}]\text{Ga-DOTA.SA.FAPi}$	8.7 ± 0.9	> 1	-	-
DATA^{5m}.SA.FAPi	1.7 ± 0.1	-	-	-
$[^{nat}\text{Ga}]\text{Ga-}$ DATA^{5m}.SA.FAPi	4.7 ± 0.3	> 1	-	-
UAMC-1110	1.80 ± 0.01	> 10	> 10	4.70 ± 0.40

3.6. ^{99m}Tc -FAPi-Based Radiotracers for Application in SPECT Imaging of Various Cancers

In preparation for submission

Summary

SPECT imaging of epithelial cancers with ^{99m}Tc -radiotracers by targeting fibroblast activation protein alpha (FAP) has a very big potential as most nuclear medicine applications involve ^{99m}Tc -labeled radiotracers and most epithelial cancers strongly express FAP. Currently, there is no established pan-tumor ^{99m}Tc -radiotracer used in clinical routine yet that would be comparable to ^{18}F -FDG in PET.

Only few research articles about ^{99m}Tc -FAPi radiotracers have been published so far. The first FAPi precursor suited for ^{99m}Tc -labeling was FAPI-34 and was designed to be labeled with the $\text{Tc}(\text{I})$ tricarbonyl complex $\text{fac-}[^{99m}\text{Tc}][\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ that has to be synthesized first from $[\text{H}_2\text{O}][^{99m}\text{Tc}]\text{O}_4^-$ with an IsoLink® labeling kit and thus is a two step-procedure [49]. First patient studies demonstrated significant tumor uptake and good tumor-to-background-ratio (TBR), making it a suitable ^{99m}Tc -FAPi radiotracer. Besides that, FAPi-based ^{68}Ga -PET radiotracers were developed like DOTA- and DATA^{5m}.SA.FAPi by MOON et al. [25] that utilized squaric acid as a linker unit for the coupling of two amines. The resulting squaramide-based (SA) radiotracers showed fast and high tumor accumulation along with fast renal clearance from non-target tissues. High TBR at early time points enabled PET imaging at early time points [35].

Based on this, new SA-based FAPi radiotracers were developed by utilizing the same FAPi targeting vector (TV) based on UAMC-1110 characterized by high affinity and selectivity. The TV was coupled via SA to N_xS_y chelators ($x + y = 4$) for direct ^{99m}Tc -labeling. MAG3 (N_3S) and the tetramine chelator N4 (N_4) were chosen as chelators that are able to build stable complexes with the $\text{Tc}(\text{V})$ core.

The organic synthesis was started with en.SA.FAPi. It was synthesized in three steps in an overall yield of 56% and was used as a synthetic platform for the subsequent coupling of the two chelators. Prior attempts in our group to synthesize the precursor compound S-Bz-MAG3.SA.FAPi failed due to the instability of the Bz-protecting group under the basic conditions that are needed for the second amidation step of the SA coupling. Using different protecting groups like the acid-labile trityl group did not work as well. Therefore, a totally new synthesis route was developed that introduced the S-Bz-mercaptoacetic acid group in the final step. Following the 3-step synthesis of en.SA.FAPi, S-Bz-MAG3.SA.FAPi was synthesized successfully in another 6-step synthesis and N4.SA.FAPi in another 4-step synthesis, respectively. Here, an alternative new synthesis route for the preparation of the N4 chelator scaffold by using *N*-Boc-ethylenediamine instead of unprotected ethylenediamine (according to ABIRAJ et al. [61]) resulted in much higher yields (70% vs. 2%).

Both compounds demonstrated high FAP affinity with subnanomolar IC_{50} value for FAP and a similar PREP/FAP selectivity index ($SI = 5000 - 6000$) which is comparable to DOTA.SA.FAPi, ^{67}Ga -DOTA.SA.FAPi and ^{67}Ga -DATA^{5m}.SA.FAPi. DPP4/FAP selectivity was even higher for

the new compounds than for DOTA.SA.FAPi and DATA^{5m}.SA.FAPi. Both compounds showed promising results *in vitro* for following *in vivo* investigations.

Subsequently, the radiolabeling of S-Bz-MAG3.SA.FAPi with technetium-99m was investigated. Tin(II)chloride was used to reduce [^{99m}Tc]Tc^{VII}O₄⁻ to [^{99m}Tc][Tc^{VO}]³⁺ *in situ*. Tartrate was used as a weak intermediate exchange ligand that is needed to intercept Tc(V) and prevent further reduction to colloidal [^{99m}Tc]Tc^{IV}O₂ in a combined bicarbonate/acetate buffer system (pH = 8.5 – 9.0). Technetium-99m was manually labeled at room temperature (RT) and at 90 °C. 100 nmol S-Bz-MAG3.SA.FAPi at RT and 20 nmol at 90 °C were needed to label 1 GBq ^{99m}Tc quantitatively which was analyzed with two radio-TLC systems and radio-HPLC. Furthermore, an automated proof-of-principle synthesis demonstrated the production of 3.0 GBq [^{99m}Tc]Tc-MAG.SA.FAPi (radiochemical yield RCY = 52%). Complex stability was investigated for one day in human serum, saline at 37 °C and pure final formulation solution at RT. The complex was almost completely stable in human serum and around 90% stable in saline and pure formulation for four hours and still motly stable after 24 hours (four half-lives).

Author Contributions

Frank Roesch supervised this study and conceptualized it with MARCEL MARTIN's support. MARCEL MARTIN planned and carried out the organic synthesis whereby Deniz Göral and Kevin Machel supported the organic synthesis of S-Bz-MAG3.SA.FAPi and N4.SA.FAPi, respectively. The radiolabeling was carried out by MARCEL MARTIN supported by Lukas Greifenstein. The radiolabeling results were analyzed and interpreted by MARCEL MARTIN. The *in vitro* studies were carried out and analyzed by Yentl Van Rymenant, Joni De Loose and Emile Verhulst and interpreted together with MARCEL MARTIN. The first draft of the manuscript was written by MARCEL MARTIN and then revised by Lukas Greifenstein, Frank Roesch and MARCEL MARTIN.

^{99m}Tc -FAPi-Based Radiotracers for Application in SPECT Imaging of Various Cancers

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Abstract

Single-photon emission computed tomography (SPECT) with technetium-99m is the most used application in diagnostic nuclear medicine and nuclear medicine in general. Despite the high demand, there is no universal oncologic $^{99\text{m}}\text{Tc}$ -SPECT tracer yet that can fulfill a similar function as, for example, ^{18}F -FDG for PET. Radiotracers that target fibroblast activation protein alpha (FAP) address cancer-associated fibroblasts (CAFs) in the tumor microenvironment (TME) that are highly expressed in most epithelial tumors. The new compounds N4.SA.FAPi and S-Bz-MAG3.SA.FAPi are based on the selective FAP inhibitor UAMC-1110 and the targeting vector is coupled to the respective chelator MAG3 and N4 via squaramide (SA). S-Bz-MAG3.SA.FAPi and N4.SA.FAPi were synthesized successfully in 9 and 7 steps, respectively. *In vitro* binding studies demonstrated high affinity for FAP and very high selectivity over the related proteases PREP and DPP4 for both compounds.

$^{99\text{m}}\text{Tc}$ -MAG3.SA.FAPi was synthesized with quantitative radiochemical conversion and high radiochemical purity in manual as well automated synthesis and showed high complex stability for several hours in different media. $^{99\text{m}}\text{Tc}$ -MAG3.SA.FAPi is a promising candidate for further studies *in vivo*.

Keywords

Fibroblast activation protein alpha (FAP); FAP inhibitors (FAPi); FAPi-based radiotracers; FAPi SPECT imaging; single-photon emission computed tomography (SPECT); technetium-99m; $^{99\text{m}}\text{Tc}$; tumor microenvironment (TME); cancer-associated fibroblasts (CAFs); squaric acid

1. Introduction

Targeting fibroblast activation protein alpha (FAP) with radiotracers has emerged as an exciting new alternative for molecular imaging in oncology and several non-oncological conditions (liver, pancreatic or lung fibrosis, arthritis, etc.).

FAP is strongly expressed in the tumor microenvironment (TME) by cancer-associated fibroblasts (CAFs) in around 90% of all epithelial tumors [1,2]. FAP is additionally expressed by cancer cells of different soft-tissue and bone sarcomas [3–6], as well as by activated fibroblasts in tissues of wound healing/scar formation, inflammation and fibrosis [7–9]. In contrast, there is no FAP expression in healthy tissue of adults which makes it a universal target for oncology and beyond. FAP is a post proline-selective serine protease of the S9b family and a type II transmembrane glycoprotein with a unique dual enzymatic activity [7,10]. It has dipeptidyl peptidase (DPP) activity like the closely related proteases DPP4, DPP8 and DPP9 although its endopeptidase activity to cleave peptides after a Gly-Pro unit preferably is more efficient where it competes with the only other post-proline selective prolyl endopeptidase (PREP) [11–13]. In particular DPP4 and PREP are ubiquitously expressed in healthy tissue [14].

The TME, also called tumor stroma, is important for the tumor supply and consists of many different components like different cell types (inflammatory, immune, vascular, CAFs) and extracellular matrix (ECM) that supports tumor growth by providing nutrients and other mechanisms [1,15]. Every epithelial lesion that is bigger than around 1 mm build an extensive TME than can make up to 90% of the total tumor mass [1,15]. CAFs are an essential part of the TME and involved in tumor proliferation, metastasis and tumor invasion by producing cytokines, chemokines, growth factors and enzymes that mainly induce ECM degradation and remodeling [16,17]. CAFs pave the way for cancer cells to invade new neighboring tissue and even distant metastatic sites [18–20].

A radiotracer targeting FAP must have a high affinity for FAP and low affinity to the related proteases. An optimized FAP inhibitor (FAPi) UAMC-1110 (Figure 1a) was reported by JANSEN et al. [21,22] in extensive structure-activity relationship (SAR) studies. It was later functionalized with a linker and DOTA chelator yielding the first FAPi-based radiotracers like FAPI-04 and FAPI-46 [23,24], followed by DOTA.SA.FAPi (Figure 1b) and DATA^{5m}.SA.FAPi that used squaramide (SA) as a linker unit [25]. SA allows for pH-controlled selective asymmetric amidation of two amines under mild conditions and has already proven to be advantageous in several radiopharmaceutical applications like PSMA inhibitor- or FAP inhibitor-based radiotracers [25–31].

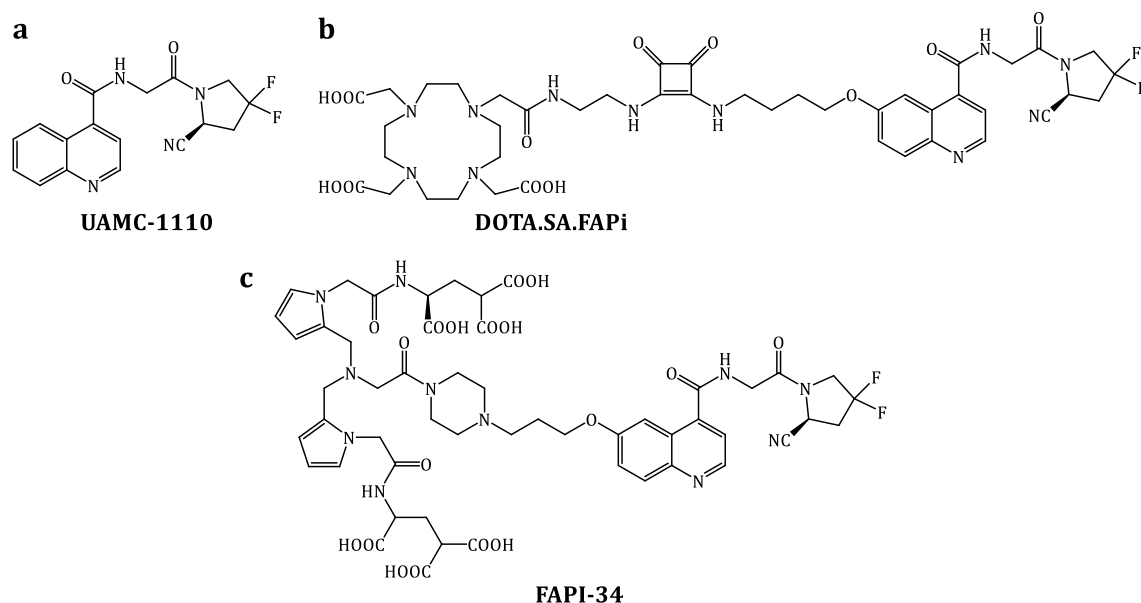


Figure 1: Structures of a) the FAP inhibitor UAMC-1110, b) the FAP ligand DOTA.SA.FAPi for ^{68}Ga -PET and c) the FAP ligand FAPI-34 for $^{99\text{m}}\text{Tc}$ -SPECT.

Further FAPi-based radiopharmaceuticals like QCP02 [32] or OncoFAP-DOTAGA [33] with slightly different FAPi-targeting vectors were designed for the application with trivalent radiometals like gallium-68 for positron emission tomography (PET) imaging. All these compounds showed fast accumulation in the TME and fast renal clearance to give high tumor-background ratios (TBR) in many cancer types after only a couple of minutes post-injection [5,34,35].

Technetium-99m is the most-used diagnostic radionuclide in nuclear medicine and is broadly available due to the $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ radionuclide generator. Despite the dominating role of $^{99\text{m}}\text{Tc}$ -SPECT there is no universal oncological radiotracer like ^{18}F -FDG for PET. There definitely is a big demand and potential for such tracers to improve medical coverage, not only in low- and middle-income countries that have a limited number of PET scanners but predominantly use SPECT scanners.

Nevertheless, the focus of the current research and the majority of the publications on FAP imaging deal with ^{68}Ga -labeled radiotracers for PET and explore its diagnostic value in many different cancer types. A lot of effort is being put into the translation of the great diagnostic performance into an optimized therapeutic counterpart. This theranostic concept is realized with FAPi dimers [30,36–41] or cyclic peptidomimetic structures [42–45] that focus on trivalent radiometals. Other PET radiotracers using fluorine-18 have also been published [46–48].

The first FAPi-based compound that was labeled with technetium-99m for single-photon emission computed tomography (SPECT) imaging was FAPI-34 (Figure 1c) which can be labeled with the technetium-99m tricarbonyl complex $\text{fac-}[^{99\text{m}}\text{Tc}][\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ [49]. Other $^{99\text{m}}\text{Tc}$ -FAPi radiotracers known to us are FL-L3- $^{99\text{m}}\text{Tc}$ [50], $^{99\text{m}}\text{Tc}$ -HFAPi [51,52], $^{99\text{m}}\text{Tc}$ -iFAP [53,54]

and the hexamer $[^{99m}\text{Tc}][\text{Tc}-(\text{CN-PEG}_4\text{-FAPi})_6]^+$ [55]. HFAPi and iFAP are labeled via HYNIC (6-hydrazinonicotinamide) strategy and iFAP utilizes an Ala-boroPro FAP targeting vector. FL-L3- ^{99m}Tc also uses a FAPi-targeting vector different to UAMC-1110 with 4-pyridinoyl instead of 4-quinolinoyl moiety.

Our approach for the new ^{99m}Tc -FAPi radiotracers was based on the squaramide linker strategy that was applied from DOTA and DATA^{5m}.SA.FAPi and on the other hand on the functionalized N₃S chelator MAG3 (mercapto acetyl triglycine) or the tetraamine chelator N4 that can form stable complexes with Tc(V). The structures of the precursors N4.SA.FAPi and S-Bz-MAG3.SA.FAPi as well as $[^{99m}\text{Tc}]\text{Tc-MAG3.SA.FAPi}$ are shown in Figure 2.

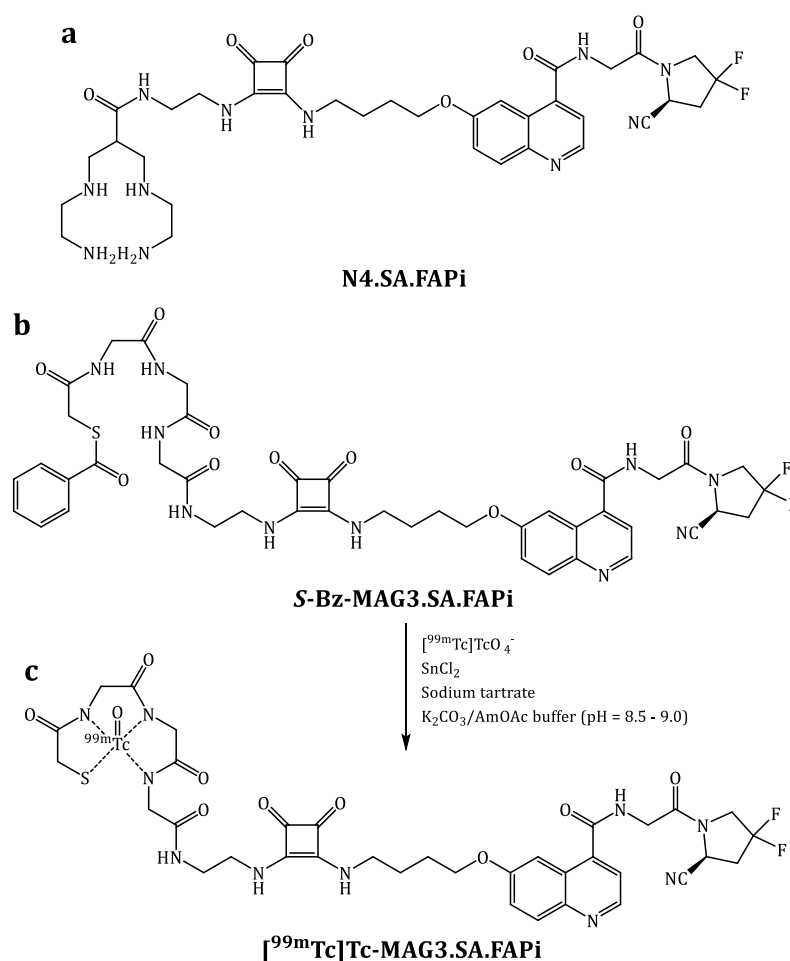


Figure 2: Structures of the FAPi precursors a) N4.SA.FAPi, b) S-Bz-MAG3.SA.FAPi and c) the new FAPi radiotracer $[^{99m}\text{Tc}]\text{Tc-MAG3.SA.FAPi}$.

The tricarbonyl strategy is a two-step reaction where the tricarbonyl complex $\text{fac-}[^{99m}\text{Tc}][\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ has to be synthesized first. Our approach is supposed to enable direct labeling either at elevated temperatures or at room temperature for potential kit-type labeling. The organic synthesis and subsequent ^{99m}Tc -radiolabeling was carried out. Furthermore, IC_{50} values for FAP and the related proteases PREP, DPP4, 8 and 9 are reported that were determined in affinity studies *in vitro*.

2. Materials and Methods

2.1. Chemicals and Equipment

Chemicals were purchased from Sigma-Aldrich and Merck (Darmstadt, Germany), TCI (Eschborn, Germany), Alfa Aesar by Thermo Fisher Scientific (Kandel, Germany), Acros Organics (Schwerte, Germany), ABCR (Karlsruhe, Germany) and VWR (Bruchsal, Germany). Dry solvents were purchased from Acros Organics, Alfa Aesar by Thermo Fischer Scientific and Merck and used directly.

(*S*)-6-(4-aminobutoxy)-*N*-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide (FAPi-NH₂*TFA) was purchased from KE Biochem Co. (Shanghai, China).

Thin-layer chromatography plates (TLCs) from Merck, coated with silica gel 60 F₂₅₄, were used. They were analyzed via UV lamp (λ = 254 and 366 nm) and staining with potassium permanganate. Silica 60 (0.040-0.063 mm or 0.063–0.200 mm particle size) was used for column chromatography.

Nuclear magnetic resonance (NMR) spectra were recorded in deuterated solvents, purchased from Deutero (Kastellaun, Germany). An Avance II 400 spectrometer (400 MHz, 5 mm BBFO sample head with z-gradient and ATM, SampleXPress 60 sample changer) or an Avance III 300 spectrometer (300 MHz, 5 mm BBFO sample head with z-gradient and ATM, BACS 60 sample changer) from Bruker (Rheinstätten, Germany) were used. The spectra were analyzed using MestReNova 14.2.0 software from Mestrelab Research (Santiago de Compostela, Spain).

Electrospray ionization mass spectrometry coupled with liquid chromatography (ESI-LC/MS) was measured with an Agilent Technologies (Santa Clara, USA) 1220 Infinity LC coupled to an Agilent Technologies 6130B Single Quadrupole LC/MS system. The separation was realized with an Agilent Zorbax SB-C18 column (21x50 mm, 1.8 μ m) and a linear gradient of acetonitrile (MeCN)/Milli-Q® water + 0.05% formic acid (flow rate: 0.5 mL/min).

LaChrom HPLC (7000 series) from Merck Hitachi with L-7100 pump, L-7400 UV detector (λ = 254 nm), a D-7000 interface and autosampler was used for semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC). A Phenomenex Synergi Max-RP C18 column (250x10 mm, 4 μ m), linear gradient of MeCN/H₂O + 0.1% trifluoacetic acid (TFA) and a flow rate of 5 mL/min was used for separation via RP-HPLC.

Only chemicals of the highest purity (TraceSelect if available) were used for ^{99m}Tc-radiolabeling. Radio-TLCs were developed with two mobile phase systems: ethyl acetate/methyl ethyl ketone (EA/MEK, (2:3)) and 0.25 M ammonium acetate (AmOAc, pH = 4.0)/methanol (MeOH) (1:1). All radio-TLCs were evaluated using a TLC miniGita scanner (Elysia-Raytest, Angleur, Belgium) with the analysis software GINA star 6.3 (Elysia-Raytest, Angleur, Belgium). Analytical radio-HPLC was performed using an analytical HPLC from Agilent (Infinity 1200) with a Ramona * radio-detector

(Elysia-Raytest, Angleur, Belgium), the column VDSpher PUR 150 C18-E 5 μm (100 $\times$ 40 mm), linear gradient 5–45% MeCN + 0.1% TFA in 10 min (flow rate: 1 mL/min) and analyzed with GINA star 6.3 (Elysia-Raytest, Angleur, Belgium).

2.2. Organic Synthesis

2.2.1. Synthesis of en.SA.FAPi

(S)-N-(2-(2-Cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)-6-(4-((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)amino)butoxy)quinoline-4-carboxamide (1, SA.FAPi)

FAPi-NH₂*TFA (134 mg, 245 μmol , 1.50 eq) was dissolved in ethanol (EtOH, 3.5 mL) and both 3,4-diethoxy-3-cyclobutene-1,2-dione (27.7 mg, 24.1 μL , 163 μmol , 1.00 eq) and triethylamine (TEA, 17.0 μL , 123 μmol , 0.75 eq) was added. The reaction was stirred at room temperature (RT) for one day. More TEA (17.0 μL , 123 μmol , 0.75 eq) was added and stirring continued at room temperature. After one day each, FAPi-NH₂*TFA (22.3 mg, 40.8 μmol , 0.25 eq) and TEA (5.6 μL , 40.5 μmol , 0.25 eq) was added two more times, respectively. The reaction solution was stirred at RT for one more day (4 days in total) before the solvents were under reduced pressure. SA.FAPi **1** was obtained after column chromatography (dichloromethane (DCM)/MeOH (100:7)) as a colorless solid (82.9 mg, 149 μmol , 92%). ¹H-NMR (400 MHz, DMSO-d₆): δ [ppm] = 9.10 (t, J = 6.0 Hz, 1H), 8.83 (d, J = 4.4 Hz, 1H), 8.00 (d, J = 9.2 Hz, 1H), 7.89 (d, J = 2.8 Hz, 1H), 7.54 (d, J = 4.4 Hz, 1H), 7.47 (dd, J = 9.2, 2.7 Hz, 1H), 5.13 (d, J = 9.2 Hz, 1H), 4.63 (p, J = 6.8 Hz, 2H), 4.39–4.06 (m, 6H), 3.57 (q, J = 6.6 Hz, 1H), 3.39 (q, J = 6.5 Hz, 1H), 3.02–2.76 (m, 2H), 1.89–1.67 (m, 4H), 1.40–1.27 (m, 3H). MS (ESI⁺): m/z (%) = 556.2 (100, [M+H]⁺), 557.2 (31, [M+H]⁺), calculated for C₂₇H₂₇F₂N₅O₆: 555.19 [M].

tert-butyl (S)-2-((2-((4-((4-((2-(2-Cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)ethyl)carbamate(2, Boc-en.SA.FAPi)
SA.FAPi **1** (92.2 mg, 166 μmol , 1.00 eq) and TEA (66.0 μL , 476 μmol , 2.87 eq.) were dissolved in EtOH (5 mL) and *N*-Boc-ethylenediamine (50.3 μL , 50.8 mg, 317 μmol , 1.91 eq.) was added and stirred at RT overnight. More TEA (66.0 μL , 476 μmol , 2.87 eq.) was added and the reaction was stirred for another day at 40 °C. The solvents were removed under reduced pressure and the crude product was purified with column chromatography (DCM/MeOH (100:5–10)) to yield Boc-en.SA.FAPi **2** (67.8 mg, 101 μmol , 61%) as a colorless solid. ¹H-NMR (300 MHz, MeOD-d₄): δ [ppm] = 8.74 (d, J = 4.5 Hz, 1H), 8.02–7.93 (m, 2H), 7.56 (d, J = 4.5 Hz, 1H), 7.45 (dd, J = 9.3, 2.7 Hz, 1H), 5.13 (dd, J = 9.3, 3.2 Hz, 1H), 4.42–4.03 (m, 6H), 3.67 (dd, J = 5.5, 3.9 Hz, 2H), 3.59–3.53 (m, 2H),

3.25–3.16 (m, 2H), 3.06–2.71 (m, 2H), 2.03–1.77 (m, 4H), 1.39 (s, 9H). MS (ESI⁺): m/z (%) = 670.3 (100, [M+H]⁺), 671.3 (39, [M+H]⁺), calculated for C₃₂H₃₇F₂N₇O₇: 669.27 [M].

(S)-6-(4-((2-((2-Aminoethyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)butoxy)-N-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide (**3**, en.SA.FAPi)

Boc-en.SA.FAPi **2** (58.3 mg, 87.0 μmol, 1.00 eq) was dissolved in DCM (3 mL) and TFA (1 mL) and was stirred at RT for 5 hours. The solvents were removed via co-distillation with MeOH (6 × 10 mL) and a yellow solid was obtained which was used in the next step without further purification. MS (ESI⁺): m/z (%) = 285.7 (100, [M+H]²⁺), 570.3 (50, [M+H]⁺), 571.3 (16, [M+H]⁺), calculated for C₂₇H₂₉F₂N₇O₅: 569.22 [M].

2.2.2. Synthesis of N4.SA.FAPi

3-((2-((tert-Butoxycarbonyl)amino)ethyl)amino)-2-(((2-((tert-butoxycarbonyl)amino)ethyl)amino)methyl)propanoic acid (**4**, Di-Boc-N4)

3-Bromo-2-(bromomethyl)propionic acid (1.05 g, 4.27 mmol) and *tert*-butyl (2-aminoethyl) carbamate (2.02 g, 12.6 mmol, 2.95 eq) were dissolved in tetrahydrofuran (THF, 2 mL). The reaction solution was stirred at RT for one day. The precipitated solid was then filtered and washed with cold THF and dried *in vacuo*. Di-Boc-N4 **4** (1.72 g, 4.25 mmol, 100%) was quantitatively obtained as a colorless solid. ¹H-NMR (400 MHz, DMSO-d₆): δ [ppm] = 7.02 (t, *J* = 5.7 Hz, 2H), 3.26–3.16 (m, 4H), 3.05–2.87 (m, 8H), 2.64–2.53 (m, 1H), 1.39 (s, 18H). MS (ESI⁺): m/z (%) = 405.3 (100, [M+H]⁺), 406.3 (20, [M+H]⁺), calculated for C₁₈H₃₆N₄O₆: 404.26 [M].

3-((tert-Butoxycarbonyl)(2-((tert-butoxycarbonyl)amino)ethyl)amino)-2-(((tert-butoxycarbonyl)(2-((tert-butoxycarbonyl)amino)ethyl)amino)methyl)propanoic acid (**5**, Tetra-Boc-N4)

Di-Boc-N4 **4** (1.70 g, 4.20 mmol, 1.00 eq) was dissolved in 1,4-dioxane (50 mL) and 1 M sodium hydroxide (46 mL, 46 mmol, 11.0 eq). Di-*tert*-butyldicarbonate (Boc₂O, 6.34 g, 29.5 mmol, 7.02 eq) was added and the reaction solution was stirred at RT overnight. 1,4-Dioxane was removed under reduced pressure and the pH of the remaining aqueous solution was adjusted to pH = 3 with 1 M hydrochloric acid. A colorless solid precipitated that was filtered after cooling. The solid was washed with cold water and yielded Tetra-Boc-N4 **5** (1.78 g, 2.94 mmol, 70%) after drying *in vacuo*. ¹H-NMR (400 MHz, MeOD-d₄): δ [ppm] = 3.60–3.15 (m, 12H), 3.14–2.96 (m, 1H), 1.49 (s, 18H), 1.45 (s, 18H). MS (ESI⁺): m/z (%) = 405.4 (63, [M–2Boc+H]⁺), 627.4 (100, [M+Na]⁺), 628.4 (33, [M+Na]⁺), calculated for C₂₈H₅₂N₄O₁₀: 604.37 [M].

Di-tert-butyl ((7-((2-((2-((4-((2-((2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)ethyl)carbamoyl)-2,2,12,12-tetramethyl-4,10-dioxo-3,11-dioxo-5,9-diazatridecane-5,9-diyl)bis(ethane-2,1-diyl))(S)-dicarbamate (6, Tetra-Boc-N4.SA.FAPi)

Tetra-Boc-N4 **5** (135 mg, 222 μ mol, 1.00 eq) and 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU, 119 mg, 313 μ mol, 1.41 eq) were dissolved in dry *N,N*-dimethylformamide (DMF, 2 mL) with *N,N*-diisopropylethylamine (DIPEA, 57.9 μ L, 340 μ mol, 1.53 eq) under argon atmosphere. The solution was stirred at RT for 30 minutes. Then, a solution of en.SA.FAPi **3** (64.3 mg, 113 μ mol, 0.51 eq) and DIPEA (122 μ L, 717 μ mol, 3.23 eq) in dry DMF (2 mL) was added and stirred overnight. HATU (57.0 mg, 150 μ mol, 0.68 eq) was added again and stirring was continued for another 6 hours. DMF was removed under reduced pressure. The residue was taken up in EA (75 mL) and washed with 1 M citric acid (15 mL) and brine (15 mL). The aqueous phase was extracted with EA (3 $\times$ 15 mL) and the combined organic phase was dried over Na₂SO₄ and EA was removed *in vacuo* again. The crude product was purified via column chromatography (CHCl₃/MeOH (100:6–10)) and Tetra-Boc-N4.SA.FAPi **6** (78.0 mg, 67.4 μ mol, 30%) was obtained as a yellow solid. MS (ESI⁺): *m/z* (%) = 530.3 (100, [M–Boc+H]²⁺), 604.3 (18, [M+Na]²⁺), calculated for C₅₅H₇₉F₂N₁₁O₁₄: 1155.58 [M].

(S)-6-(4-((2-((2-((3-((2-Aminoethyl)amino)-2-(((2-aminoethyl)amino)methyl)propanamido)ethyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)butoxy)-N-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide (7, N4.SA.FAPi)

Milli-Q® water (25 μ L), triisopropylsilane (TIPS, 25 μ L) and TFA (950 μ L) was added to Tetra-Boc-N4.SA.FAPi **6** (78.0 mg, 67.4 μ mol). The reaction was stopped after one hour at RT by adding MeOH (10 mL) to remove all the solvents via co-distillation. MeOH was added two more times. The crude product was purified via RP-HPLC (16% MeCN isocratic, *t_R* = 9–10 min) and N4.SA.FAPi **7** (23.0 mg, 30.4 μ mol, 45%) was obtained as a yellow solid (99.0% purity: cf. analytical RP-HPLC in Figure S1). ¹H-NMR (600 MHz, D₂O): δ [ppm] = 8.96 (dd, *J* = 5.4, 1.1 Hz, 1H), 8.16 (d, *J* = 9.4 Hz, 1H), 8.03 (dd, *J* = 5.5, 1.0 Hz, 1H), 7.85 (d, *J* = 2.6 Hz, 1H), 7.76 (dd, *J* = 9.4, 2.5 Hz, 1H), 5.11 (dd, *J* = 9.1, 3.7 Hz, 1H), 4.42–4.08 (m, 6H), 3.71–3.55 (m, 4H), 3.48–3.34 (m, 12H), 3.26 (dd, *J* = 13.1, 4.6 Hz, 2H), 3.12 (tt, *J* = 8.3, 4.6 Hz, 1H), 2.99–2.87 (m, 2H), 1.97–1.89 (m, 2H), 1.86–1.78 (m, 2H). ¹⁹F-NMR (400 MHz, D₂O): δ [ppm] = –76.78 (s). MS (ESI⁺): *m/z* (%) = 252.8 (85, [M+H]³⁺), 378.8 (100, [M+H]²⁺), 756.4 (42, [M+H]⁺), 757.4 (18, [M+H]⁺), 778.3 (21, [M+Na]⁺), calculated for C₃₅H₄₇F₂N₁₁O₆: 755.37 [M].

2.2.3. Synthesis of *S*-Bz-MAG3.SA.FAPi

((9H-Fluoren-9-yl)methoxy)carbonyl)glycylglycylglycine (8, Fmoc-G3)

Sodium carbonate (840 mg, 7.93 mmol, 2.50 eq) was dissolved in water (10 mL) and a solution of triglycine (G3, 600 mg, 3.17 mmol, 1.00 eq) in THF (5 mL) was added. The reaction mixture was cooled to 0 °C, 9-fluorenylmethoxycarbonyl chloride (FmocCl, 821 mg, 3.17 mmol, 1.00 eq) was added in portions. Afterwards, the reaction was allowed to warm to RT and stirring was continued for one day at RT. THF was removed under reduced pressure and after more water was added, the aqueous phase was washed with ethyl acetate (3 × 80 mL). Then, the aqueous phase was acidified with 1 M hydrochloric acid to pH = 2.0 under ice cooling. The acidified solution was extracted with ethyl acetate (4 × 100 mL) again. The organic phase was washed with brine (4x50 mL) and dried over Na₂SO₄. EA was removed under reduced pressure and Fmoc-G3 **8** (87.0 mg, 210 µmol, 7%) was obtained as a colorless solid. ¹H-NMR (300 MHz, DMSO-d₆): δ [ppm] = 8.16 (q, *J* = 6.2 Hz, 2H), 7.89 (d, *J* = 7.5 Hz, 2H), 7.71 (d, *J* = 7.4 Hz, 2H), 7.57 (t, *J* = 6.1 Hz, 1H), 7.37 (dt, *J* = 26.4, 7.3 Hz, 4H), 4.33 – 4.17 (m, 3H), 3.75 (dd, *J* = 5.9, 3.5 Hz, 4H), 3.66 (d, *J* = 5.5 Hz, 2H). MS (ESI⁺): *m/z* (%) = 412.1 (100, [M+H]⁺), 413.1 (26, [M+H]⁺), calculated for C₂₁H₂₁N₃O₆: 411.14 [M].

(9H-Fluoren-9-yl)methyl (S)-(2-((2-((2-((2-((4-((2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)ethyl)amino)-2-oxoethyl)amino)-2-oxoethyl)amino)-2-oxoethyl)carbamate (9, Fmoc-G3.SA.FAPi)

Fmoc-G3 **8** (30.9 mg, 75.1 µmol, 1.00 eq) was dissolved in dry DMF (1.5 mL) with DIPEA (15.3 µL, 90.0 µmol, 1.20 eq) under argon atmosphere and HATU (31.4 mg, 82.6 µmol, 1.10 eq) was added. The solution was stirred at RT for one hour. Then, a solution of en.SA.FAPi **3** (42.6 mg, 75.0 µmol, 1.00 eq) and DIPEA (31.9 µL, 188 µmol, 2.50 eq) in dry DMF (1.5 mL) was added and stirring at RT was continued overnight. DMF was removed *in vacuo* and after column chromatography (DCM/MeOH (10:1)) Fmoc-G3.SA.FAPi **9** (68.4 mg, 71.0 µmol, 95%) was obtained as a yellowish solid. MS (ESI⁺): *m/z* (%) = 482.2 (100, [M+H]²⁺), 963.3 (38, [M+H]⁺), 964.3 (22, [M+H]⁺), 965.3 (7, [M+H]⁺), calculated for C₄₈H₄₈ F₂N₁₀O₁₀: 962.35 [M].

(S)-6-(4-((2-((2-((2-((2-Aminoacetamido)acetamido)ethyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)butoxy)-N-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide (10, G3.SA.FAPi)

Fmoc-G3.SA.FAPi **9** (66.8 mg, 69.4 µmol, 1.00 eq) was dissolved in dry DMF (1.6 mL) and piperidine (400 µL) and the solution was stirred at RT for one hour under argon atmosphere. The solvents were removed under reduced pressure and gave **10** as a colorless solid in quantitative

yield that was used in the next step without further purification. MS (ESI⁺): *m/z* (%) = 741.3 (100, [M+H]⁺), 742.3 (33, [M+H]⁺), calculated for C₃₃H₃₈F₂N₁₀O₈: 740.28 [M].

2-(Benzoylthio)acetic acid (11, S-Bz-MA)

Thioglycolic acid (4.60 g, 3.48 mL, 50.0 mmol, 1.00 eq) and sodium hydroxide (4.40 g, 110 mmol, 2.20 eq) were dissolved in toluene/water (1:1, 20 mL each). At 0 °C, benzoyl chloride (7.00 g, 5.76 mL, 50.0 mmol, 1.00 eq) was added to the reaction mixture with a dropping funnel. The reaction mixture was then stirred for another 10 minutes at 0 °C and one hour at room temperature. The organic phase was then separated and washed with water (4 × 15 mL). The combined aqueous phases were acidified to pH = 0.6 with 37% hydrochloric acid (10 mL). The colorless precipitate was filtered and washed with water (10 mL) and 1 M hydrochloric acid (1 mL). The crude product was then recrystallized from EA twice (85 mL and 15 mL) to yield S-Bz-MA **11** (4.80 g, 24.5 mmol, 49%) as colorless crystals. ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 8.01 – 7.94 (m, 2H), 7.61 (ddt, *J* = 8.7, 7.0, 1.3 Hz, 1H), 7.47 (ddt, *J* = 7.9, 6.6, 1.2 Hz, 2H), 3.93 (s, 2H). MS (ESI⁺): *m/z* (%) = 197.1 (22, [M+H]⁺), 214.0 (72, [M+NH₄]⁺), 219.0 (100, [M+Na]⁺), calculated for C₉H₈O₃S: 196.02 [M].

2,5-Dioxopyrrolidin-1-yl 2-(benzoylthio)acetate (12, S-Bz-MA-NHS)

S-Bz-MA **11** (500 mg, 2.55 mmol, 1.00 eq), *N*-hydroxysuccinimide (NHS, 322 mg, 2.80 mmol, 1.10 eq) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC*HCl, 586 mg, 3.06 mmol, 1.20 eq) were dissolved in dry DCM (15 mL) under argon atmosphere. The solution was stirred at RT for two hours. More NHS (58.7 mg, 0.51 mmol, 0.20 eq) and EDC*HCl (97.8 mg, 0.51 mmol, 0.20 eq) was added and stirring continued for one more day. DCM (50 mL) was added and the organic phase was washed with 0.1 M hydrochloric acid (3 × 50 mL) and brine (5 mL). The organic phase was dried over Na₂SO₄, filtered, and DCM was removed. The colorless crude product was recrystallized from EA (10 mL) and S-Bz-MA-NHS **12** was obtained as a colorless crystalline solid (423 mg, 1.44 mmol, 57%). ¹H-NMR (300 MHz, CDCl₃): δ [ppm] = 8.02 – 7.95 (m, 2H), 7.65 – 7.58 (m, 1H), 7.52 – 7.45 (m, 2H), 4.17 (s, 2H), 2.84 (s, 4H). MS (ESI⁺): *m/z* (%) = 179.0 (100, [M-NHS+H]⁺), 311.1 (69, [M+NH₄]⁺), 316.0 (45, [M+Na]⁺), calculated for C₁₃H₁₁NO₁₃S: 293.04 [M].

(S)-6-(4-((2-((2-(2-(2-(2-Aminoacetamido)acetamido)acetamido)ethyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)butoxy)-N-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide (13, S-Bz-MAG3.SA.FAPi)

G3.SA.FAPi **10** (51.4 mg, 69.4 μmol, 1.00 eq) and S-Bz-MA-NHS **12** (20.4 mg, 69.4 μmol, 1.00 eq) were dissolved in dry THF (2 mL) and dry DMF (0.5 mL) under argon atmosphere. After one day at RT more S-Bz-MA-NHS **12** (10.2 mg, 34.7 μmol, 0.50 eq) in dry THF (3 mL) and DMF (1 mL) was added and after stirring at RT for two more days the solvents were removed under reduced pressure. The crude product was purified via RP-HPLC (27% MeCN isocratic, *t_R* = 12–13 min) and

S-Bz-MAG3.SA.FAPi **13** (14.1 mg, 15.3 μ mol, 22%) was obtained as a yellow solid (95.2% purity: cf. analytical RP-HPLC in Figure S2). ^1H -NMR (600 MHz, DMSO- d_6): δ [ppm] = 9.13 (t, J = 6.0 Hz, 1H), 8.84 (d, J = 4.3 Hz, 1H), 8.53 (t, J = 5.7 Hz, 1H), 8.23 (t, J = 5.8 Hz, 1H), 8.14 (t, J = 5.9 Hz, 1H), 8.03 – 7.88 (m, 4H), 7.74 – 7.68 (m, 1H), 7.60 – 7.54 (m, 2H), 7.49 (dd, J = 9.2, 2.7 Hz, 1H), 7.39 (s, 1H), 5.15 (dd, J = 9.5, 2.7 Hz, 1H), 4.51 – 4.43 (m, 1H), 4.40 – 4.30 (m, 2H), 4.25 (d, J = 5.9 Hz, 2H), 4.21 – 4.10 (m, 4H), 3.89 (s, 2H), 3.81 – 3.74 (m, 4H), 3.71 – 3.49 (m, 6H), 3.24 (s, 2H), 2.99 – 2.79 (m, 2H), 1.89 – 1.81 (m, 2H), 1.78 – 1.70 (m, 2H). ^{19}F -NMR (600 MHz, DMSO- d_6): δ [ppm] = –75.50 (s). MS (ESI $^+$): m/z (%) = 460.2 (82, $[\text{M}+\text{H}]^{2+}$), 919.2 (100, $[\text{M}+\text{H}]^+$), 920.2 (50, $[\text{M}+\text{H}]^+$), 921.2 (19, $[\text{M}+\text{H}]^+$), calculated for $\text{C}_{42}\text{H}_{44}\text{F}_2\text{N}_{10}\text{O}_{10}\text{S}$: 918.29 [M].

2.3. Radiosynthesis ($^{99\text{m}}\text{Tc}$ -Radiolabeling)

$[\text{}^{99\text{m}}\text{Tc}]\text{TcO}_4^-$ was eluted from a Tekciv $^{99}\text{Mo}/^{99\text{m}}\text{Tc}$ -generator (GE Healthcare, Chicago, Illinois, United States) with 5 – 10 mL isotonic NaCl solution (0.9%, saline).

A stock solution and **13** (1 mM in $\text{H}_2\text{O}/\text{EtOH}$, 1:1) was prepared and stored at –20 °C before use. Tin(II) chloride dihydrate ($\text{SnCl}_2 \cdot 2 \text{H}_2\text{O}$) solution was prepared freshly each day. SnCl_2 -solution was prepared as a 4 mg/mL $\text{SnCl}_2 \cdot 2 \text{H}_2\text{O}$ solution in 0.01 M hydrochloric acid with the addition of ascorbic acid (1 mg/mL).

2.3.1. Manual Labeling of *S*-Bz-MAG3.SA.FAPi **13**

The labeling was carried out similarly to the method previously published by WANG et al. [56].

A labeling buffer (0.5 M potassium bicarbonate, 0.25 M sodium acetate and 0.18 M ammonium hydroxide, pH = 8.5 – 9.0) was prepared and disodium tartrate dihydrate was dissolved in the labeling buffer (c = 50 mg/mL).

The mixture of 0.25 M sodium acetate (pH = 7, 200 – 250 μ L), tartrate in labeling buffer I (40 – 50 μ L) and the precursor **13** (10 – 100 nmol, 10 – 100 μ L 1 mM stock solution) was prepared and the freshly prepared SnCl_2 solution (10 μ L) was added shortly before labeling. $[\text{}^{99\text{m}}\text{Tc}]\text{TcO}_4^-$ (0.5 – 1.0 GBq, 200 – 400 μ L) was added and the reaction was carried out at RT or at 90 °C.

Reaction kinetics were investigated by taking samples after 5, 15 and 30 min and were analyzed via radio-TLCs. Two mobile phase systems were used: ethyl acetate/methyl ethyl ketone (EA/MEK, 3:2) and 0.25 M ammonium acetate (pH = 4.0)/methanol (AmOAc/MeOH, 1:1). The radio-TLCs were analyzed by integrating the spots at baseline (R_f = 0.0) and on top of the TLC (R_f > 0.7). With the EA/MEK system the respective product and “colloidal $^{99\text{m}}\text{Tc}$ ” ($[\text{}^{99\text{m}}\text{Tc}]\text{TcO}_2$) stay on the baseline and “free $^{99\text{m}}\text{Tc}$ ” ($[\text{}^{99\text{m}}\text{Tc}]\text{TcO}_4^-$) moves up. With AmOAc/MeOH, $[\text{}^{99\text{m}}\text{Tc}]\text{TcO}_2$ is on the baseline and the product as well as $[\text{}^{99\text{m}}\text{Tc}]\text{TcO}_4^-$ are on the top. The radiochemical conversion (RCC) is calculated as follows:

$$\text{RCC [\%]} = \text{EA/MEK (Baseline)} - \text{AmOAc/MeOH (Baseline)} \quad (1)$$

Analytical radio-HPLCs of the samples taken after 30 minutes were recorded for every labeling to determine radiochemical purity (RCP) and double-check RCC. Additionally, selected radio-HPLCs were measured at some of the earlier time points.

2.3.2. Automated Labeling of *S-Bz-MAG3.SA.FAPi 13*

An automated synthesis was carried out using the All-In-One mini synthesis module (Trasis, Ans, Belgium) with a disposable cassette. Subsequently, an Oasis HLB Plus cartridge was used to agitate the final compound. Elution of the product was performed with 0.5 mL ethanol. The product was then formulated via a sterile filter with 10 mL of saline. The setup is schematically shown in Figure S3.

A mixture of 0.25 M sodium acetate (pH = 7, 500 μL), tartrate in labeling buffer (100 μL), **13** (100 nmol, 100 μL 1 mM stock solution) and SnCl_2 -solution (25 μL) was prepared and put into the buffer vial and $[^{99\text{m}}\text{Tc}]\text{TcO}_4^-$ (ca. 5.8 GBq, 2.0 mL) was added using a syringe. The reaction was heated for 15 minutes at 90 $^\circ\text{C}$ and purified via Oasis HLB Plus cartridge and sterile-filtered afterward. $^{99\text{m}}\text{Tc}$ -**13** (3.0 GBq, RCY = 52%, RCP = 85%) was obtained and the final product was analyzed via radio-HPLC and the two radio-TLC systems (see above: EA/MEK and AmOAc/MeOH).

2.3.3. Complex Stability Measurements of $[^{99\text{m}}\text{Tc}]\text{Tc-MAG3.SA.FAPi } ^{99\text{m}}\text{Tc-13}$

After **13** (20 nmol) was radiolabeled with $^{99\text{m}}\text{Tc}$ (1.0 GBq) for 30 minutes at 90 $^\circ\text{C}$ (RCC = 92.5%, RCP = 75.9%), the stability in human serum (HS) and saline was investigated (n = 3) by adding 75 MBq of the reaction solution to 500 μL HS and saline (≈ 150 MBq/mL) and incubating for one day at 37 $^\circ\text{C}$. Additionally, the complex stability in the final formulation ($\approx 0.5 - 1.0$ GBq/mL) was investigated for one day at RT. Samples were taken after 0.5, 1, 2, 4, and 24 hours, respectively. The complex stability was determined via radio-TLC with EA/MEK as the mobile phase. Additional radio-TLCs of the sample after 24 hours were developed using AmOAc/MeOH and radio-HPLCs were measured after 1, 4 and 24 hours.

2.4. In Vitro-Inhibition Assays (IC_{50} Measurements)

DPP8 (dipeptidyl peptidase VIII), DPP 9 (dipeptidyl peptidase IX) and rhFAP (recombinant human fibroblast activation protein) were expressed and purified as previously described [57,58]. DPP4 (dipeptidyl peptidase IV) was purified from human seminal plasma [59]. Human recombinant PREP (prolyl endopeptidase) was expressed in BL21(DE3) cells and purified using immobilized co-chelating chromatography (GE healthcare), followed by anion-exchange chromatography on a 1 mL Mono Q column (GE healthcare) [57].

IC_{50} (FAP) measurements of the samples were performed using Z-Gly-Pro-7-amino-4-methylcoumarine (AMC, Bachem) as the substrate at a concentration of 50 μ M at pH 8 in 0.05 M Tris-HCl buffer, 1 mg/mL BSA and 140 mM NaCl.

IC_{50} (PREP) measurements of the samples were carried out using *N*-succinyl-Gly-Pro-AMC (Bachem) as the substrate at a concentration of 250 μ M at pH 7.4 in 0.1 M K-phosphate, 1 mM EDTA, 1 mM DTT and 1 mg/mL BSA.

IC_{50} values for DPP4, DPP8 and DPP9 were determined using Ala-Pro-*para*nitroanilide (*p*NA) as the substrate at the respective final concentrations of 25 μ M (DPP4), 300 μ M (DPP8) or 150 μ M (DPP9) at pH 7.4 (0.05 M HEPES-NaOH buffer with 0.1 % Tween-20, 0.1 mg/mL BSA and 150 mM NaCl).

At least eight concentrations of inhibitors were tested. The final DMSO concentration was kept constant during the experiment to exclude any effects. Inhibitors were pre-incubated with the enzyme for 15 minutes at 37 °C, afterwards the substrate was added and the velocities of AMC release were measured kinetically at λ_{ex} = 380 nm and λ_{em} = 465 nm (or 405 nm for *p*NA) for at least 10 minutes at 37 °C.

All IC_{50} measurements were performed using the Infinite 200 instrument from Tecan Group Ltd. (Männedorf, Switzerland) and analyzed using Magellan software. The data were fitted using a nonlinear fit model in GraFit7.

3. Results and Discussion

3.1. Organic Synthesis

3.1.1. Synthesis of en.SA.FAPi

The FAPi-targeting vector is supposed to be coupled to the respective chelators via squaramide (SA).

Squaric acid can be used to synthesize asymmetric squaramides. In aqueous solution this can be realized by pH control. At neutral pH, only the monoamide is formed and more basic conditions (pH = 9) are needed to get the diamide. This allows asymmetric amidation with two different amines. Alternatively, the reaction can also be carried out in organic solvents such as methanol or ethanol with the addition of an organic base like triethylamine (TEA). For the first part of the reaction only small amounts of base (around 1 eq) are sufficient whereas higher excess of base, and possibly heating, is required to couple the second amine [25,26,31,60].

At first, the compound en.SA.FAPi **3** was synthesized according to Figure 3.

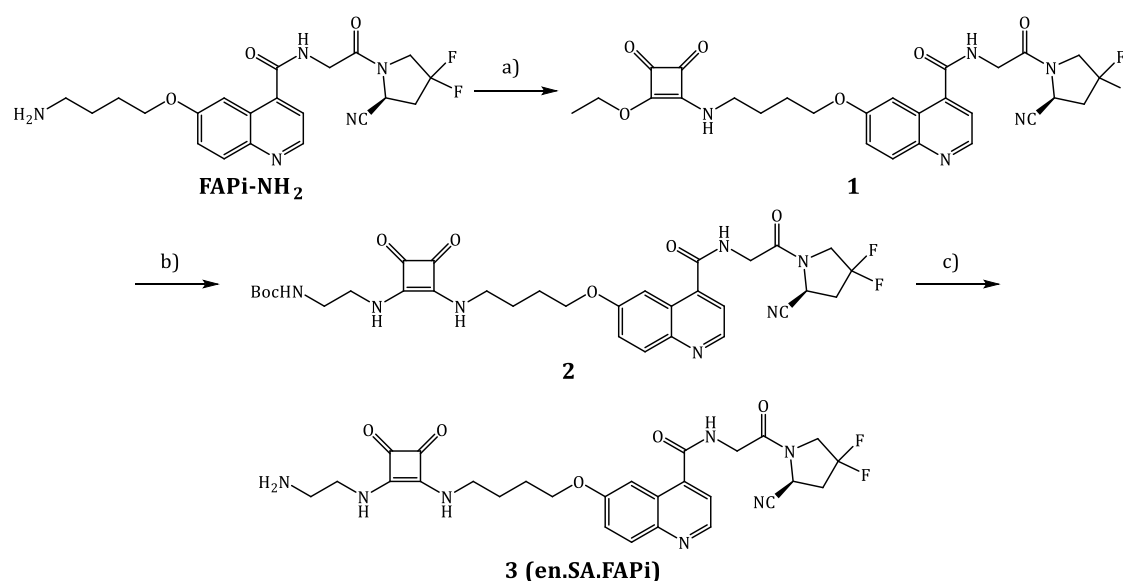


Figure 3: Synthesis of en.SA.FAPi (**3**): a) 3,4-diethoxy-3-cyclobutene-1,2-dione, TEA (1.50 eq), EtOH, RT, 4 d, 92%; b) N-Boc-ethylenediamine, TEA (5.75 eq), EtOH, RT–40 °C, 2 d, 61%; c) TFA/DCM (1:3), RT, 5 h, 100%.

At first, FAPi-NH₂ was coupled with diethyl squarate to yield FAPi.SA **1**. The reaction was started with TEA (0.9 eq) and FAPi-NH₂ (1.5 eq) but more of both had to be added (1.5 eq TEA and 2.0 eq FAPi-NH₂) to reach a quantitative conversion of diethyl squarate. N-Boc-ethylenediamine was then coupled to **1** with an excess of TEA (5.75 eq) and heated at 40 °C. Finally, the Boc-protective group was cleaved with 25% TFA in DCM to obtain en.SA.FAPi **3** in an overall yield of 56%.

The coupling order might be exchanged in the future to first couple N-Boc-ethylenediamine to diethyl squarate, followed by FAPi-NH₂ coupling afterwards.

3.1.2. Synthesis of N4.SA.FAPi

For the synthesis of the N4 chelator, a procedure according to the work from ABIRAJ et al. [61] was tried first. However, tetra-Boc-N4 **5** was obtained with a very low yield of only 2% (data not shown). Problems resulted from the excess ethylenediamine and from getting all four amine groups protected with Boc. Above all, the secondary amines were harder to protect probably due to steric hindrance. N4.SA.FAPi **7** was instead synthesized according to a new route as shown in Figure 4.

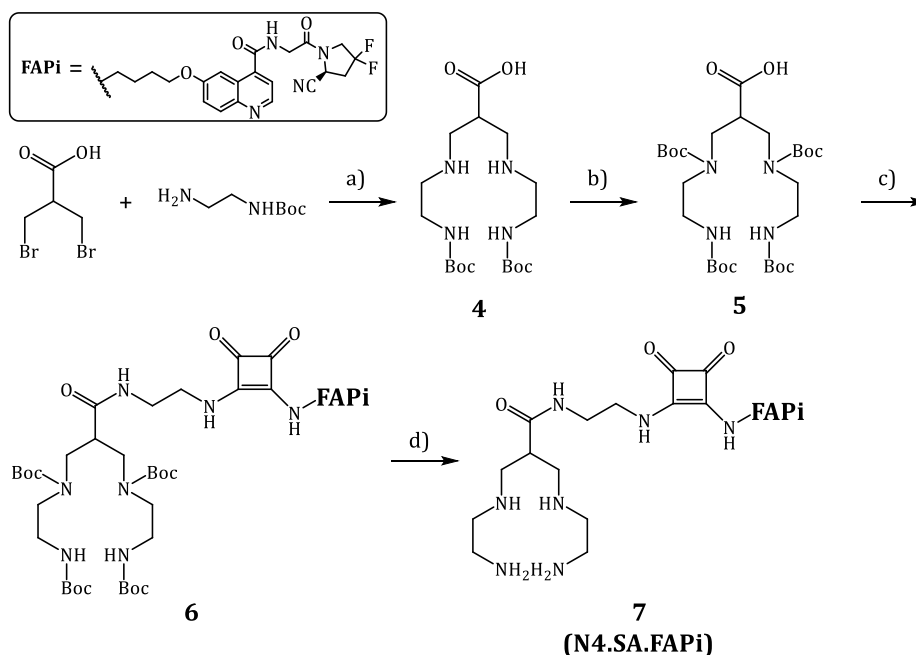


Figure 4: N4.SA.FAPi (**7**) synthesis scheme: a) THF, RT, 1 d, 100%; b) Boc_2O , 1,4-dioxane/1 M NaOH (1:1), RT, 1 d, 70%; c) 1. HATU, DIPEA, DMF, RT, 30 min; 2. en.SA.FAPi **3**, DIPEA, DMF, RT, 1 d, 30%; d) TFA/TIPS/ H_2O (95:2.5:2.5), RT, 1 h, 45%.

N-Boc-ethylenediamine was used instead of unprotected ethylenediamine. Di-Boc-N4 intermediate **4** was obtained in quantitative yield. Subsequently, only the two secondary amines had to be protected which was done with excess di-tert-butyl dicarbonate (Boc_2O , 7.0 eq) to yield Tetra-Boc-N4 **5** with an overall yield of 70% compared to 2% with the previous route. Afterwards, the chelator was coupled to en.SA.FAPi **3** with HATU and gave the protected precursor **6** (30%) after column chromatography. After deprotection in TFA/TIPS/ H_2O (95:2.5:2.5) and semipreparative RP-HPLC N4.SA.FAPi **7** was obtained in 45% and an overall yield of 9% (4 steps). Theoretically, there are other possibilities like a synthesis route similar to DOTA.SA.FAPi and DATA^{5m}.SA.FAPi [25]. In this case, the protected chelator **5** would be coupled with N-Boc-ethylenediamine first, then coupled with diethyl squarate followed by FAPi- NH_2 before the chelator is deprotected in the final step.

Since en.SA.FAPi **3** was needed as a building block for the synthesis of S-Bz-MAG3.SA.FAPi **13** (see next chapter), it was chosen to utilize building block **3** for the preparation of N4.SA.FAPi **7** as well.

3.1.3. Synthesis of *S*-Bz-MAG3.SA.FAPi

The chelator *S*-Bz-MAG3 was synthesized at first following an unpublished route. Then, amide coupling with *N*-Boc-ethylenediamine or the asymmetric squaramide coupling was tested but the basic conditions led to the cleavage of the benzoyl-protective group of the thiol. *S*-Trityl-MAG3 was also tried as a protective group that is stable under basic conditions but did not yield any product in subsequent squaramide coupling.

Due to these previous experiences, the synthesis route was changed in a way that the benzoyl-protected thiol group was introduced into the molecule in the final step as shown in Figure 5.

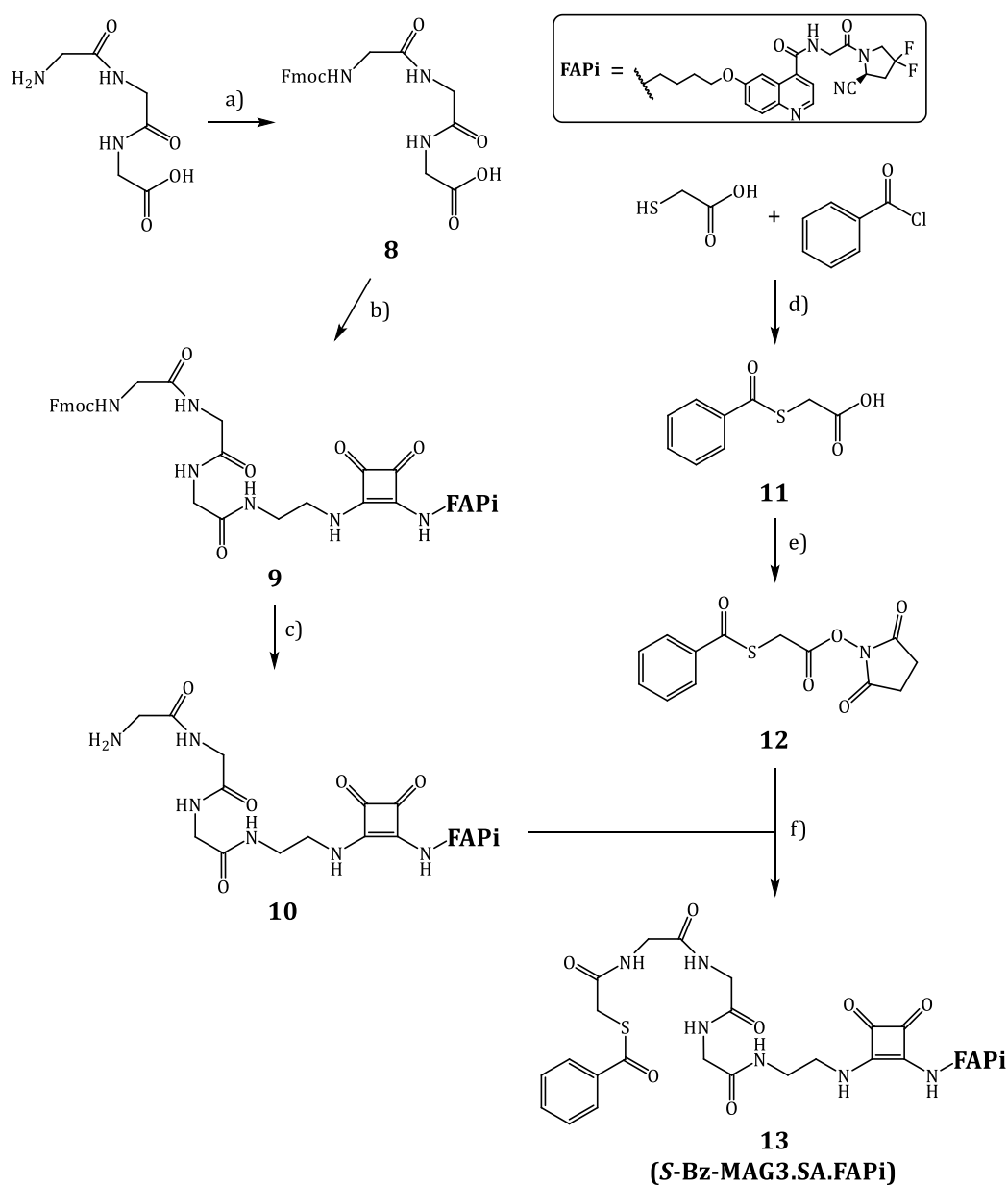


Figure 5: *S*-Bz-MAG3.SA.FAPi (**13**) synthesis scheme: a) FmocCl, Na₂CO₃, H₂O/THF (1:2), 0 °C–RT, 1 d, 7%; b) HATU, DIPEA, DMF, RT, 1 h; 2. en.SA.FAPi **3**, DIPEA, DMF, RT, 1 d, 95%; c) piperidine/DMF (1:4), RT, 1 h, 100%; d) NaOH, toluene/water (1:1), 0 °C–RT, 1.5 h, 49%; e) NHS, EDC·HCl, DCM, RT, 1 d, 57%; f) THF/DMF (4:1), RT, 3 d, 22%.

Triglycine (G3) was protected with a Fmoc protective group first. The previously synthesized building block en.SA.FAPi **3** was then coupled with HATU and DIPEA in DMF. Deprotection of the Fmoc-protected amine yielded the intermediate G3.SA.FAPi **10**.

In parallel, *S*-Bz-MA **11** was synthesized from thioglycolic acid and benzoyl chloride under basic conditions (NaOH) as described by BRANDAU et al. [62]. The carboxylic acid was supposed to be converted to an activated NHS ester. The original synthesis protocol from BRANDAU et al. [62] with NHS and *N,N'*-dicyclohexylcarbodiimide (DCC) was not optimal because it was not possible to separate *N,N'*-dicyclohexylurea (DCU) from the product *S*-Bz-MA-NHS **12**. However, with EDC*HCl the resulting urea was separated completely by washing the organic DCM phase with diluted hydrochloric acid and brine yielding **12** after recrystallization.

12 and **10** were coupled in the absence of base and two test reactions were carried out in 0.1 M phosphate-buffer (pH = 8.0)/DMF (1:5) and THF/DMF. The aqueous reaction did show some problems to dissolve the reagents which is why more DMF needed to be added. Furthermore, the purification was less convenient, and conversion analyzed via LC/MS was inferior. The reaction was eventually carried out in THF/DMF (4:1) and *S*-Bz-MAG3.SA.FAPi **13** was obtained after semipreparative RP-HPLC purification with 22%.

3.2. Radiosynthesis of [^{99m}Tc]Tc-MAG3.SA.FAPi (^{99m}Tc-13)

Cleavage of the *S*-Bz-protecting group must happen *in situ* just like the reduction of [^{99m}Tc]TcO₄⁻ to [^{99m}Tc][Tc^{VO}]³⁺. The radiolabeling of *S*-Bz-MAG3.SA.FAPi **13** with technetium-99m was based on WANG et al. [56]. Tartrate was used as the weak exchange ligand and tin(II) chloride as the reducing agent. The reaction kinetics were investigated at RT and at 90 °C with two different precursor amounts at each temperature and are depicted in Figure 6.

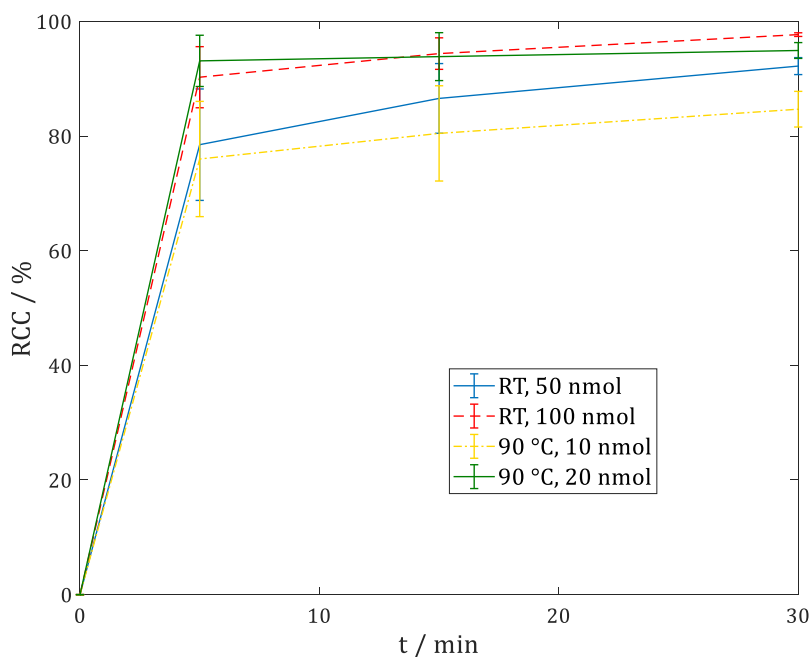


Figure 6: Reaction kinetics (radiochemical conversion (RCC) in %) of [^{99m}Tc]Tc-MAG3.SA.FAPi ^{99m}Tc -**13** at RT or 90 °C with 1.0 GBq ^{99m}Tc and 10–100 nmol **13**. All reactions were carried out in triplicate ($n = 3$).

S-Bz-MAG3.SA.FAPi **13** can be labeled at room temperature. High RCC > 90%, but no quantitative RCC, can be achieved with 50 nmol **13** as there is still some free ^{99m}Tc after 30 minutes. In contrast, no [^{99m}Tc]TcO $_4^-$ is left with 100 nmol after 30 minutes and the RCC is already > 90% after 5 minutes and can be confirmed via radio-HPLC (Figure S4).

As expected, labeling at 90 °C leads to much faster labeling kinetics and less precursor **13** needed. 10 nmol is not enough for quantitative radiolabeling and some formation of colloidal [^{99m}Tc]TcO $_2$ is observed. In contrast, 20 nmol **13** gives quantitative RCC = $97.6 \pm 0.5\%$ after 5 minutes with no substantial formation of [^{99m}Tc]TcO $_2$ (cf. Table S1). The RCC decreases slightly when the reaction times are too long (RCC = $95.7 \pm 1.6\%$ after 30 min). Radio-HPLCs as shown in Figure S5 also show that the RCP decreases with longer reaction times at 90 °C. In fact, an additional peak at 6.0 min occurs that increases over time. N $_x$ S $_y$ -complexes of the [^{99m}Tc][Tc $^{\text{VO}}$] $^{3+}$ core can have two stereoisomers (*syn*- and *anti*-conformation). For example, the peak with a retention time (t_R) of 7.0 minutes might be the kinetic product whereas the other isomer ($t_R = 6.0$ min) is the thermodynamic product as this peak ($t_R = 6.0$ min) seems to increase over time.

Besides the aim for a “kit-type” production automated synthesis are very helpful in a routine production environment. Therefore, translation to an automated system was performed using the Trasis All-in-one mini synthesis module. 100 nmol **13** were labeled with 5.8 GBq ^{99m}Tc at 90 °C for 15 minutes. 3.0 GBq ^{99m}Tc -**13** (RCY = 52%) were obtained after purification via Oasis HLB plus cartridge and subsequent sterile filtration. Figure 7 shows the analytical radio-HPLC as well as both radio-TLCs. Slight formation of two side products occurred again ($t_R = 6.0$ and 7.5 min). Maybe a shortening of the reaction to 5–10 minutes could help to minimize this.

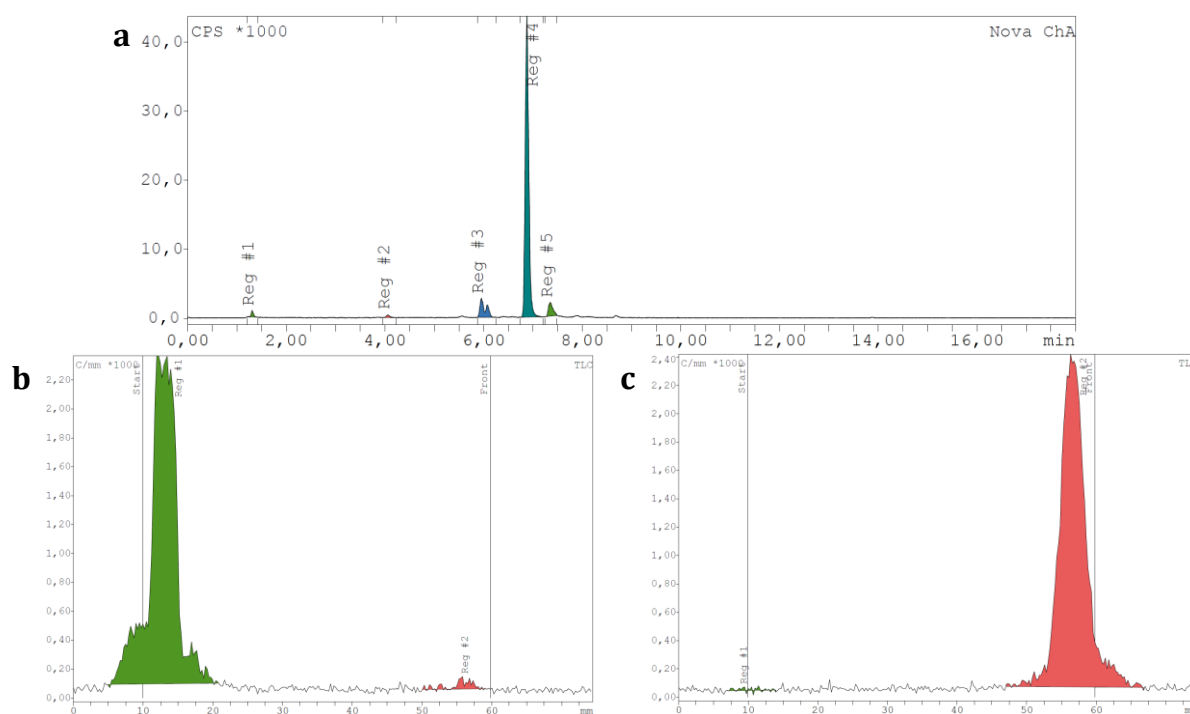


Figure 7: Radiolabeling analytics of automated synthesis of $[^{99m}\text{Tc}]\text{Tc-MAG3.SA.FAPi } ^{99m}\text{Tc-13}$ ($t_R = 7.0$ min, $\text{RCP} = 85.1\%$) according to labeling protocol III at 95°C for 15 min with 100 nmol **13** and 5.8 GBq ^{99m}Tc . a) Radio-HPLC (5-45% MeCN + 0.1% TFA in 10 min), b) EA/MEK radio-TLC with EA/MEK(Baseline) = 98.2% and c) AmOAc/MeOH radio-TLC with AmOAc/MeOH(Baseline) = 0.4%. The RCC that results out of b) and c) with equation (1) is 97.8%.

The complex stability of $^{99m}\text{Tc-13}$ was investigated in three different media: human serum (HS), saline and the pure final formulation. The product was incubated in HS and saline at 37°C as well as the final formulation at room temperature for 24 hours, respectively. The results of the radio-TLC analysis are shown in Figure 8.

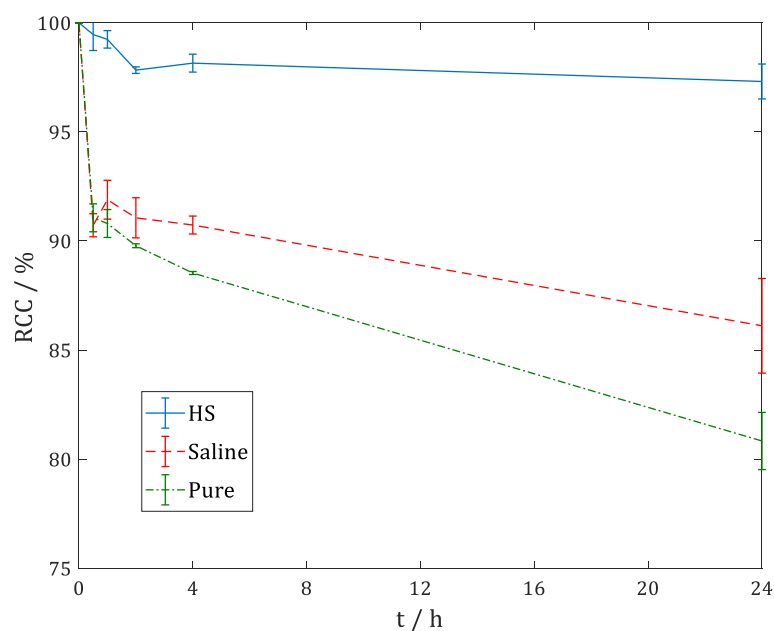


Figure 8: Complex stability of [^{99m}Tc]Tc-MAG3.SA.FAPi ^{99m}Tc -**13** in human serum (HS) and saline (ca. 150 MBq/mL) at 37 °C and the pure final formulation (ca. 0.5–1.0 GBq/mL) at RT over 24 hours ($n = 3$, respectively).

Additional analytical radio-HPLCs were measured. Figure S6 shows exemplary radio-HPLCs after incubation for 24 hours for all three media.

Surprisingly, the complex was more stable in HS (TLC: $97.3 \pm 0.8\%$; HPLC: 74.8% after 24 h) than in saline (TLC: $86.1 \pm 2.2\%$; HPLC: 69.2% after 24 h). Even in the final formulation (“pure”), where the activity concentration is much higher (ca. 0.5–1.0 GBq/mL), the complex was still stable after incubation for 24 hours at room temperature (TLC: $80.8 \pm 1.3\%$; HPLC: 64.1%). The quite fast drop after the first hour could be due to the relatively long time (at least 30 minutes) between the end of previous radiolabeling and the start of the complex stability investigation. But overall, the complex can be considered stable for several hours in different media and also at higher concentrations which makes it suitable for *in vivo* applications.

3.3. In Vitro-Inhibition Assays

The IC_{50} values were determined in *in vitro* affinity studies for FAP and the related proteases PREP, DPP4, DPP8 and DPP9. Table 1 summarizes the results of the IC_{50} measurements for FAP as well the respective selectivity index (SI) to the other proteases with $SI = IC_{50}(\text{respective protease})/IC_{50}(\text{FAP})$. For comparison, Table 1 includes analogue data for other SA-conjugated FAP inhibitors DOTA- and DATA^{5m}.SA.FAPi as well as the initial FAP inhibitor UAMC-1110. The IC_{50} values for PREP, DPP4, 8 and 9 are listed in Table S2.

Table 1: IC_{50} values for FAP and the respective selectivity index (SI) of different FAP inhibitors. The data for [^{nat}Ga]Ga- and DOTA- and DATA^{5m}.SA.FAPi from MOON et al. [25,30] and for the initial FAP inhibitor UAMC-1110 from JANSEN et al. [22] and MOON et al. [30].

Compound	IC_{50} / nM	SI			
	FAP	PREP/FAP	DPP4/FAP	DPP8/FAP	DPP9/FAP
N4.SA.FAPi (7)	0.67 ± 0.06	5478	11104	6433	6821
S-Bz-MAG3.SA.FAPi (13)	0.46 ± 0.05	5957	2348	109	35
DOTA.SA.FAPi	0.9 ± 0.1	6000	-	-	-
[^{nat}Ga]Ga-DOTA.SA.FAPi	1.4 ± 0.2	6214	> 714	-	-
DATA^{5m}.SA.FAPi	0.8 ± 0.2	2113	-	-	-
[^{nat}Ga]Ga- DATA^{5m}.SA.FAPi	0.7 ± 0.1	6714	> 1429	-	-
UAMC-1110	0.43 ± 0.02	4186	23256	> 23256	10930

The two new compounds **7** and **13** have subnanomolar IC_{50} (FAP) values and have a very high selectivity for FAP over PREP (> 5000) and DPP4 (> 10,000 and 2348 respectively). The different SI values show that N4.SA.FAPi and S-Bz-MAG3.SA.FAPi have comparable PREP selectivity to DOTA.SA.FAPi, [^{nat}Ga]Ga-DOTA.SA.FAPi and [^{nat}Ga]Ga-DATA^{5m}.SA.FAPi, compounds that have already proven to be suitable FAP imaging agents for application in ^{68}Ga -PET. The DPP4 selectivity is comparable (**13**) or even higher (**7**) than of [^{nat}Ga]Ga-DOTA.SA.FAPi and [^{nat}Ga]Ga-DATA^{5m}.SA.FAPi. PREP and DPP4 are expressed ubiquitously and high selectivity for these two proteases is crucial for suitable pharmacodynamic properties. The DPP8 and DPP9 selectivity of **7** is also very high (SI > 5000, respectively) whereas it is lower for **13**. DPP8 and DPP9 are only expressed intracellularly and thus do not compete with FAP while the tracer circulating in the body/blood plasma [13]. It only comes into play when the compound should be internalized by a FAP-expressing cell. Here, the higher DPP8 and DPP9 affinity might lead to decreased efflux from the cell if the cell is also DPP8- and/or DPP9-positive and the compound might bind to DPP8 or DPP9.

No equivalent data to these studies have been reported for other compounds like HFAPi or iFAP, especially regarding selectivity to the related proteases, that would allow for direct comparison [52,54]. Extensive SAR studies on FAP inhibitors by the group of PIETER VAN DER VEKEN [21,22,63,64] demonstrated higher affinity and selectivity for compounds based on (4-

quinolinoyl)-glycyl-2-cyanopyrrolidine rather than boronic acid warheads (like [^{99m}Tc]Tc-iFAP [54]) or using other aromatic units, e.g. 4-pyridinoyl like in [^{99m}Tc]Tc-HFAPi or [^{99m}Tc]Tc-FL-L3 [50,52]. Therefore, it might be assumed that radiotracers utilizing these less optimal FAP inhibitors as targeting vectors will probably also have lower FAP affinity and selectivity.

Overall, the results show that the new FAP ligands might be suitable imaging agents for ^{99m}Tc -SPECT of CAFs-expressing tissue such as different epithelial cancers.

4. Conclusion

The new compounds N4.SA.FAPi and S-Bz-MAG3.SA.FAPi contain a fibroblast activation protein alpha (FAP) inhibitor targeting vector coupled to the respective chelator via squaramide (SA). The compounds are designed to be radiolabeled with technetium-99m for oncologic application in SPECT imaging as they are addressing FAP-expressing cancer-associated fibroblasts (CAFs) that are highly expressed in the tumor microenvironment (tumor stroma) of over 90% of all epithelial cancer types. FAP is a pan-tumor target and suitable radiotracer have the potential for broad application in many cancer types such as breast, lung, gastric, colorectal, prostate, pancreatic or ovarian cancer [1,2]. On top, many different types of sarcomas cancer cells directly express FAP. Despite the great potential for a universal oncologic SPECT radiotracer targeting FAP only few ^{99m}Tc -FAPi radiotracers have been published so far [49–51]. The FAPi-based ^{99m}Tc -radiotracers that are already published either use the two-step tricarbonyl strategy [49], the HYNIC strategy [51–54] or utilize a different targeting vector [50] or both [53,54]. We believe there is both a clinical demand and a significant potential for new and optimized ^{99m}Tc -FAPi radiotracers that allow for SPECT imaging at early time points.

Both precursors N4.SA.FAPi and S-Bz-MAG3.SA.FAPi were successfully synthesized in 7 and 9 steps, respectively. The high affinity and selectivity for FAP over the related proteases prolyl endopeptidase (PREP) and dipeptidyl peptidase 4 (DPP4) was demonstrated in *in vitro* binding affinity studies with subnanomolar $IC_{50}(\text{FAP})$ for both precursors. Whereas the selectivity for FAP over DPP8 and DPP9 was also good for N4.SA.FAPi it was lower for S-Bz-MAG3.SA.FAPi, yet this appears to be less critical since these two proteases are only expressed intracellularly. The promising *in vitro* results suggest that these compounds can be new interesting ^{99m}Tc -radiotracers for *in vivo* application in FAPi-SPECT imaging.

The radiochemical synthesis of $[\text{}^{99m}\text{Tc}]\text{Tc-MAG3.SA.FAPi}$ was investigated. Quantitative radiochemical conversion (RCC) was achieved at room temperature and elevated temperatures which makes it potentially suitable for instant-kit type labeling, beyond a first proof-of-principle automated radiosynthesis that was demonstrated within this study. The complex stability in human serum was high. The complex was also mostly stable in saline as well the final formulation for several hours. The radiochemical synthesis of $[\text{}^{99m}\text{Tc}]\text{Tc-N4.SA.FAPi}$ still needs to be evaluated. In conclusion, $[\text{}^{99m}\text{Tc}]\text{Tc-MAG3.SA.FAPi}$ deserves further (pre)clinical investigations based on the suitable binding properties *in vitro* and ^{99m}Tc -radiolabeling properties.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Organic Synthesis

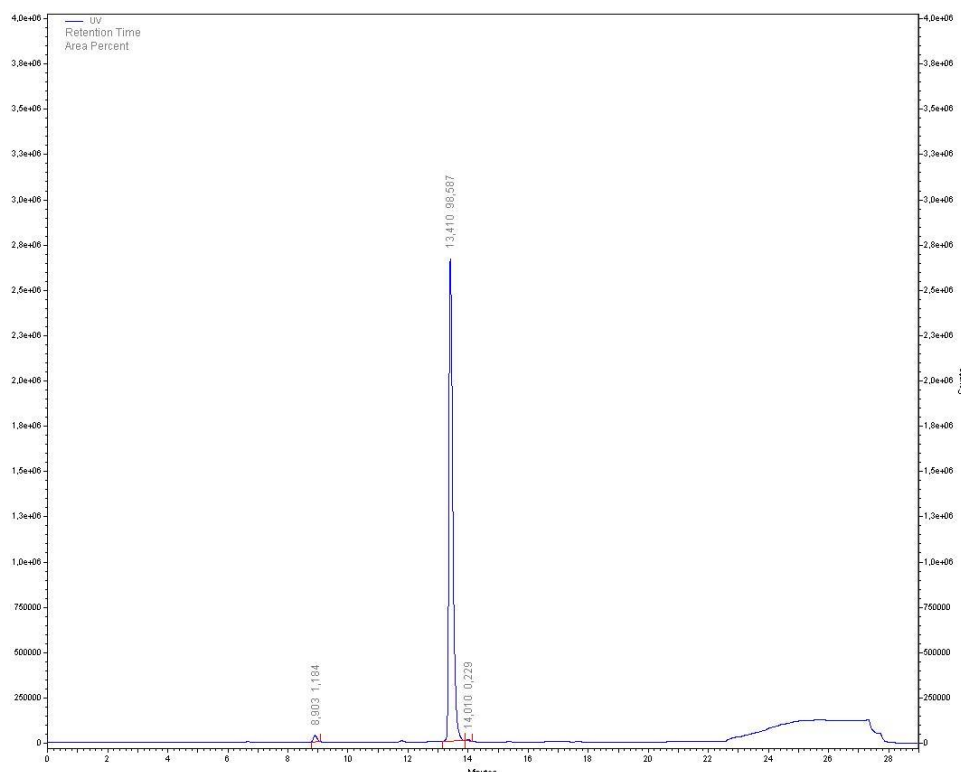


Figure S1: Analytical RP-HPLC of N4.SA.FAPi **7** ($t_R = 13.4$ min), 10-30% MeCN + 0.1% TFA (linear gradient) in 20 min, 5 mL/min: purity = 99.0%.

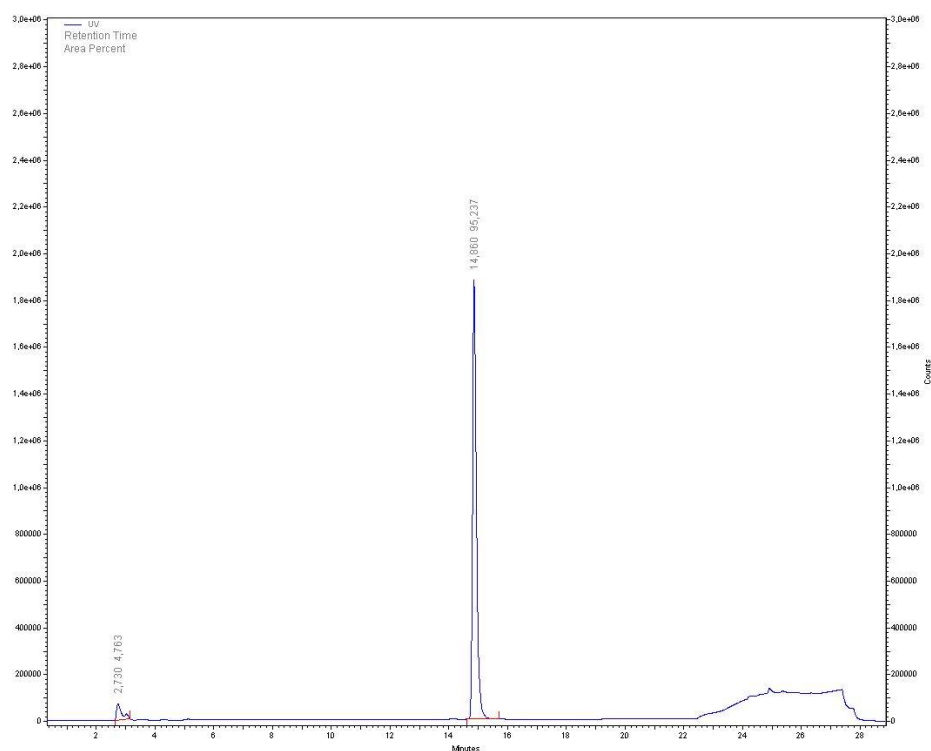
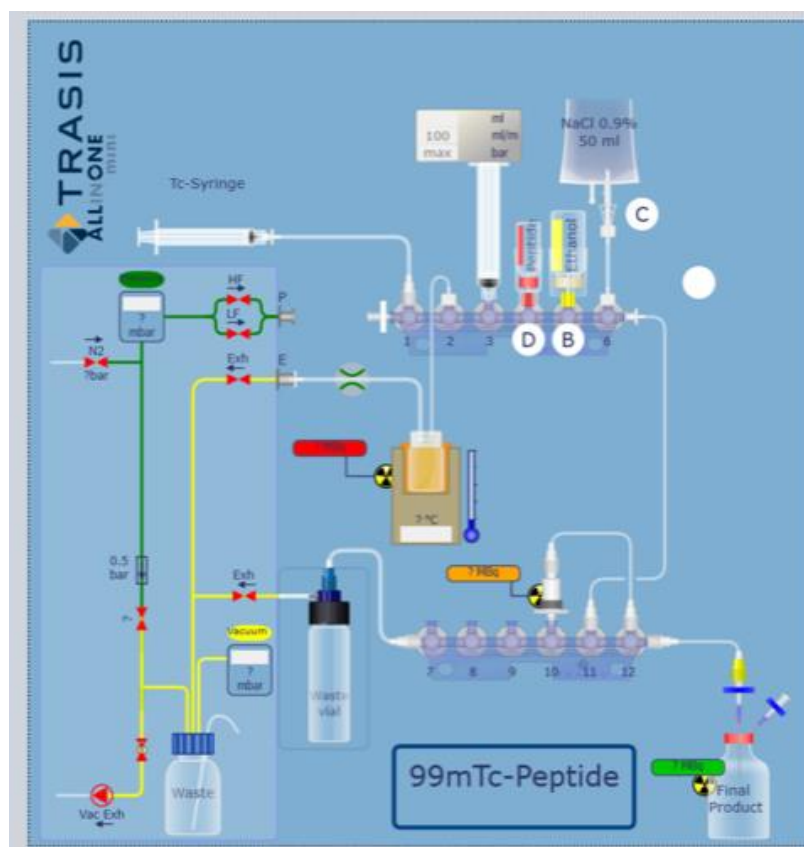


Figure S2: Analytical RP-HPLC of S-Bz-MAG3.SA.FAPi **13** ($t_R = 14.9$ min), 20-40% MeCN + 0.1% TFA (linear gradient) in 20 min, 5 mL/min: purity = 95.2%.

The following Figures and Tables show additional data to the labeling studies.



Chromatogram of Nova ChA showing five peaks labeled Reg #1 to Reg #5. The y-axis is CPS * 1000 (0.00 to 6.00) and the x-axis is time in minutes (0.00 to 16.00). Reg #4 is the largest peak at approximately 6.8 minutes.

Figure S4: Exemplary analytical radio-HPLC of [^{99m}Tc]Tc-MAG3.SA.FAPi ^{99m}Tc-**13** (t_R = 7.0 min, RCP = 95.7%) with 5-45% MeCN + 0.1% TFA in 10 min after radiolabeling according to labeling protocol III at RT for 30 minutes.

Table S1: Radiochemical conversion (RCC in %) of ^{99m}Tc -radiolabeling of *S*-Bz-MAG3.SA.FAPi **13** according to labeling protocol III. RCC was calculated according to equation (1) and each labeling was carried out in triplicate (except where noted).

Reaction temperature	<i>n</i> [nmol]	<i>t</i> [min]	EA/MEK (Baseline) [%]	AmOAc/MeOH (Baseline) [%]	RCC [%]
RT	50	5	79.4 ± 9.7	1.2 ± 0.2	78.5 ± 9.7
		15	87.4 ± 6.3	0.9 ± 0.3	86.6 ± 6.1
		30	94.2 ± 0.5	2.0 ± 1.1	92.2 ± 1.5
RT	100	5	91.4 ± 5.7	1.1 ± 0.4	90.3 ± 5.3
		15	95.3 ± 3.2	0.9 ± 0.5	94.4 ± 2.8
		30	98.1 ± 0.4	0.4 ± 0.4	97.7 ± 0.3
90 °C	10	5	91.5 ± 5.4	15.5 ± 4.7	76.0 ± 10.0
		15	99.4 ± 0.4	18.9 ± 7.9	80.5 ± 8.3
		30	94.4 ± 0.6	9.7 ± 3.3	84.7 ± 3.1
90 °C	20	5	99.8 ± 0.1	2.2 ± 0.6	97.6 ± 0.5
		15	99.4 ± 0.2	1.4 ± 0.4	98.0 ± 0.3
		30	98.4 ± 0.7	2.6 ± 0.9	95.7 ± 1.6
90 °C (5.8 GBq, n = 1)	100	15	98.2	0.4	97.8

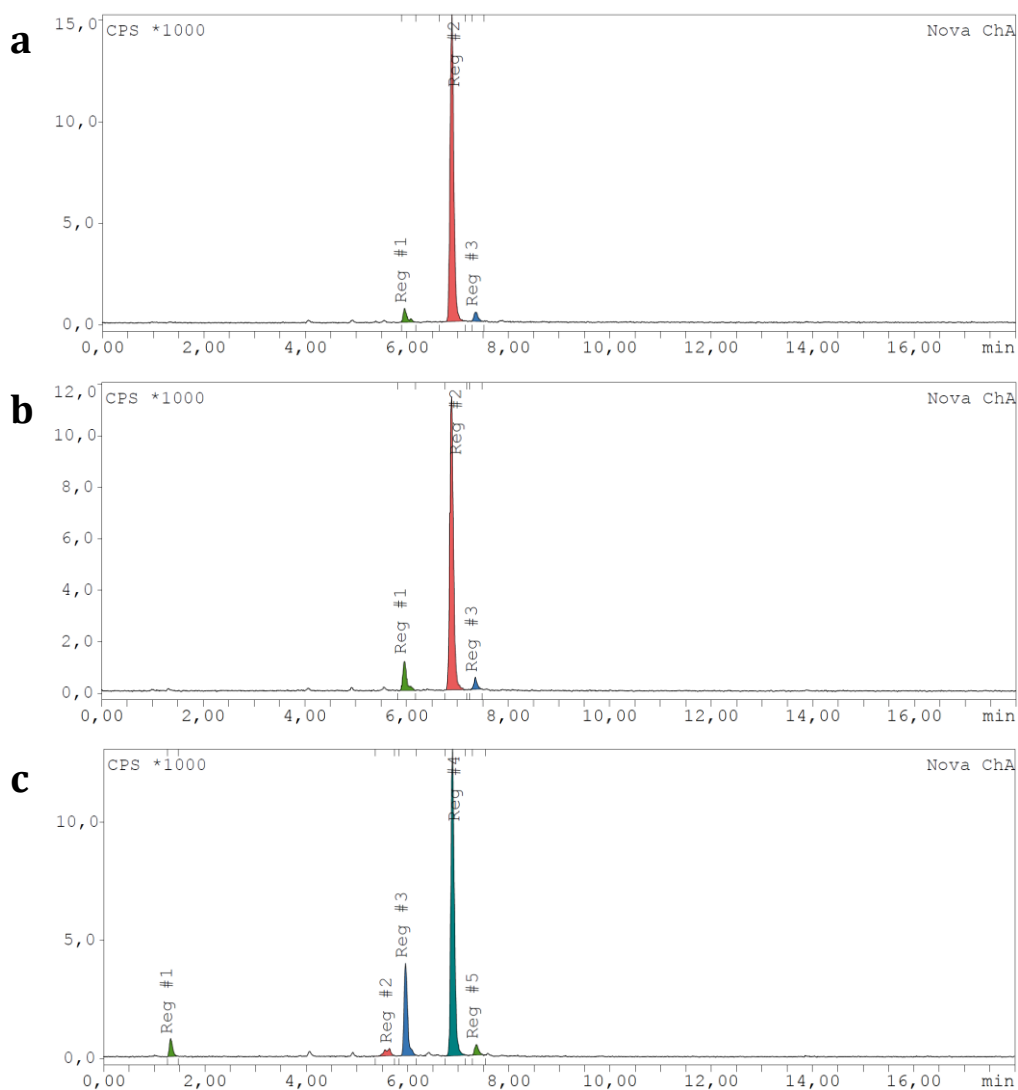


Figure S5: Exemplary analytical radio-HPLC after radiolabeling $[^{99m}\text{Tc}]\text{Tc-MAG3.SA.FAPi } ^{99m}\text{Tc-13}$ ($t_R = 7.0$ min) according to labeling protocol III at 90 °C for a) 5 min (RCP = 92.4%), b) 15 min (RCP = 87.4%) and c) 30 min (RCP = 70.0%). All measurements were carried with 5-45% MeCN + 0.1% TFA in 10 min.

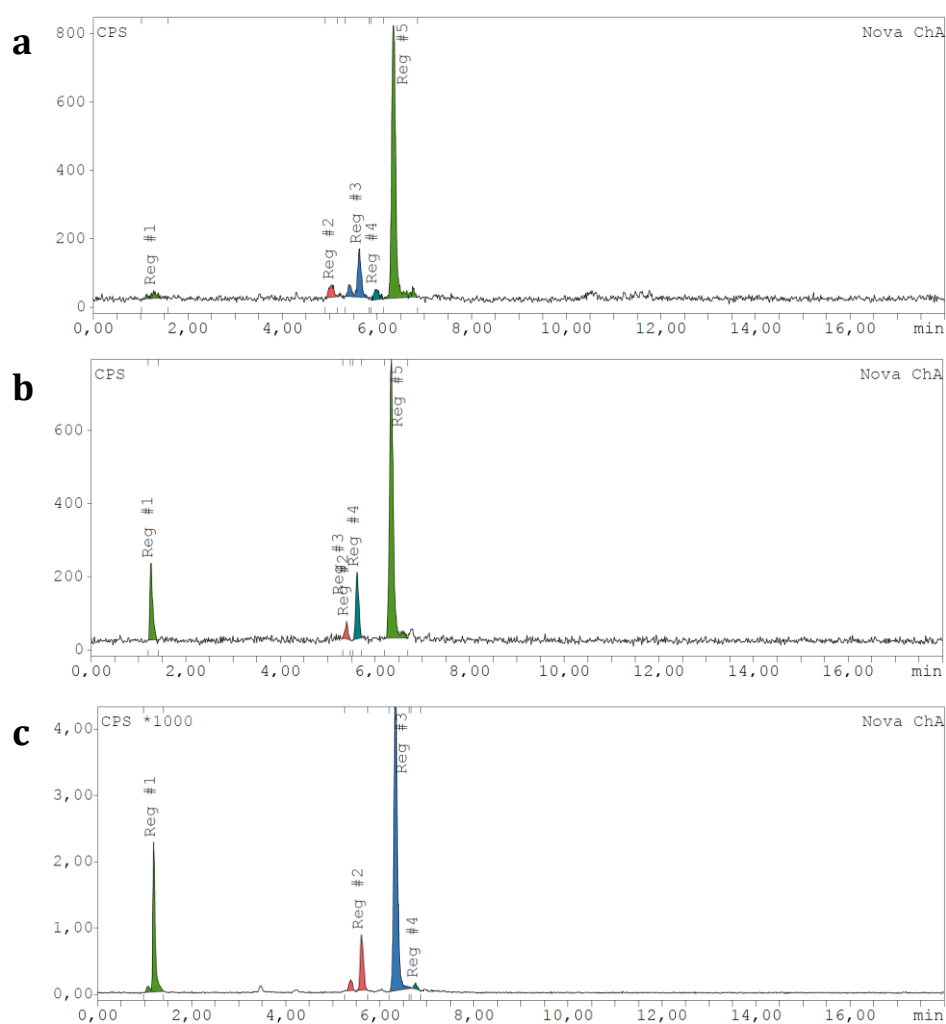


Figure S6: Exemplary analytical radio-HPLC after incubation of $[^{99m}\text{Tc}]\text{Tc-MAG3.SA.FAPi } ^{99m}\text{Tc-13}$ ($t_R = 7.0$ min) for 24 hours to determine complex stability a) in human serum (HS) at 37 °C, b) in saline at 37 °C (both ca. 150 MBq/mL) and c) in the final formulation (0.5–1.0 GBq/mL) at RT. All measurements were carried with 5-45% MeCN + 0.1% TFA in 10 min.

In Vitro-Inhibition Assays

Table S2: IC_{50} values for FAP and respective selectivity index ($SI = IC_{50}(\text{respective protease})/IC_{50}(\text{FAP})$) of different FAP inhibitors. The data for $[^{nat}\text{Ga}]\text{Ga-}$ and DOTA- and $\text{DATA}^{5m}.\text{SA.FAPi}$ from MOON et al. [25,30] and for the initial FAP inhibitor UAMC-1110 from JANSEN et al. [22] and MOON et al. [30].

Compound	$IC_{50} / \mu\text{M}$			
	PREP	DPP4	DPP8	DPP9
N4.SA.FAPi (7)	3.67 ± 0.51	7.44 ± 0.43	4.31 ± 0.77	4.57 ± 0.39
S-Bz-MAG3.SA.FAPi (13)	2.74 ± 0.99	1.08 ± 0.14	0.050 ± 0.011	0.016 ± 0.003
DOTA.SA.FAPi	5.4 ± 0.3	-	-	-
$[^{nat}\text{Ga}]\text{Ga-DOTA.SA.FAPi}$	8.7 ± 0.9	> 1	-	-
$\text{DATA}^{5m}.\text{SA.FAPi}$	1.7 ± 0.1	-	-	-
$[^{nat}\text{Ga}]\text{Ga-}$ $\text{DATA}^{5m}.\text{SA.FAPi}$	4.7 ± 0.3	> 1	-	-
UAMC-1110	1.80 ± 0.01	> 10	> 10	4.70 ± 0.40

4. Discussion and Summary

In the beginning of this chapter, the state of research on emerging radiotheranostics addressing cancer-associated fibroblasts (CAFs) by FAP inhibitor (FAPi)-based radiopharmaceuticals will be discussed. This includes a short summary of the aspects that were introduced in chapter 1.5. “FAPi-based Radiopharmaceuticals”. This will be comprehensively expanded by newest developments and strategies including recently published radiopharmaceuticals (chapter 4.1.). Subsequently, the results of this work (chapter 4.2. and 4.3) will be discussed and compared to those current results on radiotheranostics targeting FAP.

Molecular imaging of the tumor microenvironment (TME) by addressing the cancer-associated fibroblasts (CAFs) that strongly express FAP has made a major leap since 2018. At this point, the first highly affine and selective FAPi-based radiotracers with the leading candidate [^{68}Ga]Ga-FAPI-04 were published by the group of HABERKORN (Heidelberg University) [1]. This was followed by the second leading candidate from Heidelberg [^{68}Ga]Ga-FAPI-46 [2], the radiotracers [^{68}Ga]Ga-DOTA.SA.FAPi and [^{68}Ga]Ga-DATA^{5m}.SA.FAPi from the group of ROESCH (JGU Mainz) [3], and also FAPI-42 and FAPI-74 for ^{18}F -PET [4]. All these compounds have proven to be high-performance PET radiotracers with fast tumor uptake and rapid renal clearance from non-target tissues (including blood) to obtain PET images with high tumor-to-background ratio (TBR) at early time points [1–3,5–7]. Their clinical impact was reported to be on par or superior to the most-used oncological PET radiotracer [^{18}F]F-FDG in a variety of studies and cancer types up to this point [5,7–19]. When searching for “FAP” in *SciFinder* (accessed on May 31, 2023), almost 100 publications are displayed when filtering for “PET” in 2022 alone compared to 53 the year before. This demonstrates the high and rising interest in molecular PET imaging with pan-tumor diagnostic FAPi radiotracers.

Most of these monomeric FAPi radiotracers are chelator-based (e.g. DOTA or DOTA derivatives) and thus designed with a theranostic approach in mind. Theoretically, this shall enable a straightforward theranostic translation by simply exchanging the diagnostic trivalent radiometal (e.g. gallium-68) by a therapeutic one. Established therapeutic trivalent radiometals are the β -emitters yttrium-90 and lutetium-177 or the α emitter actinium-225. This strategy was realized very successfully with other inhibitor-based radiopharmaceuticals. For example, prostate specific membrane antigen (PSMA) inhibitors like [^{177}Lu]Lu-PSMA-617 (Pluvicto®) were recently approved by the EMA and FDA as a commercial drug for use in late stage metastasized castration-resistant prostate cancer [20–23].

In contrast, the translation of the diagnostic variant into the therapeutic counterpart did not lead to satisfactory properties/results with the abovementioned monomeric FAPi-based

radiopharmaceuticals. As it turned out, the main problem is the insufficient residence time in the tumor lesion. The FAPi monomers are not only rapidly washed out from non-target tissue such as the blood, which is desirable for high TBR in PET imaging, but also from the tumor. This results in a biological half-life of 2 and 8 hours for [^{177}Lu]Lu-FAPi-04 and -46, respectively [1,2]. Their corresponding effective half-life of max. half a day does not fit the relatively long physical half-life of the eligible therapeutic radiometals yttrium-90 ($t_{1/2} = 64.0$ h), lutetium-177 ($t_{1/2} = 6.65$ d) and actinium-225 ($t_{1/2} = 10.0$ d).

4.1. Current State of Research: Variety of CAF-Targeting Radiopharmaceuticals

Several strategies are currently pursued to prolong tumor retention towards significantly improved radiotheranostic application and FAP-targeting radioligand therapy (RLT).

Two of those strategies were already discussed in the chapter 1.5.2. “Emerging FAPi-based Radiotheranostics”. On the one hand, the optimization of the linker and spacer units by the Heidelberg group resulted in FAPi-46 which showed slightly improved tumor retention, but still not long enough for optimal FAPi-based RLT.

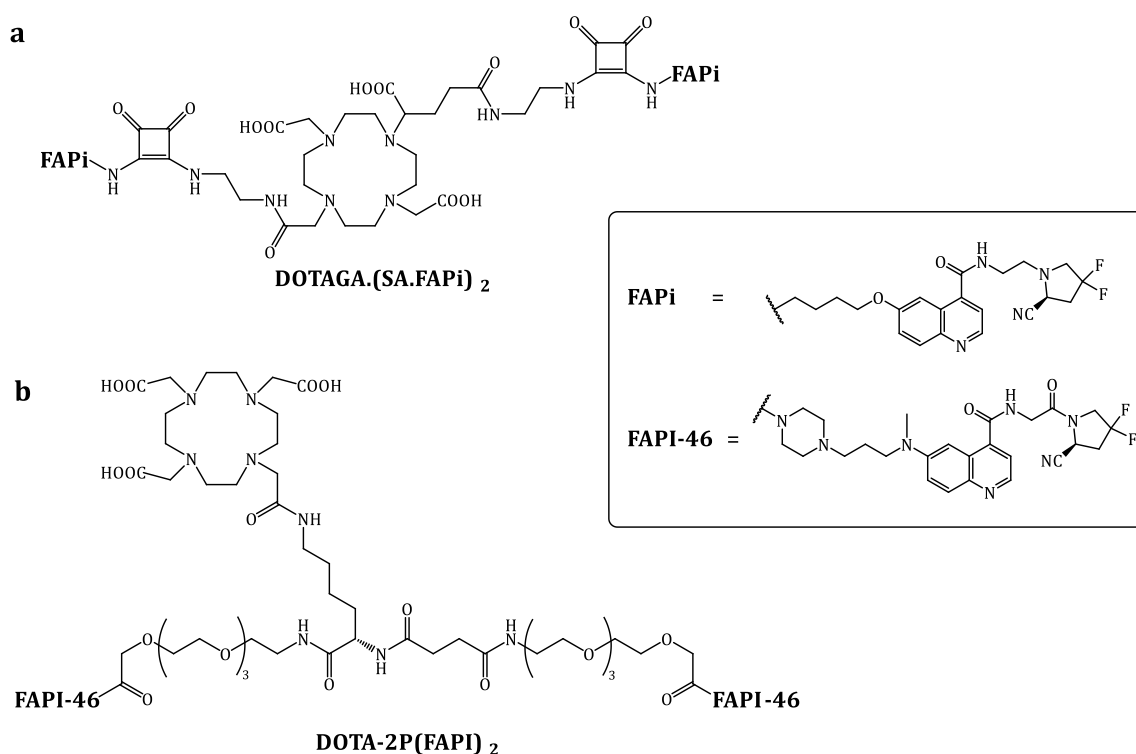
On the other hand, the precursor design was adjusted to enable radiolabeling with other therapeutic radionuclides with shorter physical half-life. Compounds belonging to this category are [^{211}At]At-FAPi-04, using the α emitter astatine-211 ($t_{1/2} = 7.0$ h), and FAPi-34, that can be radiolabeled with the long-range β^- emitter rhenium-188 ($t_{1/2} = 17.0$ h) in addition to technetium-99m for SPECT. Besides the early preclinical stage of investigation, the clinical use of these therapeutic FAPi ligands appears to be limited.

The following chapter 4.1.1. will focus on FAPi homodimers with two identical TVs. Subsequently, chapter 4.1.2. will focus on FAPi heterodimers with two different TVs, 4.1.3. on FAPi-based compounds with additional albumin-binding moiety and 4.1.4. on cyclic peptide strategies.

4.1.1. FAPi-based Homodimers for Radioligand Therapy (RLT)

The third strategy is to synthesize radiotracers with several targeting vectors (TVs), called homodimers when two identical TVs are being utilized. The concept of small molecule dimeric ligands exists in pharmacology for several decades [24–26]. Having multiple identical TVs increases the probability of ligand-enzyme interaction and is increasing the avidity of the compound but it can also have substantial effects on enzyme affinity and selectivity [24,27]. This strategy has been translated into radiopharmaceuticals in recent years. Multiple cyclic RGD-

containing peptides like c[RGDfK] in one molecule were coupled to a TRAP chelator in [^{68}Ga]Ga-TRAP(RGD) $_3$, a PET radiotracer to address $\alpha_v\beta_3$ integrins [28,29]. Later, Zia et al. [30] demonstrated higher and prolonged tumor uptake of a ^{64}Cu -labeled PSMAi-dimer compared to the respective monomer. [^{64}Cu]Cu-SarbisPSMA is a linear homodimer consisting of two PSMAi TVs ($2 \times \text{KuE}$) and a sarcophagine (Sar) chelator optimized for $^{64/67}\text{Cu}$ -radiolabeling. In 2021, Moon et al. [31] from the group of Roesch (JGU Mainz) synthesized the first homodimeric FAPI-based radiotracer DOTAGA.(SA.FAPI) $_2$ (cf. Figure 1a). The linear homodimer consisted of two TVs FAPI-NH $_2$ coupled to the central DOTAGA chelator via squaramide linkers (cf. chapter 1.5.2.) [31].



Increased and prolonged uptake in the tumor lesions was observed with [^{177}Lu]Lu-DOTAGA.(SA.FAPI) $_2$. A pilot study on 15 patients with radioiodine-refractory differentiated thyroid cancer (RR-DTC) has demonstrated an effective half-life of 88 hours with an interquartile range (IQR) of 86.6 – 99 hours, in contrast to 8 and 14.5 hours for [^{177}Lu]Lu-FAPI-04 and -46, respectively [32]. The median radiation dose to tumor lesions was 11 Gy/GBq (IQR: 4 – 90 Gy/GBq) and much higher compared to [^{177}Lu]Lu-PSMA-617 (mean: 4.17 Gy/GBq), for example [33]. [^{177}Lu]Lu-DOTAGA.(SA.FAPI) $_2$ showed hepatobiliary excretion and the colon was found to be the critical organ regarding the absorbed radiation dose. The treatment was well tolerated and no grade III/IV renal, hepatic and hematotoxicity occurred. Only 1/15 patients had grade I diarrhea that was gone after one day. Other organs like salivary glands, kidneys and bone marrow received

lower radiation doses. Promising treatment responses (4/15 partial response, 3/15 stable disease) was observed after a median follow-up of 7 months [32].

Following our strategy, other groups have adopted this approach since then and developed several FAPI homodimers since 2022. ZHAO et al. [34] synthesized a dimer DOTA-2P(FAPI)₂ (cf. Figure 1b) that consisted of two FAPI-46 targeting vectors with additional PEG₃ spacers between TV and a central linker unit (lysine) that couples both TVs and the chelator DOTA. The *IC*₅₀ value was somewhat higher (3.68 ± 1.82 nM) than for FAPI-46 (2.06 ± 1.84 nM) but not significantly as it was within the error of margin [34]. This indicates that the TVs do not bind simultaneously and increase avidity but rather proximity effects might play a role (for further discussion: cf. chapter 4.2.5). ⁶⁸Ga-PET in hepatocellular cancer patient-derived xenografts and a patient with metastasized thyroid cancer indicated a two-fold increase in tumor uptake 4 h p.i. The human PET scan showed low TBR even at 4 h p.i. due to high blood pool activity, e.g. tumor-to-kidney ratio was 1.3:1. In addition, relatively high uptake was found in pancreas, liver, kidneys and thyroid. [⁶⁸Ga]Ga-DOTA-2P(FAPI)₂ was termed “inferior” to the monomeric [⁶⁸Ga]Ga-FAPI-46 for PET/CT imaging [35].

Unpublished murine SPECT data on [¹⁷⁷Lu]Lu-DOTA-2P(FAPI)₂ were presented at the online “ICPO Theranostics FAP Summit 2022” (Nov. 4 – 5, 2022). Tumor uptake until 96 hours at minimum and first murine treatment response data were reported (HT-1080.hFAP tumor cell model), indicating superiority of [¹⁷⁷Lu]Lu-DOTA-2P(FAPI)₂ versus [¹⁷⁷Lu]Lu-FAPI-46 [36]. Overall, the results on tumor uptake and retention confirmed the findings by MOON et al. [31] that dimerization is a promising strategy for FAP-targeted RLT.

The monomeric OncoFAP-DOTAGA (cf. Figure 2a) of Philochem AG, published by MILLUL et al. [37], was introduced in chapter 1.5.1. It utilizes a differently functionalized quinoline moiety, a 8-aminoquinoline for subsequent amide coupling instead of 6-(4-amino)butoxyquinoline as in FAPI-NH₂ (cf. Figure 1 and 2). Small animal PET with [⁶⁸Ga]Ga-OncoFAP-DOTAGA in various cancer types demonstrated very high uptake (SUV up to 20 %ID/g) in primary and metastatic lesions and high contrast PET images. Yet fast washout from tumor lesions of SK-RC52.hFAP mice could be seen with ⁶⁸Ga-PET (1 vs. 3 h p.i.) as well as *ex vivo* biodistribution studies with ¹⁷⁷Lu-OncoFAP-DOTAGA (3 vs. 6 h p.i.) [37,38].

Subsequently, the Philochem group also switched to a dimeric molecule design with BiOncoFAP-DOTAGA (cf. Figure 2b) published by GALBIATI et al. [39]. Monomer and dimer both showed high hydrophilicity ($\log D_{7.4} = -4.02 \pm 0.22$ and -3.60 ± 0.31) and subnanomolar *IC*₅₀ values (0.46 nM and 0.19 nM for the respective ^{nat}Lu-complex). Regarding the *in vitro* affinity studies it has to be noted that (i) the *IC*₅₀ value of the unfunctionalized TV OncoFAP was found to be 16.8 nM in a previous study by MILLUL et al. [37], thus the big difference/improvement of roughly two orders

of magnitude of the chelator-based compound compared to the sole TV is surprising; (ii) the assay conditions used in these studies are different from this work (e.g. performed at RT) and (iii) no selectivity data over the related proteases, especially PREP and DPP4, were reported.

In vivo studies on HT-1080.hFAP tumor-bearing mice resulted in more persistent and even higher tumor uptake of [^{177}Lu]Lu-BiOncoFAP-DOTAGA (20 %ID/g after 24 h) in contrast to the monomer [^{177}Lu]Lu-OncoFAP-DOTAGA (5 %ID/g after 24 h). The high tumor-to kidney ratio (ca. 10:1 at 4 h p.i.) seemed to be much better than of [^{68}Ga]Ga-DOTA-2I(FAPI) $_2$ (1.3:1 4 h p.i.) [34,35,39]. [^{177}Lu]Lu-BiOncoFAP-DOTAGA achieved tumor shrinkage after a single administration in HT-1080.hFAP tumor bearing mice. Interestingly, the anti-tumor effect of the ^{177}Lu -monomer was quite similar and only slightly inferior to the ^{177}Lu -dimer [39].

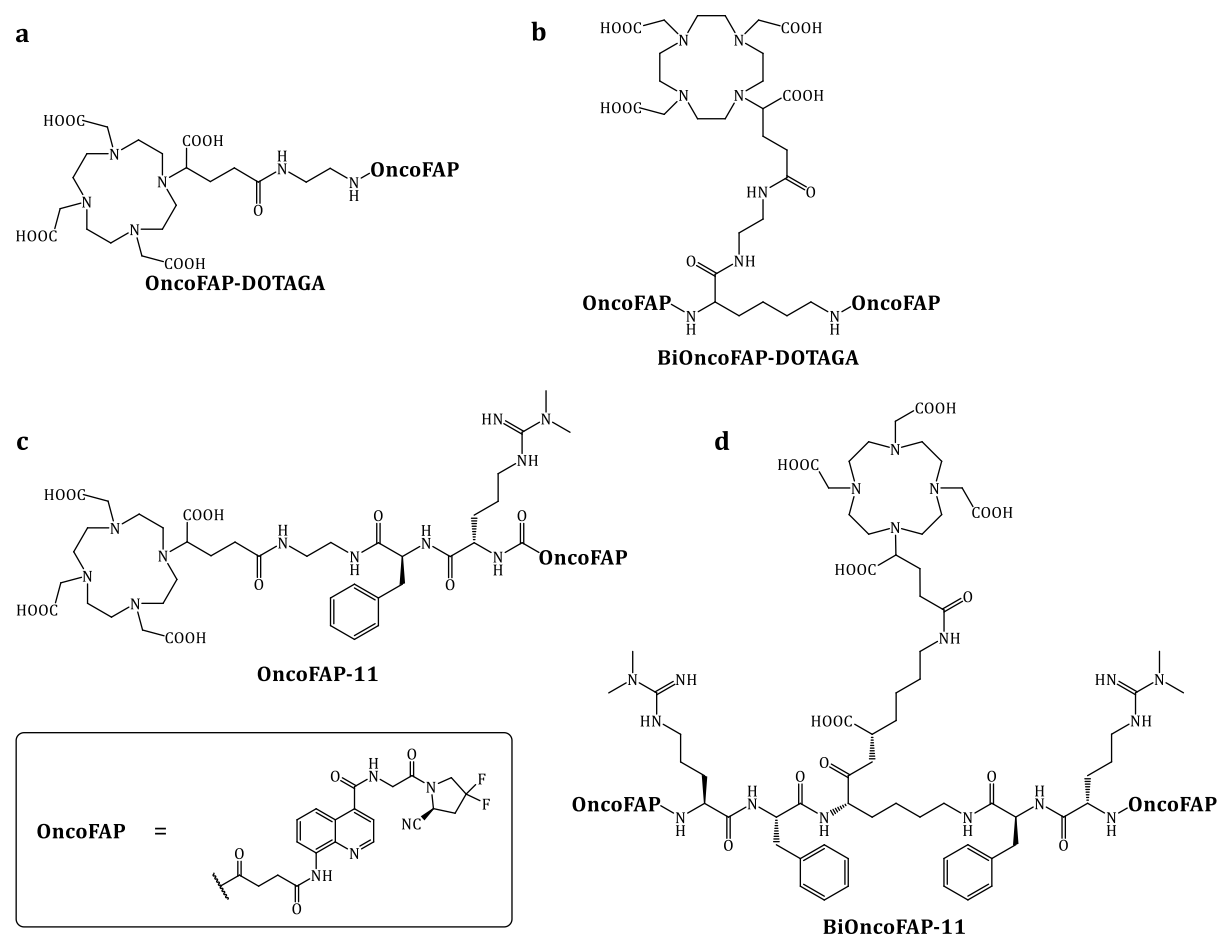


Figure 2: Structures of the Philochem compounds a) OncoFAP-DOTAGA, b) BiOncoFAP-DOTAGA, c) OncoFAP-11 and d) BiOncoFAP-11.

Recently, the Philochem group published a study where the spacer unit was optimized. The $IC_{50}(\text{FAP}) = 30 \pm 7$ pM for the new OncoFAP-11 is 30-fold lower compared to OncoFAP ($IC_{50}(\text{FAP}) = 0.90 \pm 0.07$ nM) in a head-to-head comparison [40]. A high DPP4 selectivity ($SI > 1000$) was reported but no PREP selectivity was investigated. The spacer of OncoFAP-11 (cf. Figure 2c) consists of a short L-Phe and a *N,N*-dimethyl-L-arginine peptide sequence. The new compound

the tumor uptake is more than twice as high as 4 h p.i. (> 30 %ID/g). Surprisingly, however, [^{177}Lu]Lu-ND-bisFAPi is mostly washed out after 72 hours (3–4 %ID/g). These pharmacokinetics result in a 4- to 5-fold higher absorbed dose in the A549.FAP tumor lesion as with [^{177}Lu]Lu-FAPi-04. Although organs like kidney and bone marrow also have somewhat higher radiation exposure of 2.5- and 1.5-fold, respectively, the absorbed dose ratio of tumor-to-kidney/bone marrow is significantly better for [^{177}Lu]Lu-ND-bisFAPi as for [^{177}Lu]Lu-FAPi-04. Tumor did grow slower upon treatment with [^{177}Lu]Lu-ND-bisFAPi compared to [^{177}Lu]Lu-FAPi-04 and much slower compared to the saline control group. However, this did not manifest itself in significant differences in overall survival in the FAP-positive A549 lung cancer xenograft [41].

Recently, the FAPi-04 dimer [^{68}Ga]Ga- and [^{177}Lu]Lu-DOTA-Suc-Lys-(FAPi-04)₂ (cf. Figure 3b) was investigated [42]. The hydrophilic compound ($\log D_{7.4} \approx -3.0$ for ^{68}Ga - and ^{177}Lu -complex) did show slightly higher and prolonged tumor uptake in *ex vivo* biodistribution studies in FAP-positive tumor-bearing mice compared to the monomer but was mostly washout after 1 – 3 days p.i. [42]. Treatment with [^{177}Lu]Lu-(FAPi-04)₂ led to slightly inhibited tumor growth but no remission was observed.

Since the shift from monomers to dimers did show a significant improvement of tumor retention and first results indicate that this will have beneficial effects on anti-tumor efficacy, the idea to go further from dimers to multimers is not far-fetched. DOTA-2I(FAPi)₂ was altered into a tetrameric derivative DOTA-4P(FAPi)₄ [36]. This resulted in higher background and increased uptake especially in the kidneys but not to substantially higher or prolonged tumor uptake in small animal studies. Treatment responses with a HT-1080-FAP xenograft tumor model showed efficacy of the ^{177}Lu -labeled tetramer being almost identical to the dimer [34,36].

4.1.2. Heterodimers

Beside homodimers, there is also the possibility of utilizing heterodimers with two different TVs, i.e. combining one FAPi TV with another type of TV. For example, not all metastasized prostate cancers, especially in recurring disease, are PSMA positive but only around 90%. Many of the remaining 10% are FAP positive. The aim of this strategy is to minimize false-negative results in PSMA-PET [43].

Two PSMA-FAPi-heterodimers were developed using a PSMA-617 like PSMA inhibitor TV and linker unit in combination with a UAMC-110 like FAP inhibitor TV with FAPi-04-like piperazine linker (PSMA-FAPi-02) or without piperazine linker (PSMA-FAPi-01) as shown in Figure 4 [43]. A NOTA chelator was conjugated to the two TVs for [^{18}F]F-AlF₂⁺ radiolabeling. The new radiotracers showed selective binding and high cell-internalization in FAP-positive A549 cells and

PSMA-positive 22Rv1 cells, respectively. Murine *in vivo* studies showed significant tumor uptake and selective blocking in both cell types as well as rapid renal clearance in contrast to monomeric ^{18}F -FAPi radiotracers (cf. chapter 1.5.1.) [43].

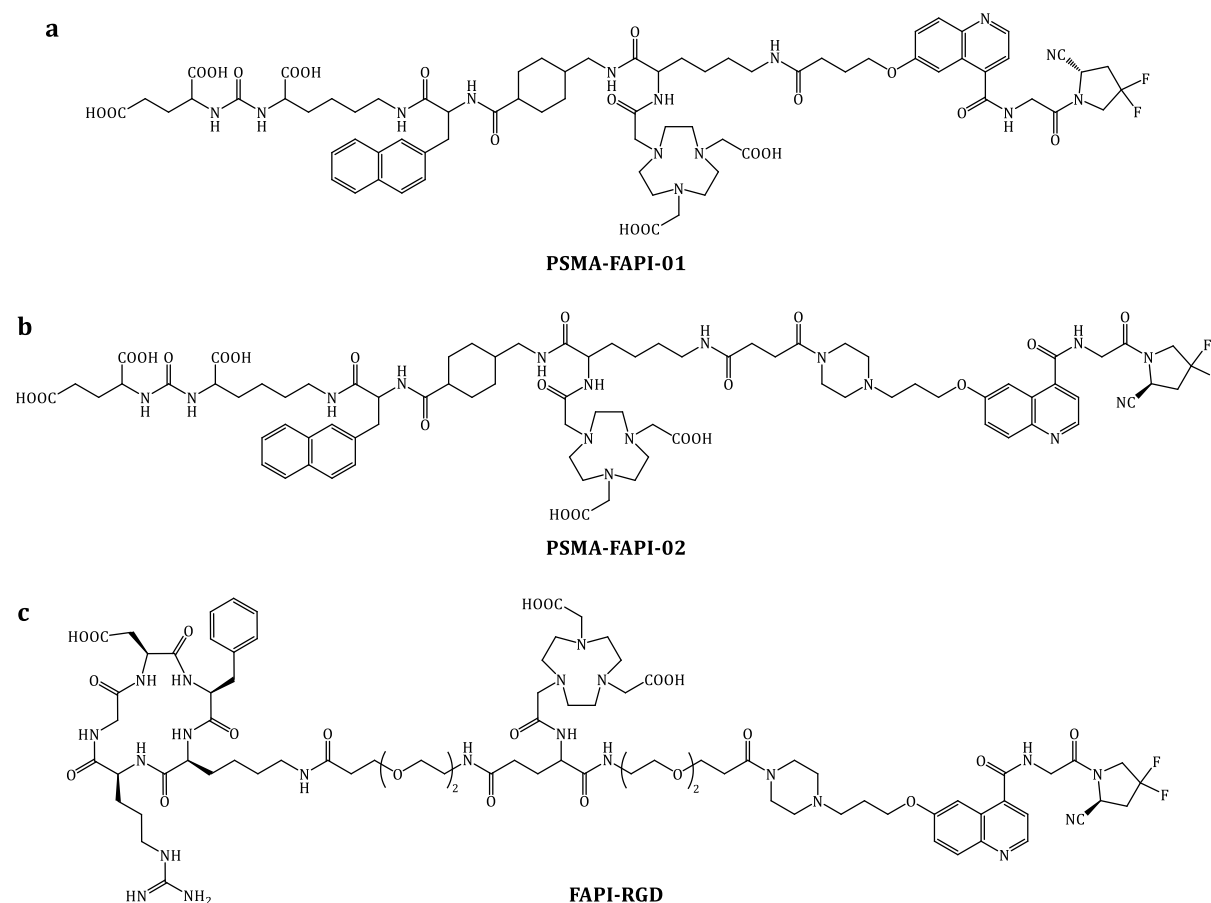


Figure 4: Structures of the FAPi heterodimers a) PSMA-FAPI-01, b) PSMA-FAPI-02 and c) the FAPI-RGD heterodimer.

Furthermore, a FAPI-RGD heterodimer with NOTA chelator was developed (cf. Figure 4c) to simultaneously target FAP-expressing CAFs and $\alpha_v\beta_3$ integrin-expressing cells like vascular cells (endothelial cells) or macrophages in the TME [44]. It was labeled with gallium-68 and was first compared to ^{18}F -FDG in 6 patients and with ^{18}F -FDG and ^{68}Ga -FAPI-46 in a subsequent study in 22 patients [44,45]. ^{68}Ga -FAPI-RGD was superior to both compared PET radiotracers with regard to tumor uptake, TBR and thus also regarding tumor lesion detection rate [44,45].

Just recently, another heterodimer DOTA-FAPI-RGD was published with low nanomolar affinity for both FAP ($IC_{50} = 6.8 \pm 0.6$ nM) and $\alpha_v\beta_3$ integrin ($IC_{50} = 2.1 \pm 0.4$ nM) and first preclinical *in vitro* cell und murine *in vivo* studies in U87MG (FAP and $\alpha_v\beta_3$ -positive) as well as A549 (FAP-positive) and PC-3 ($\alpha_v\beta_3$ -positive) showed selective and high uptake and TBR of ^{68}Ga -DOTA-FAPI-RGD in biodistribution studies [46].

The pharmacokinetics of heterodimers might be altered compared to monomers because of increased “TV-concentration” in proximity to the oncological targets which increases (re)binding

probability and might lead to longer tumor residence times as a result [47]. Since the published FAPi heterodimers to date were only investigated as ^{18}F - and ^{68}Ga -labeled radiotracer, the effect on pharmacokinetics is still not clarified.

4.1.3. Albumin Binders

Structurally, radiopharmaceuticals with additional albumin binding moieties can also be seen as heterodimers since they have one TV addressing tumor cells or CAFs while addressing the serum albumin in the blood with another targeting moiety. This concept has already been applied to PSMA- and SSTR2-targeting radiotracers like PSMA-617, DOTA-TATE or bisphosphonates like BPAMD [48–50]. The most common albumin binding units are Evans Blue (EB) and 4-(*p*-iodophenyl)butyric acid.

Similarly, 4-(*p*-chlorophenyl)butyric acid has been utilized in the first published FAPi-albumin binder heterodimer Alb-FAPtp-01 (cf. Figure 5a) [51]. A different FAP-targeting structure with tryptophan instead of quinoline and proline with normal carboxylic acid instead of nitrile warhead seemed to bind to DPP4. Hence, there was no or low DPP4 selectivity. [^{68}Ga]Ga-Alb-FAPtp-01 showed higher uptake than FAPI-04 and seemed to be retained in the glioma xenograft lesion longer but already after 3 – 5 h p.i. most of the activity was cleared from the tumor lesion and the tumor-to-blood ratio was ca. 1:1 at all times [51].

A structure similar to FAPI-04 as coupled to 4-(*p*-iodophenyl)butyric acid in TEFAPI-06 and Evans Blue (EB) in TEFAPI-07 (cf. Figure 5b and c) [52]. Tumor uptake lasted at least 6 days whereas [^{177}Lu]Lu-FAPI-04 could not be detected after more than 24 hours. TEFAPI-06 and -07 did differ in terms of blood circulation where the Evans blue derivative [^{177}Lu]Lu-TEFAPI-07 was cleared from the blood faster, but tumor uptake was still comparable to [^{177}Lu]Lu-TEFAPI-06 after several days. However, the kidney uptake was significantly higher for TEFAPI-07 and no clearance was observed. Pancreatic cancer PDX model demonstrated higher efficacy of the albumin-binding compounds compared to [^{177}Lu]Lu-FAPI-04. [^{177}Lu]Lu-TEFAPI-06 and -07 showed similar treatment response with significantly delayed tumor growth [52].

The ^{68}Ga - and ^{177}Lu -labeled FAPi-albumin binder heterodimers FAPI-C12 and FAPI-C16 using C12 and C16 fatty acids (cf. Figure 5d and e) showed remarkable differences and were compared to [^{177}Lu]Lu-FAPI-04 as [53]. ^{68}Ga - and ^{177}Lu -labeled FAPI-C16 displayed the slowest pharmacokinetics with long blood circulation on the one hand, but long tumor residence time on the other hand whereas [^{177}Lu]Lu-FAPI-04 consistent with previous studies showed a very fast PK profile. The PK profile of [^{68}Ga]Ga- and [^{177}Lu]Lu-FAPI-C12 was somewhere in between. While [^{68}Ga]Ga-FAPI-C16 was the least suitable of the three for PET imaging, [^{177}Lu]Lu-FAP-C16 showed the best

efficacy in therapeutic murine studies on HT-1080.FAP tumor-bearing mice even when lower doses were used [53].

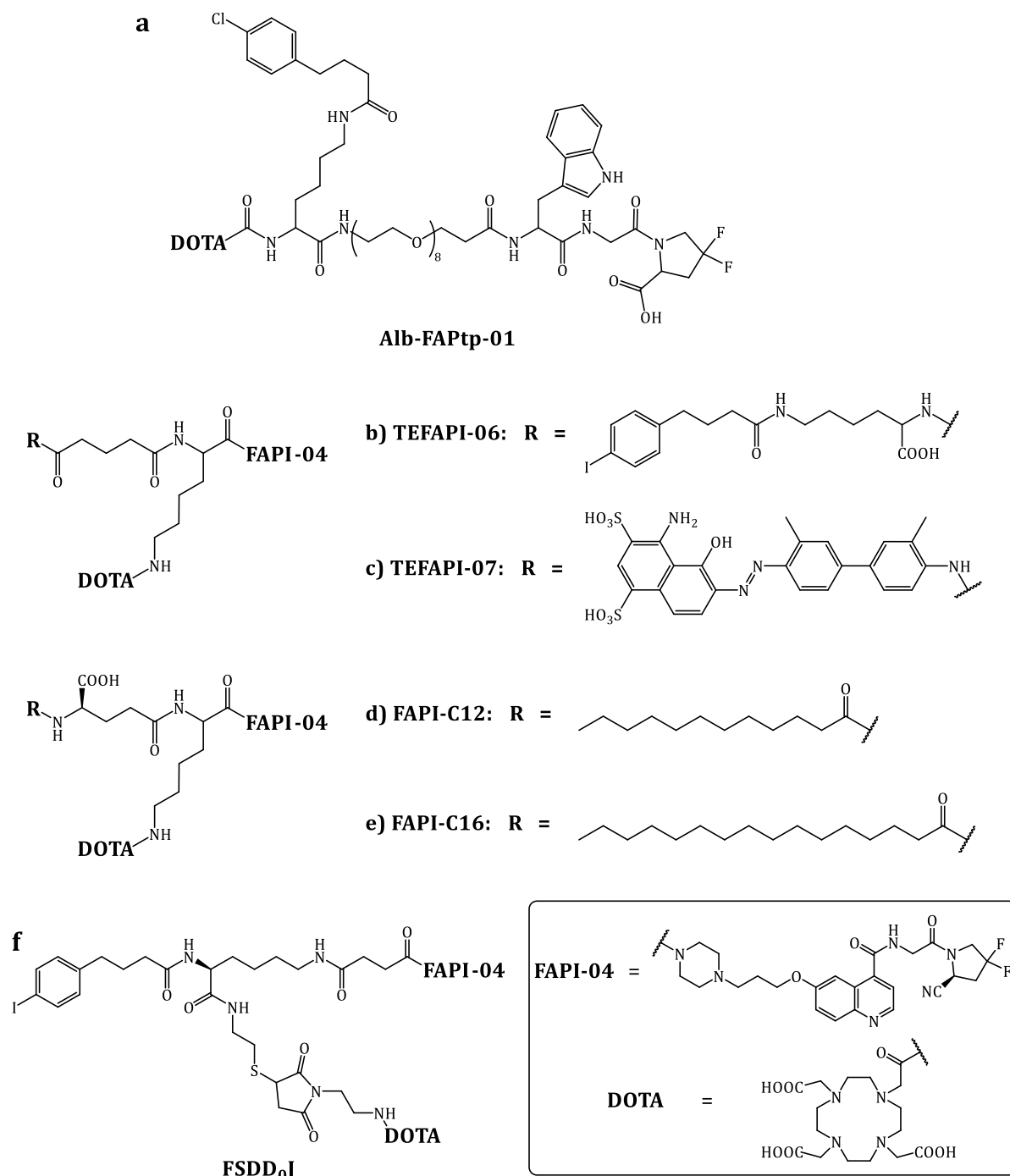


Figure 5: Structures of the FAP-albumin binder heterodimers: a) Alb-FAPtp-01, b) TEFAPI-06, c) TEFAPI-07, d) FAPI-C12, e) FAPI-C16, f) FSDD₀J.

Another EB-derivative with a non-fluorinated FAPI-02-like TV and different PEG_n spacer units ([¹⁷⁷Lu]Lu-EB-FAPI-B1 – 4 with n = 0 – 3) had lower IC₅₀ than FAPI-02 (16.8 nM vs. 10.9 nM) and no selectivity data were reported [54]. Therapeutic *in vivo* studies with U87MG glioma cells using [¹⁷⁷Lu]Lu-EB-FAPI-B1 showed some anti-tumor effect similar to the results above.

4-(*p*-iodophenyl) butyric acid was also utilized in another set of compounds the leading candidate FSDD₀I (cf. Figure 5f). Interestingly, the other candidates with an additional polar peptidic spacer of one or three aspartic acid units (FSDD₁I and FSDD₃I) had a clear negative effect on tumor retention and area under the curve [55]. [¹⁷⁷Lu]Lu-FSDD₀I uptake started to substantially decrease after 24 – 48 hours p.i. in *ex vivo* biodistribution studies with hepatocellular carcinoma PDX model. No therapeutic efficacy was evaluated yet [55].

Overall, FAPi-albumin binder “heterodimers” are still in the phase of preclinical evaluation and only murine *in vivo* studies are reported until now. Some of them showed promising therapeutic results.

The general drawback with these kinds of compounds is that they circulate in the bloodstream much longer which may result in higher doses to off-target tissues. Since they lack an additional tumor targeting moiety unlike homodimers, the ratio of radiation dose to tumor versus healthy tissue remains roughly the same for most of these kind of compounds known to date [48,51,52,54,56–59]. Therefore, the absorbed dose Gy per GBq injected activity is maximized which can reduce the activity needed to achieve the same therapeutic effect as with monomers. The activity is usually the most expensive portion of the radiopharmaceutical production and costs can be lowered significantly, e.g. when using [¹⁷⁷Lu]Lu-EB-PSMA-617 instead of [¹⁷⁷Lu]Lu-PSMA-617 [49,60,61]. But without additional pharmacological optimization like spacer/linker modifications, this approach seems to not be optimal for FAP-targeted RLT as the binding affinity/avidity to the oncological target is not substantially improved compared to a monomer.

4.1.4. Peptides

A cyclic peptide FAP-2286 (cf. Figure 6a) was identified by 3B Pharmaceuticals GmbH (Berlin) [62]. Binding to human FAP gave dissociation constants K_D of 1.1 ± 0.5 nM for FAP-2286 and $0.4 - 1.4$ nM for the different metal complexes (^{nat}Ga, ^{nat}In, ^{nat}Lu). A cell-based competitive binding assay gave IC_{50} values of $1.5 - 3.4$ nM for human FAP. IC_{50} values were in the micromolar range for DPP4 (> 10 μM) and PREP (> 1 μM) resulting in a roughly 10³-fold selectivity over PREP and 10⁴-fold over DPP4 [62].

SPECT imaging with [¹¹¹In]In-FAP-2286 showed fast accumulation and persistent tumor uptake for up to two days, fast renal clearance yet relatively low kidney uptake for a cyclic peptide e.g. when compared to DOTA-TATE. A human PET imaging study with [⁶⁸Ga]Ga-FAP-2286 indicated superiority to [¹⁸F]F-FDG and to be on par with [⁶⁸Ga]Ga-FAPI-46 in many cancer types [63].

The therapeutic efficacy of a single low and high dose [¹⁷⁷Lu]Lu-FAP-2286 (30 and 60 MBq) was investigated in a FAP-positive HEK tumor model and a sarcoma PDX model. The HEK tumor model showed almost complete remission with high doses whereas the sarcoma xenograft only showed

delayed tumor growth. [^{177}Lu]Lu-FAP-2286 was clearly superior to [^{177}Lu]Lu-FAPI-46 in the HEK tumor cell model. The median overall survival (OS) was not reached after over 40 days whereas the median OS was 27.5 days with [^{177}Lu]Lu-FAPI-46 and 16.5 days in the control group. The absorbed tumor dose was calculated to be around 10-fold higher for [^{177}Lu]Lu-FAP-2286 in this small animal study.

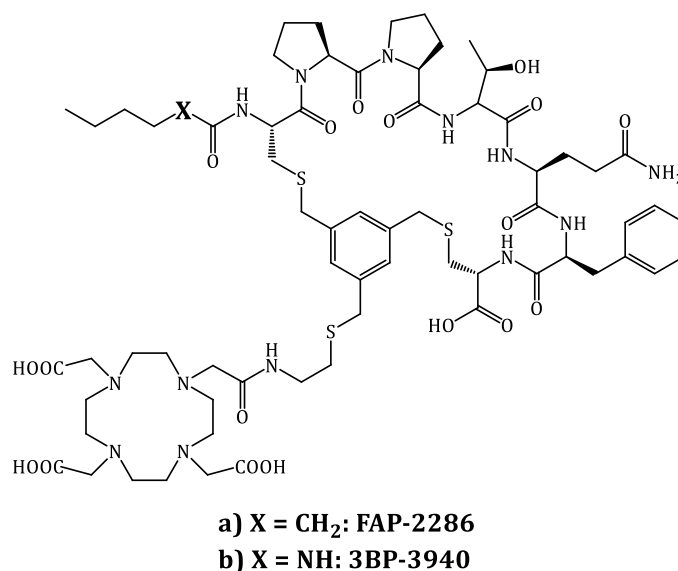


Figure 6: Cyclic peptides targeting FAP: a) FAP-2286 ($X = \text{CH}_2$) and b) 3BP-3940 ($X = \text{NH}$).

First in-human compassionate use of [^{177}Lu]Lu-FAP-2286 in 11 patients with various adenocarcinomas demonstrated substantial and sustained tumor uptake with effective half-lives of 44 ± 25 h in 13 bone metastases (3.0 ± 2.7 Gy/GBq absorbed dose) and 32 h in 1 liver metastasis (ca. 0.4 Gy/GBq absorbed dose) [64]. “The whole-body effective dose was 0.07 ± 0.02 Gy/GBq” [64]. The absorbed kidney and bone marrow doses were comparable to [^{177}Lu]Lu-PSMA-617. The absorbed doses for the kidney were 1.0 ± 0.6 Gy/GBq, similar to [^{177}Lu]Lu-PSMA-617 and relatively low when compared to the cyclic peptide [^{177}Lu]Lu-DOTA-TATE where the absorbed doses are in the same range only with Lys/Arg kidney protection. Nevertheless, there were some more severe grade III adverse events in 3/11 patients (27%). Despite the high and prolonged tumor uptake the clinical response was not very high as only 1/11 patients showed a partial response and 3/11 patients reported pain relief. Further investigations are currently running in the LuMIERE phase I/II study [64,65].

The big difference between treatment response of [^{177}Lu]Lu-FAP-2286 in murine HEK and sarcoma tumor model already indicated that the small animal HEK tumor model might not be best suited as the results seem to be not very meaningful when compared to the clinical outcomes so far. Again, it is a major challenge in radiotheranostics targeting FAP to have a reliable and meaningful murine xenograft model because the TME is addressed (except for sarcomas) and it is

very difficult to model the complex composition of a tumor lesion with its TME including all the different cell types (cf. chapter 1.1. “Tumor Microenvironment”).

The radiolabeling of a second-generation peptide 3BP-3940 (cf. Figure 6b) with various trivalent radiometals (^{68}Ga , ^{90}Y , ^{177}Lu , ^{225}Ac) along with the first clinical applications were presented at conferences last year [66,67]. The first clinical results of [^{177}Lu]Lu-FAP-2286 probably led to the conclusion that other radionuclides with higher-energy particle emission (^{90}Y) or α emitters with higher LET (^{225}Ac) might be necessary for improved treatment response. The new peptide has improved pharmacological properties with minimized kidney dose while retaining long tumor residence time. RLT of 3BP-3940 labeled with several radiometals and combinatorial “TANDEM Peptide-Targeted Radionuclide Therapy (PTRT)” with simultaneous administration of [^{177}Lu]Lu- and [^{225}Ac]Ac-3BP-3940 or [^{90}Y]Y- and [^{225}Ac]Ac-3BP-3940 was reported in 10 patients. The simultaneous administration of ^{90}Y - and ^{225}Ac -labeled 3BP-3940 seems to be more promising than using ^{177}Lu -labeled FAP-peptides only, but still only 1 in 10 patient showed partial response [66,67]. No grade III or IV adverse events were reported [66].

For the FAP-targeting peptides one may conclude so far that (i) the very promising murine *in vivo* could not be translated into clinical responses yet and (ii) longer follow-up, more patients and prospective studies are required to better assess and determine therapeutic efficacy of these novel cyclic peptides. In particular the varying therapeutic effects of the different radiometals need to be further evaluated.

4.2. Next-Generation FAPi-Based Homodimers – Results of Project 1 and 2 and Comparison with Literature

Projects 1 and 2 focused on the preparation and investigation of novel homodimeric FAPi-based radiopharmaceuticals with a next-generation molecular design involving a central linker unit, two FAPi TVs and a bifunctional chelator DO3A or DOTAGA in terminal *exo*-position. Different (central) linker and spacer units were used to investigate the effect of these derivatizations in structure-property relationship studies on radiolabeling, protease binding *in vitro* and finally the *in vivo* properties like tumor uptake/retention and biodistribution including PK/PD profile.

This was directed towards an improved application in FAP-targeted RLT with lutetium-177, yttrium-90 and FAP-targeted TAT with actinium-225. Figure 7 shows the chemical structures of the synthesized FAPi homodimers (Figure 7a and b: project 1 and Figure 7c–g: project 2).

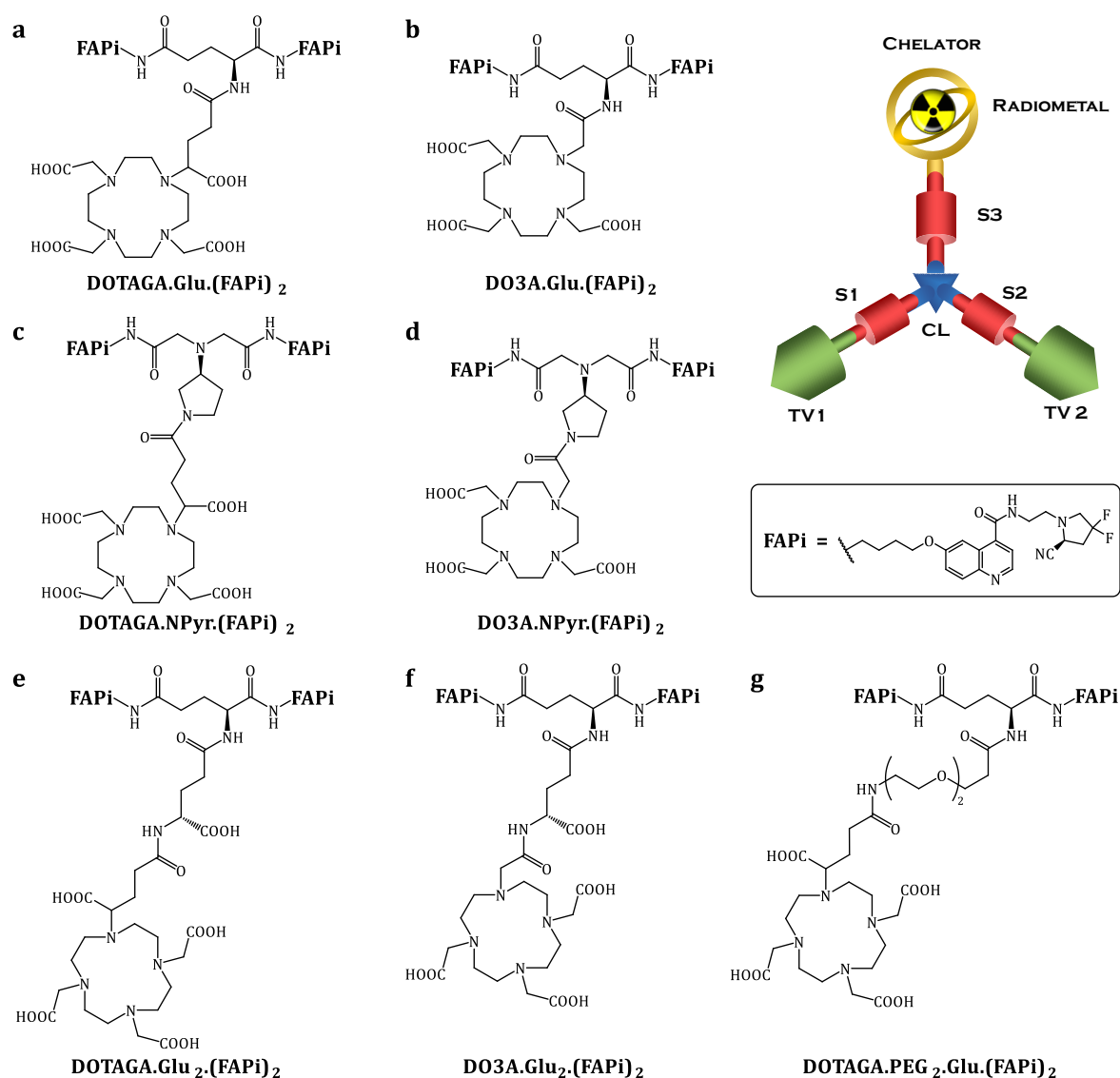


Figure 7: Chemical structures of the novel-generation FAPi homodimers a) DOTAGA.Glu.(FAPi)₂, b) DO3A.Glu.(FAPi)₂, c) DOTAGA.NPyr.(FAPi)₂, b) DO3A.NPyr.(FAPi)₂, e) DOTAGA.Glu₂.(FAPi)₂, f) DO3A.Glu₂.(FAPi)₂ and g) DOTAGA.PEG₂.(FAPi)₂.

The following sections are divided thematically. The different sections will be organic synthesis, radiolabeling, complex stability, lipophilicity, FAP affinity, FAP selectivity (over the related proteases PREP and the DPPs), cell binding (internalization and externalization), small animal and patient studies.

The results of project 1 and 2 will be summarized at the beginning of each chapter and subsequently discussed and compared to the literature state of research. The literature comparison will be focused on other homodimeric FAPi-based radiopharmaceuticals.

4.2.1. Organic Synthesis

Peptide-like chemistry – usage and cleavage of protecting groups like Boc and Fmoc and formation of amide bonds with different coupling agents like HOBt, HBTU, HATU, NHS, EDC etc. was utilized a lot in the preparation of these compounds.

The first set of compounds with glutamate (Glu) as the central linker unit contained DO3A.Glu.(FAPi)₂ and DOTAGA.Glu.(FAPi)₂ and were both synthesized in a 4-step organic synthesis starting from FAPi-NH₂ in an overall yield of 20%, respectively.

Five more precursors were synthesized in project 2 and each compound was synthesized in a six-step procedure. The alternative central linker unit NPyr, based on 3-aminopyrrolidine, was chosen because of the similarity to the piperazine linkers in known radiotracers like FAPI-04 or FAPI-46. The precursors DO3A- and DOTAGA.NPyr.(FAPi)₂ were prepared in an overall yield of 4% over 6 steps, respectively. In this case, the DOTAGA coupling had to be performed via NHS ester route since using HATU like for the other DOTAGA derivatives did not result in any product formation. DO3A- and DOTAGA.Glu₂.(FAPi)₂ consisted of an additional Glu unit with a free carboxylic acid group. Glu acted as a polar spacer and the overall yield was 7% and 12%, respectively. The diethylene glycol (PEG₂) unit of DOTAGA.PEG₂.Glu.(FAPi)₂ was the longest spacer used mainly to minimize potential steric interference and increase flexibility of the spacer and attached chelator. The synthesis of DO3A.PEG₂.Glu.(FAPi)₂ was tried several times and with several conditions and coupling reagents but no product could be isolated. DOTAGA.PEG₂.Glu.(FAPi)₂ was successfully prepared with 17% overall yield.

During the last deprotection step (*tert*-butyl ester cleavage of the chelators) a side-product was regularly identified that was proposed to be formed by hydrolysis of the nitrile group under the harsh reaction conditions (almost pure TFA). Better yields could be achieved by adding acetonitrile to the reaction in the synthesis of DOTAGA.Glu₂.(FAPi)₂. The deprotection of DOTAGA(*t*Bu)₄.PEG₂.Glu.(FAPi)₂ was also performed with MeCN and gave the highest yields of the five compounds for the last two reaction steps. Nevertheless, the yields might be further improved and these reactions definitely deserve to be investigated in more detail to further improve yields of FAPi homodimers.

4.2.2. Radiolabeling

Reaction kinetics, radiochemical conversion (RCC) and purity (RCP) were investigated in subsequent radiolabeling studies with gallium-68 and lutetium-177. DO3A.Glu.(FAPi)₂ and DOTAGA.Glu.(FAPi)₂ were additionally radiolabeled with yttrium-90 and actinium-225, respectively, and the results are summarized in Table 1.

Radiolabeling was always performed at 95 °C since the precursors contained the macrocyclic bifunctional chelators DO3A and DOTAGA. Fast ⁶⁸Ga-radiolabeling with quantitative RCC was achieved using 1 M HEPES buffer (pH = 4.5 – 5.5). This was superior to labeling in 1 M AmOAc buffer. Here, 20 nmol precursor was needed to reach RCC of around 90% whereas 10 nmol was enough to achieve quantitative RCC of ca. 100 MBq ⁶⁸Ga in HEPES buffer. It seems that the bidentate HEPES works more efficiently as a weak exchange ligand and facilitates subsequent formation of the stable DOTAGA complex. Final products [⁶⁸Ga]Ga-DO3A.Glu.(FAPi)₂ and [⁶⁸Ga]Ga-DOTAGA.Glu.(FAPi)₂ had a RCP > 99% after C18 cartridge purification. The results are similar to [⁶⁸Ga]Ga-DOTAGA.(SA.FAPi)₂ where quantitative RCC could be achieved with 10 nmol/100 MBq ⁶⁸Ga in HEPES buffer [31].

⁶⁸Ga-radiolabeling studies of project 2 gave > 90% RCC for all compounds except for DOTAGA.NPyr.(FAPi)₂ (max. ≈ 85%) and RCP > 97% after C18 cartridge purification. The three DOTAGA derivatives were labeled in three buffer systems: 1 M HEPES (pH = 5.5), 1 M AmOAc and NaOAc (pH = 4.5). In agreement with previous radiolabeling investigations on FAPi homodimers [31,68], HEPES buffer was superior for DOTAGA.NPyr.(FAPi)₂ and DOTAGA.Glu₂.(FAPi)₂. In contrast, DOTAGA.PEG₂.Glu.(FAPi)₂ was the first FAPi homodimer of our group that showed equal radiolabeling capabilities in HEPES and AmOAc buffer.

¹⁷⁷Lu-radiolabeling was investigated with all compounds except DO3A.Glu₂.(FAPi)₂. ¹⁷⁷Lu-radiolabeling worked very fast and efficiently even with low precursor amounts per activity for all compounds. For example, DOTAGA.Glu.(FAPi)₂ gave 98–99% RCC with just 10 nmol/GBq in 5 minutes. Similar results were obtained for the other ¹⁷⁷Lu-radiotracers with quantitative RCC > 99%. The radiolabeled products exhibited a radiochemical purity (RCP) > 99% as quantified via two radio-TLC systems and radio-HPLC without any purification needed. Only DOTAGA.PEG₂.Glu.(FAPi)₂ showed slightly lower RCC and RCP values with citrate radio-TLC (RCC = 90 – 97%) and some discrepancy with the other analytical methods (AmOAc/MeOH radio-TLC and radio-HPLC: RCC/RCP > 98%). No additional peak was identified in radio-HPLC and the smearing on the citrate radio-TLC was interpreted to be most likely caused by protonation equilibrium of [¹⁷⁷Lu]Lu-DOTAGA.PEG₂.Glu.(FAPi)₂. DO3A.Glu.(FAPi)₂ was additionally labeled with yttrium-90 and yielded similar results as the ¹⁷⁷Lu-labeling with RCC > 99%.

Table 1: Summary of the radiolabeling results with the FAPI homodimers that were investigated in this work.

Complex	RCC/RCY *1 [%] (reaction time [min]) at 95 °C	RCP *2 [%]	Complex Stability [%] *3	
			HS	PBS
[⁶⁸ Ga]Ga-DOTAGA.(SA.FAPi) ₂	98–99% (5–10 min, 10–20 nmol)	—	≈ 91%	≈ 98%
[⁶⁸ Ga]Ga-DOTAGA.Glu.(FAPi) ₂	95.7 ± 0.1 (5 min)	99.1 (95.2)	96.5 ± 0.6	95.9 ± 0.1
[¹⁷⁷ Lu]Lu-DOTAGA.Glu.(FAPi) ₂	99.8 ± 0.1 (10 min)	99.8	99.4 ± 0.1	99.1 ± 0.1
[²²⁵ Ac]Ac-DOTAGA.Glu.(FAPi) ₂ *4	94.3 ± 2.1 (15 min)	100	85.5 ± 1.7	45.7 ± 0.2 (95.4 ± 1.0)
[⁶⁸ Ga]Ga-DO3A.Glu.(FAPi) ₂	97.5 ± 0.1 (5 min)	98.7 (98.7)	98.1 ± 0.3	98.9 ± 0.2
[¹⁷⁷ Lu]Lu-DO3A.Glu.(FAPi) ₂	99.4 ± 0.1 (15 min)	99.9	98.9 ± 0.1	94.7 ± 2.0
[⁹⁰ Y]Y-DO3A.Glu.(FAPi) ₂	99.4 ± 0.3 (15 min)	—	98.5 ± 0.6	98.2 ± 0.3
[⁶⁸ Ga]Ga-DOTAGA.NPyr.(FAPi) ₂	85.3 ± 2.4 (10 min)	97.4 (82.3)	93.4 ± 0.2	91.8 ± 0.7
[¹⁷⁷ Lu]Lu-DOTAGA.NPyr.(FAPi) ₂	99.7 ± 0.1 (5 min)	99.4	99.2 ± 0.1	99.4 ± 0.0
[⁶⁸ Ga]Ga-DO3A.NPyr.(FAPi) ₂	97.0 ± 0.5 (10 min)	99.6 (97.9)	96.8 ± 0.3	95.6 ± 1.0
[¹⁷⁷ Lu]Lu-DO3A.NPyr.(FAPi) ₂	99.5 ± 0.2 (10 min)	100	99.0 ± 0.3	98.1 ± 0.6
[⁶⁸ Ga]Ga-DOTAGA.Glu ₂ .(FAPi) ₂	95.7 ± 3.6 (10 min)	97.6 (92.9)	92.1 ± 1.0	95.1 ± 1.5
[¹⁷⁷ Lu]Lu-DOTAGA.Glu ₂ .(FAPi) ₂	99.5 ± 0.2 (5 min)	99.3	99.0 ± 0.1	99.1 ± 0.0
[⁶⁸ Ga]Ga-DO3A.Glu ₂ .(FAPi) ₂	93.7 ± 3.6 (30 min)	98.3 (95.2)	79.4 ± 2.7	87.6 ± 1.4
[⁶⁸ Ga]Ga-DOTAGA.PEG ₂ .(FAPi) ₂	94 – 95 (10 min, AmOAc or HEPES)	98.7 (93.1)	94.6 ± 0.2	97.4 ± 0.7
[¹⁷⁷ Lu]Lu-DOTAGA.PEG ₂ .(FAPi) ₂	96.9 ± 0.4 (30 min)	98.8	98.2 ± 0.1	98.5 ± 0.1

*1 Reaction was carried out in triplicate at least ($n \geq 3$) and in 1 M HEPES buffer (pH = 5.5) except for ²²⁵Ac-labeling (0.1 M Na-ascorbate (pH = 7.0) // *2 RCP of ⁶⁸Ga-labeled products (via radio-HPLC) before C18 cartridge purification is given in brackets. // *3 After incubation at 37 °C for two half-lives (⁶⁸Ga: 120 min, ¹⁷⁷Lu: 14 d, ⁹⁰Y: 6 d, ²²⁵Ac: 20 d). // *4 RCC: Radio-TLC after 1 d; RCP: determined via γ spectroscopy; Complex stability in final formulation (saline+10% EtOH + Na-ascorbate) after incubation for 20 d at RT in brackets.

Furthermore, [²⁵Ac]Ac-DOTAGA.Glu.(FAPi)₂ showed very good radiolabeling properties with almost quantitative RCC of around 95% after 15 minutes at 95 °C with 30 nmol precursor per MBq. This was quantified via radio-TLC (TLC-imaging after 24 hours) and γ-spectroscopy. After C18 cartridge purification the RCP was over 98%, determined with both analytical methods again. This is significantly better compared to DOTAGA.(SA.FAPi)₂ and DO3A.Glu.(FAPi)₂ where RCC did not exceed 50% (data unpublished).

The radiolabeling of the FAPi homodimers from project 2 with actinium-225 and yttrium-90 could not be investigated yet. ⁹⁰Y-radiolabeling is most probably going to yield similar results than ¹⁷⁷Lu-radiolabeling due to the high similarity of the radiometals. ²²⁵Ac-radiolabeling, especially of a compound like DOTAGA.PEG₂.Glu.(FAPi)₂ that showed improved ⁶⁸Ga-radiolabeling properties, would be very interesting but could not be realized due to the limited availability of actinium-225.

4.2.3. Complex Stability

The complex stability of every radiolabeled product was determined by incubation at 37 °C in human serum (HS) and phosphate-buffered saline (PBS) over two half-lives. So, the incubation was carried out for 120 minutes for ⁶⁸Ga-, 14 days for ¹⁷⁷Lu-, 6 days for ⁹⁰Y- and 20 days for ²²⁵Ac-labeled radiotracers. The concentration was ≈ 20 MBq/mL except for [²²⁵Ac]Ac-DOTAGA.Glu.(FAPi)₂. The results are summarized in Table 1 in the right column (see above).

The complex stabilities of the ⁶⁸Ga-radiolabeled products were > 90% for 120 minutes in both media for all compounds except for [⁶⁸Ga]Ga-DO3A.Glu₂.(FAPi)₂. In this case, the complex stability might be improved by using D-Glu instead of L-Glu if this might be due to more pronounced enzymatic proteolysis. [⁶⁸Ga]Ga-DOTAGA.Glu.(FAPi)₂, -DO3A.Glu.(FAPi)₂, -DO3A.NPyr.(FAPi)₂ and -DOTAGA.PEG₂.(FAPi)₂ were all at least 95% stable in both media and no or only negligible radiolysis occurred and is slightly more stable than [⁶⁸Ga]Ga-DOTAGA.(SA.FAPi)₂ (≈ 91% in HS).

Practically no decomposition of the ¹⁷⁷Lu-complexes was observed and complex stabilities were > 98% after 14 days. Only [¹⁷⁷Lu]Lu-DO3A.Glu.(FAPi)₂ showed slight release of lutetium-177 in PBS (94.7 ± 2.0).

²²⁵Ac-radiolabeling was carried out in 0.1 M sodium ascorbate buffer (pH = 7.0). High RCC = 90–95% for DOTAGA.Glu.(FAPi)₂, and RCP > 98% after C18 cartridge purification were determined via radio-TLC and high-resolution γ-spectroscopy. Stability of [²²⁵Ac]Ac-DOTAGA.Glu.(FAPi)₂ was high in HS (> 80% after 20 d) as well as in PBS for a couple of days (ca. 90% after 2 d). Stability could be further increased by diluting the final formulation and adding more scavenger sodium ascorbate and 10% EtOH to the saline solution (> 90% after 20 d).

4.2.4. Lipophilicity

Furthermore, the lipophilicity of the ^{68}Ga - and ^{177}Lu -labeled derivatives was also investigated. The results are summarized in Table 2 and compared to $\log D_{7.4}$ values known from other literature radiotracers.

Table 2: Summary of the $\log D_{7.4}$ values of FAPI homodimers that were determined in this work as well as literature $\log D_{7.4}$ values of FAPI radiotracers from ROESCH and other groups.

Source	Complex	$\log D_{7.4}$
This Work	$[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu.}(\text{FAPI})_2$	-2.48 ± 0.05
	$[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu.}(\text{FAPI})_2$	-2.77 ± 0.10
	$[^{68}\text{Ga}]\text{Ga-DO3A.Glu.}(\text{FAPI})_2$	-2.08 ± 0.07
	$[^{177}\text{Lu}]\text{Lu-DO3A.Glu.}(\text{FAPI})_2$	-1.77 ± 0.10
	$[^{68}\text{Ga}]\text{Ga-DOTAGA.NPyr.}(\text{FAPI})_2$	-2.52 ± 0.11
	$[^{177}\text{Lu}]\text{Lu-DOTAGA.NPyr.}(\text{FAPI})_2$	-2.57 ± 0.14
	$[^{68}\text{Ga}]\text{Ga-DO3A.NPyr.}(\text{FAPI})_2$	-2.43 ± 0.10
	$[^{177}\text{Lu}]\text{Lu-DO3A.NPyr.}(\text{FAPI})_2$	-2.31 ± 0.06
	$[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu}_2(\text{FAPI})_2$	-2.46 ± 0.08
	$[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu}_2(\text{FAPI})_2$	-2.71 ± 0.10
	$[^{68}\text{Ga}]\text{Ga-DO3A.Glu}_2(\text{FAPI})_2$	-2.59 ± 0.13
	$[^{68}\text{Ga}]\text{Ga-DOTAGA.PEG}_2(\text{FAPI})_2$	-2.54 ± 0.06
	$[^{177}\text{Lu}]\text{Lu-DOTAGA.PEG}_2(\text{FAPI})_2$	-2.48 ± 0.10
Literature (ROESCH group)	$[^{68}\text{Ga}]\text{Ga-DOTA.SA.FAPI [3]}$	-2.68 ± 0.06
	$[^{68}\text{Ga}]\text{Ga-DOTAGA.}(\text{SA.FAPI})_2 \text{ [31]}$	-2.02 ± 0.06
	$[^{177}\text{Lu}]\text{Lu-DOTAGA.}(\text{SA.FAPI})_2 \text{ [69] }^{\ast 1}$	-1.71 ± 0.01
Literature (other groups)	$[^{177}\text{Lu}]\text{Lu-BiOncoFAP-DOTAGA [39]}$	-3.60
	$[^{177}\text{Lu}]\text{Lu-ND-bisFAPI [41]}$	-2.55 ± 0.04
	$[^{177}\text{Lu}]\text{Lu-DOTA-Suc-Lys-}(\text{FAPI-04})_2 \text{ [42]}$	-3.05 ± 0.08
	$[^{68}\text{Ga}]\text{Ga-Alb-FAPtp-01 [51]}$	-2.88
	$[^{177}\text{Lu}]\text{Lu-EB-FAPI-B1 [54]}$	-1.90 ± 0.12
	$[^{68}\text{Ga}]\text{Ga-FSDDoI [55]}$	-1.18 ± 0.02

^{$\ast 1$} Data submitted (provided by Eleni Gourni from University Clinic for Nuclear Medicine, Bern)

For many FAPI homodimers like DOTA-2P(FAPI)₂, (Bi)OncoFAP-11, or the albumin binders TEFAP106/07 and FAPI-C12/C16 no lipophilicity data are available.

The compounds that have published lipophilicity data, can be roughly divided into three categories.

The first category are more lipophilic radiolabeled complexes with $\log D_{7.4}$ values of -2.1 or higher. The albumin binder $[^{68}\text{Ga}]\text{Ga-FSDD}_0\text{I}$ stands out as the compound with the highest lipophilicity (-1.18 ± 0.02) where the aromatic 4-(*p*-iodophenyl)butyric acid albumin binding moiety is probably contributing substantially to this together with the rather lipophilic linker and spacer units. The other albumin binder with Evans Blue (EB) unit is also more lipophilic than most of the other compounds. $[^{68}\text{Ga}]\text{Ga-}$ (-2.02 ± 0.06) and $[^{177}\text{Lu}]\text{Lu-DOTAGA}(\text{SA.FAPi})_2$ (-1.71 ± 0.01) and one compound of this work, $[^{68}\text{Ga}]\text{Ga-}$ (-2.08 ± 0.07) and $[^{177}\text{Lu}]\text{Lu-DOTAGA}(\text{SA.FAPi})_2$ (-1.77 ± 0.10) can also be classified to this category.

All the other compounds of this work are in the range between -2.31 ($[^{177}\text{Lu}]\text{Lu-DO3A.NPyr}(\text{FAPi})_2$) and -2.77 ($[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu}(\text{FAPi})_2$). The literature $[^{177}\text{Lu}]\text{Lu-DOTA-Suc-Lys}(\text{FAPi-04})_2$, $[^{177}\text{Lu}]\text{Lu-ND-bisFAPi}$ and $[^{68}\text{Ga}]\text{Ga-Alb-FAPtp-01}$ can also be put into this category with high hydrophilicity ($-\log D_{7.4} = 2.3 - 3.0$). Interestingly, the two chelators of ND-bisFAPi (NOTA and DOTA with a total of 7 carboxylic acid groups) do not results in a substantially more hydrophilic compounds when compared to compounds of this work (3–5 carboxylic acid groups).

The very low $\log D_{7.4}$ value of $[^{177}\text{Lu}]\text{Lu-BiOncoFAP-DOTAGA}$ (-3.60) stands out from all the other values. The analysis was done slightly different (gamma counter and not via radio-TLC as in this work) but should not have substantially influenced the results. The quinoline moiety of the FAPi TV is linked slightly different in OncoFAP but does not contain functional groups with obviously very hydrophilic properties. The same is true for the linker and spacer moieties. BiOncoFAP-DOTAGA is the only literature compound with DOTAGA chelator which might explain the higher hydrophilicity compared to a DOTA compound (one more carboxylic acid). But it cannot explain the big difference to the DOTAGA compounds of this work or ND-bisFAPi.

All labeled novel-generation FAPi homodimers exhibited higher hydrophilicity than the linear homodimer DOTAGA.(SA.FAPi)₂. $[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu}(\text{FAPi})_2$ (-2.77 ± 0.10) was the most hydrophilic, surprisingly even more than DOTAGA.Glu₂(FAPi)₂ that contains an additional carboxylic acid (Glu spacer).

High hydrophilicity seems to be important for favorable pharmacological properties such as avoiding liver uptake and hepatobiliary excretion, especially with FAP-targeting radiotracers. Several examples like $[^{68}\text{Ga}]\text{Ga-FSDD}_0\text{I}$ and $[^{177}\text{Lu}]\text{Lu-DOTAGA}(\text{SA.FAPi})_2$ have shown that more lipophilic tracers tend to be excreted hepatobiliary.

Therefore, lipophilicity seems to be a good first indicator to estimate pharmacological properties of the radiotracer *in vivo*. Overall, these results of this work are promising as the hydrophilic

nature of the new compounds might lead to less liver and colon uptake and reduced absorbed doses in these organs compared to [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$.

4.2.5. FAP Affinity

All homodimeric FAP inhibitors of this work were investigated for their respective binding affinity to FAP as well as to the other closely related proteases PREP, DPP4, DPP8 and DPP9 (cf. chapter 4.2.6.). The compounds were compared to the FAP inhibitor UAMC-1110 developed by the group of PIETER VAN DER VEKEN (University Antwerp, Belgium) as the reference compound of the assay [70]. All the compounds from our group (MOON et al. [3,31,71]) like DOTAGA.(SA.FAPi) $_2$ can be directly compared to the results of this work since all the compounds were measured with the same assay at the Department of Pharmaceutical Sciences (University Antwerp, Belgium) in the group of INGRID DE MEESTER. A summary of all IC_{50} (FAP) values of this work and from former works in our group is given in Table 3 (left).

DOTAGA.Glu.(FAPi) $_2$ (0.26 ± 0.04 nM) and [$^{\text{nat}}\text{Lu}$]Lu-DOTAGA.Glu.(FAPi) $_2$ (0.33 ± 0.02 nM) exhibited the lowest IC_{50} values for FAP of all the compounds from our group so far. The FAP inhibition potency of the precursor and the $^{\text{nat}}\text{Lu}$ -complex was 3.5-fold and 4.7-fold higher compared to DOTAGA.(SA.FAPi) $_2$ and its $^{\text{nat}}\text{Lu}$ -complex, respectively. The affinity seems to be even slightly even higher than of UAMC-1110 (0.43 ± 0.02 nM). The IC_{50} values for the other novel homodimeric FAPi precursors of this work are all between 0.31 and 0.60 nM and thus lower than DOTAGA.(SA.FAPi) $_2$.

Literature results of binding to cell membrane-bound FAP in competitive assays cannot be directly compared to the results of this work. However, some of them were measured in comparison with reference compounds that can help to assess the results. For example, the potency of EB-FAPi-B1 ($IC_{50} = 16.5$ nM) was lower compared to FAPi-02 ($IC_{50} = 10.9$ nM) [54]. [$^{\text{nat}}\text{Ga}$]Ga-FSDDoI ($IC_{50} = 14.2 \pm 11.6$ nM) also showed higher IC_{50} than [$^{\text{nat}}\text{Ga}$]Ga-FAPi-04 ($IC_{50} = 3.5 \pm 0.5$ nM) [55].

The OncoFAP-derived compounds of the Philochem group all demonstrated subnanomolar IC_{50} values, the new generation of compounds even in the picomolar range. Direct comparison between OncoFAP-DOTAGA ($IC_{50} = 0.90 \pm 0.07$ nM) and OncoFAP-11 ($IC_{50} = 30 \pm 7$ pM) in the same study demonstrated 30-fold higher potency of the new inhibitor OncoFAP-11 with optimized linker and spacer unit. Strangely, the incubation was carried out at RT and not at 37 °C [39,40].

No binding affinity data were published for DOTA-Suc-Lys-(FAPi-04) $_2$ at all. The affinity data of literature compounds are summarized in Table 3 (right).

Table 3: IC_{50} (FAP) value of all homodimeric FAP inhibitors of this work as well as other FAP inhibitors known from literature.

Compounds of our group	IC_{50} / nM FAP	Compounds of other groups	IC_{50} / nM FAP
UAMC-1110 [31,70]	0.43 ± 0.02	DOTA-2P(FAPi) ₂ * ¹	3.68 ± 1.82
DOTA.SA.FAPi [3]	0.9 ± 0.1	OncoFAP-DOTAGA [39]	0.40
[^{nat} Ga]Ga-DOTA.SA.FAPi [3]	1.4 ± 0.2	[^{nat} Lu]Lu-OncoFAP-DOTAGA [39]	0.46
DATA ^{5m} .SA.FAPi [3]	0.8 ± 0.2	BiOncoFAP-DOTAGA [39]	0.17
[^{nat} Ga]Ga-DATA ^{5m} .SA.FAPi [3]	0.7 ± 0.1	[^{nat} Lu]Lu-BiOncoFAP-DOTAGA [39]	0.19
DOTAGA.(SA.FAPi) ₂ [31]	0.92 ± 0.06	OncoFAP-11 [40]	0.030 ± 0.007
[^{nat} Lu]Lu-DOTAGA.(SA.FAPi) ₂ [31]	1.54 ± 0.15	BiOncoFAP-11 [40]	0.043 ± 0.004
DOTAGA.Glu.(FAPi) ₂	0.26 ± 0.04	ND-bisFAPi [41]	0.25 ± 0.05
[^{nat} Lu]Lu-DOTAGA.Glu.(FAPi) ₂	0.33 ± 0.02	Alb-FAPtp-01 [51]	$K_D = 18.5 \pm 2.4 \mu M$
DO3A.Glu.(FAPi) ₂	0.60 ± 0.04	TEFAPi-06 [52] * ¹	12.24 ± 0.65
DOTAGA.NPyr.(FAPi) ₂	0.50 ± 0.03	TEFAPi-07 [52] * ¹	17.02 ± 1.43
DO3A.NPyr.(FAPi) ₂	0.48 ± 0.04	FAPi-C12 [53] * ¹	6.80 ± 0.58
DOTAGA.Glu ₂ .(FAPi) ₂	0.48 ± 0.06	FAPi-C16 [53] * ¹	5.06 ± 0.69
DO3A.Glu ₂ .(FAPi) ₂	0.31 ± 0.18	EB-FAPi-B1 [54] * ¹	16.5
DOTAGA.PEG ₂ .(FAPi) ₂	0.37 ± 0.03	[^{nat} Ga]Ga-FSDD ₀ I [55] * ¹	14.2 ± 11.6
		FAP-2286 [62] * ²	2.7 ± 0.9 ($K_D = 1.1 \pm 0.5$)
		[^{nat} M]M FAP-2286 * ²	1.5 – 3.4
		(M = Ga, In, Lu) [62]	($K_D = 0.2 - 1.4$)

*¹ IC_{50} value was determined in competitive cell-based assay.

*² K_D value was measured by direct binding to FAP, IC_{50} value in competitive cell-based assay.

It has to be noted that the subnanomolar IC_{50} values are at the lower limit of what the assay can accomplish.

Affinity data for the most published homo- and heterodimers are available and can be found on the right side of Table 3. The comparability of IC_{50} values that were measured with different assay conditions is limited because the outcomes depend on parameters like ligand and enzyme

concentration, assay volume incubation time and temperature etc. Additionally, some groups determine the IC_{50} value with the enzyme directly while other use FAP-positive cells where FAP is membrane-bound. Having better comparable values like K_i values would be beneficial in the future.

The difference between affinity and avidity is another important aspect with dimeric/multimeric inhibitors. Affinity is defined as the interaction strength of one ligand binding to one protein (enzyme/receptor). The stronger protein-ligand interaction of dimers is a result of higher avidity, also called functional affinity, which is the sum of all interactions, in this case between one dimeric ligand and two proteins. Higher avidity can manifest itself in different ways. One factor is the “forced proximity effect” of reversibly binding ligands/targeting vectors as in the case of carbonitrile warhead-FAP binding [47]. After dissociation of one complex, the TV still remains in close proximity to the now free active site because it is simultaneously bound to another active site and “fixed” in this position. This increases the probability for the second TV to bind again. This has been reported to contribute to prolonged target residence times of dimeric ligands [47]. For simultaneous binding to two neighboring proteases, the dimeric ligands usually need to be bigger (several kDa) and longer to cover the distance between the two active sites. Avidity effects have also been observed of homodimers like amyloid precursor protein inhibitors (APPI) with mesotrypsin, a soluble monomeric enzyme that does not cluster [72]. The increased avidity of the dimer was associated with higher number of TVs (“TV-concentration”) close to the active site which enhances binding probability even when none of the TVs are bound to the enzyme [72].

The mechanism on how the homodimeric and homomultimeric FAP inhibitors interact with FAP and CAFs, respectively, in such a way that the tumor retention is significantly prolonged is still unknown. It is questionable if the homodimers can bind at two active sites simultaneously when comparing the size of the molecules with the size of the enzyme FAP. It seems more likely that FAPi homodimers owe their improved tumor retention to the higher TV-concentration close to the active site compared to a monomer. This contributes to higher avidity since rebinding probability is increased as proposed by GALBIATI et al. [39] and ZHAO et al. [34]. Similar observations were already made with other targets and targeting vectors [72–74], even in the context of radiotracers with RGD dimers and multimers [29,75–78]. On the other hand, it has to be noted that FAP forms homodimers on the membrane surface where the distance between the active sites is relatively small [79]. Therefore, a forced proximity effect with two inhibitors binding simultaneously cannot be ruled out. Further studies have to take a closer at this mechanism. Uncompetitive or non-competitive inhibition cannot be excluded, too. However, it seems unlikely that dimers would behave very different from monomers in that respect. Additionally or alternatively, slow diffusion (cell externalization, tissue washout) might lead to more rebinding [47].

When interpreting binding affinity or IC_{50} values, competitive inhibition via MICHAELIS-MENTEN kinetics is assumed which describes the association and dissociation of **one** protein-ligand complex. In a competitive inhibition scenario, the IC_{50} is always higher than K_i (cf. CHENG-PRUSOFF equation in chapter 1.3.4.) but the values are similar when the substrate concentration is low. Since several interactions can happen with dimers, an effective or “apparent” affinity (or IC_{50} value) is measured that does not necessarily reflect the binding kinetics/mechanism of the dimer. Attempts to consider this have been made for certain situations by expanding the MICHAELIS-MENTEN equation [72,80]. But the application of such extended model is meaningful only if the binding mechanism of FAPi dimers will have been understood. However, several effects seem to be valid for dimers in general. The forced proximity effect, higher TV-concentration and slow diffusion all lead to increased target residence time and steepened saturation curves. Due to slower kinetics/longer residence time it might take longer to reach the protein-ligand equilibrium. If IC_{50} measurements are not given enough time to reach equilibrium state, the potency of the inhibitor might be underestimated [47].

In the case of FAPi monomers and FAPi homodimers, it can be observed that the IC_{50} values do not differ drastically as they all are in the same order of magnitude and roughly in the range of 0.2 – 1.5 nM. The SA-based monomers DOTA.SA.FAPi and DATA^{5m}.SA.FAPi and the SA-based homodimer DOTAGA.(SA.FAPi)₂ have IC_{50} values in the same range. The novel FAPi dimers without SA group all show slightly higher affinity.

All metal complexes show very similar FAP affinity to their respective precursor. Of the four compounds where data on the metal complexes are available, three have slightly higher and one roughly the same IC_{50} values (within margin of error). Data on (Bi)OncoFAP-DOTAGA precursor and ^{nat}Lu-complex as well as FAP-2286 and its various metal complexes (Ga, In, Lu) confirm that the affinities for FAP do not differ significantly.

4.2.6. FAP Selectivity

The dipeptidyl peptidase DPP4 is ubiquitously expressed in the human body and known to be upregulated in diabetes type II. Its tumorigenic role is not quite clear and seems to be tumor-promoting or -suppressive depending on the context [81,82]. PREP is an endopeptidase that preferably cleaves smaller peptides, is also widely expressed in healthy tissue and seems to exist in membrane-bound and intracellular form [83–85]. DPP8 and DPP9 are only expressed intracellularly and thus play a minor role in selectivity of FAPi radiotracers [81].

FAP has dual enzymatic activity: dipeptidyl peptidase and endopeptidase activity [86,87]. Therefore, not only the binding affinity for FAP, but also the selectivity over the competitive proteases DPPs, especially DPP4 and PREP is crucial for a selective accumulation of the FAP-

targeting radiotracer with low off-target binding. Therefore, IC_{50} value of the synthesized compounds of this work were also determined for all those related proteases. Table 4 summarizes the selectivity index SI , the ratio of IC_{50} value for the respective protease to the IC_{50} value for FAP, for each compound and compares it to values known from literature.

Table 4: Selectivity index (SI) as the ratio of IC_{50} value of the related proteases PREP, DPP4, DPP8 and DPP9 to $IC_{50}(FAP)$ including all homodimeric FAP inhibitors of this work and FAP inhibitors from literature with available data.

Compound	SI			
	PREP/FAP	DPP4/FAP	DPP8/FAP	DPP9/FAP
UAMC-1110 [31,70]	4186	23256	23256	10930
DOTA.SA.FAPi [3]	6000	—	—	—
[^{nat} Ga]Ga-DOTA.SA.FAPi [3]	6214	> 714	—	—
DATA ^{5m} .SA.FAPi [3]	2113	—	—	—
[^{nat} Ga]Ga-DATA ^{5m} .SA.FAPi [3]	6714	> 1429	—	—
DOTAGA.(SA.FAPi) ₂ [31]	424	435	456	174
[^{nat} Lu]Lu-DOTAGA.(SA.FAPi) ₂ [31]	364	409	266	117
DOTAGA.Glu.(FAPi) ₂	2269	4577	112	319
[^{nat} Lu]Lu-DOTAGA.Glu.(FAPi) ₂	1292	1967	661	557
DO3A.Glu.(FAPi) ₂	1667	900	1717	1583
DOTAGA.NPyr.(FAPi) ₂	1454	5312	211	702
DO3A.NPyr.(FAPi) ₂	3899	8187	437	417
DOTAGA.Glu ₂ .(FAPi) ₂	1306	2854	84	478
DO3A.Glu ₂ .(FAPi) ₂	1308	3288	237	415
DOTAGA.PEG ₂ .(FAPi) ₂	1351	6581	63	159
OncoFAP-11 [40]	—	> 1000	—	—
Alb-FAPtp-01 [51]	—	No DPP4 selectivity	—	—
FAP-2286 [62]	≈ 1000	≈ 10 ⁴	—	—

Again, the values of our group and of UAMC-1110 can be directly compared as the data were acquired under the same assay conditions. The SA-based FAPi monomers from our group showed high PREP selectivity, except DATA^{5m}.SA.FAPi even slightly higher than UAMC-1110 ($SI(\text{PREP}) = 4186$). In comparison, the $SI(\text{PREP})$ for DOTAGA.(SA.FAPi)₂ was 424 and 364 for [natLu]Lu-DOTAGA.(SA.FAPi)₂. The $SI(\text{PREP})$ were in a similar range and 435 and 409 for the precursor and the natLu-complex, respectively. The improved FAP affinity of the novel FAPi homodimers was accompanied with higher selectivity. PREP and DPP4 selectivity was > 1000 for DOTAGA.Glu.(FAPi)₂ and its natLu-complex. The PREP and DPP4 selectivity of DO3A.Glu.(FAPi)₂ was between the values for DOTAGA.(SA.FAPi)₂ and DOTAGA.Glu.(FAPi)₂.

The second-generation FAPi homodimers of project 2 showed similar results with $SI(\text{PREP and DPP4}) > 1000$. It is worth mentioning that DO3A.NPyr.(FAPi)₂ showed higher PREP and DPP4 selectivity compared to DOTAGA.Glu.(FAPi)₂. Furthermore, DOTAGA.NPyr.(FAPi)₂ and DOTAGA.PEG₂.Glu.(FAPi)₂ exhibited higher DPP4 selectivity.

Figure 7 illustrates the $IC_{50}(\text{FAP})$ values plotted against the PREP/FAP selectivity index (Figure 7a) and the DPP4/FAP SI (Figure 7b) of UAMC-1110 and the FAPi homodimers of our group. The further the marker is to the upper right, the higher the FAP potency and selectivity of the compound. UAMC-1110 shows better selectivity than the FAPi homodimers but the affinity is on par or even slightly higher for some of the compounds.

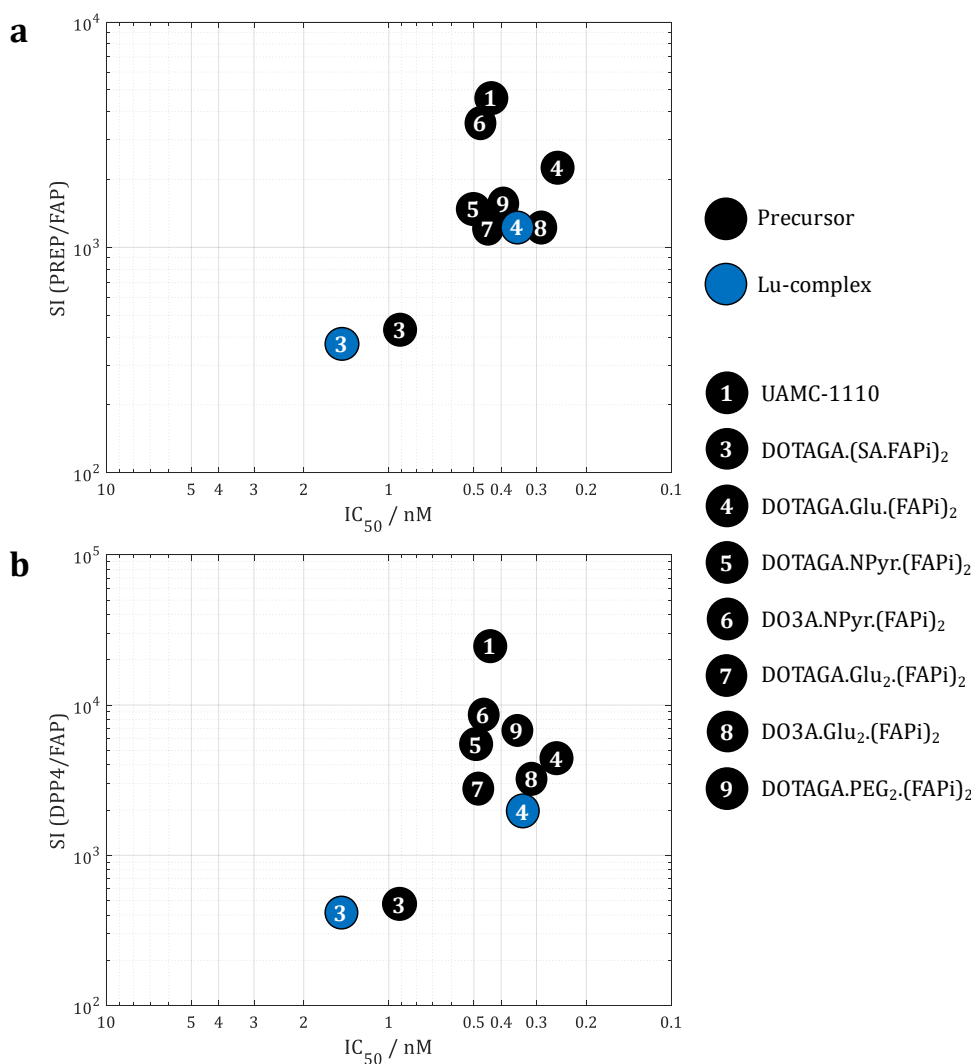


Figure 7: $IC_{50}(\text{FAP})$ values of UAMC-1110, DOTAGA.(SA.FAPi)₂ and all the FAPi homodimers of this work plotted against a) the PREP/FAP selectivity index (SI) and b) the DPP4/FAP SI.

With the exception of DO3A.Glu.(FAPi)₂, all FAPi homodimers have SI values for DPP8 and 9 below 1000. Interestingly, the DPP8 selectivity of DOTAGA.Glu₂.(FAPi)₂ and DOTAGA.PEG₂.Glu.(FAPi)₂ was even below 100. [^{nat}Lu]Lu-DOTAGA.Glu.(FAPi)₂ is slightly superior to [^{nat}Lu]Lu-DOTAGA.(SA.FAPi)₂ in this respect (DPP8: 661 vs. 266; DPP9: 557 vs. 117).

Again, the experience made in our group is that the *in vitro* binding properties of precursor and respective metal-complex do not differ a lot and this is also true for the other proteases (PREP and DPPs). Therefore, the selectivity of the respective metal complex is often similar to the precursor. The slightly decreased selectivity is often a direct result of slightly higher $IC_{50}(\text{FAP})$ values as the SI is calculated by the ratio of $IC_{50}(\text{PREP/DPP4})$ to $IC_{50}(\text{FAP})$. The small deviation manifests itself visually in Figure 7 by small distances between the precursor and metal complex marker as can be seen with DOTAGA.(SA.FAPi)₂ **3** and DOTAGA.Glu.(FAPi)₂ **4** (black: precursor, blue: Lu-complex). The same applies for the FAPi monomers investigated in our group (cf. Figure 16 in chapter 4.3.6.).

The selectivity data proving selective binding to FAP over other proteases (especially PREP and DPP4) are a central aspect that is missing for almost all compounds investigated by other groups. DPP4 selectivity ($> 10^3$), but surprisingly no PREP selectivity, was investigated for OncoFAP-11 [40]. In the publication on FAP-2286, PREP (10^3) and DPP4 (10^4) selectivity was reported [62]. In the only other publication where selectivity was discussed, the FAPi-albumin binder heterodimer [^{68}Ga]Ga-Alb-FAPtp-01 lacked DPP4 selectivity besides having low micromolar affinity ($K_D = 16.8 \pm 2.4 \mu\text{M}$) [51].

4.2.7. Cell Binding Studies

The first-generation monomers of the Heidelberg group (FAPi-04, FAPi-46 etc.) were reported to be almost completely internalized into HT-1080 cells ($> 90\%$) [1,2]. Some publications on dimers reported selective binding and blocking studies with a non-radioactive competitor [34,52,53,55]. Cellular uptake was low for some radiotracers, e.g. [^{68}Ga]Ga-Alb-FAPtp-01 (ca. 2% after 120 min) and [^{177}Lu]Lu-EB-FAPi-B1 (ca. 10% after 24 h) in U87MG cells, respectively. Li et al. [41] demonstrated high cell uptake retention of around 70% after 24 hours with a low efflux of [^{177}Lu]Lu-ND-bisFAPi [41]. The Philochem and 3BP groups did derivatizations of the respective compounds with fluorophores for staining via confocal microscopy and found fluorescent signals inside the cell (HEK-FAP [62] or SK-RC-52.hFAP [37,39,40]) and competitive inhibitor blocked the uptake. PUGLIOLI et al. [40] further looked at time-dependent dissociation from cell-bound [^{177}Lu]Lu-OncoFAP-11 and -BiOncoFAP-11. Both were stronger bound to the cells and much less dissociation compared to [^{177}Lu]Lu-OncoFAP-DOTAGA occurred [40].

Cell binding as well as internalization and externalization studies were performed with [^{177}Lu]Lu-DOTA.SA.FAPi and [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$ and were submitted for presentation at EANM 2023 by the group of GOURNI (University Clinic for Nuclear Medicine, Bern) [69]. Binding to hTERT PF179T CAFs with high FAP expression yielded dissociation constants K_D of $0.82 \pm 0.22 \text{ nM}$ and $1.35 \pm 0.68 \text{ nM}$ for monomer and dimer, respectively. The radiotracers were internalized to 20% (monomer) and 15% (dimer) after one hour with respect to total activity. Subsequent efflux studies demonstrated delayed externalization for the dimer. Half of [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$ was still internalized after one day compared to the one hour time point whereas only 13% were left in the case of [^{177}Lu]Lu-DOTA.SA.FAPi [69]. Analogous experiments with [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ and [^{177}Lu]Lu-DO3A.Glu.(FAPi) $_2$ are being carried out and first preliminary results (data unpublished) indicate similar behavior of the new dimers compared to [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$. These preliminary results already indicate that the big difference observed in tumor retention might be at least partially due to delayed externalization.

Beyond that, it is also possible that the homodimeric FAPi-based radiotracers interact with different proteases or other biological macromolecules.

DPP8 and DPP9 selectivity was < 1000 for all compounds except DO3A.Glu.(FAPi)₂. DPP8 and DPP9 are only expressed intracellularly [88,89]. Therefore, binding before cell internalization that would compete with binding to FAP cannot take place. On the contrary, it might be possible that once the FAPi radiotracer is internalized it might bind to DPP8 and/or DPP9 and thus delay externalization. As mentioned above, [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ and [¹⁷⁷Lu]Lu-DO3A.Glu.(FAPi)₂ have been and are still investigated in in- and externalization studies with CAFs. Here, it would be interesting to observe a difference in externalization behavior between DO3A.Glu.(FAPi)₂ and DOTAGA.Glu.(FAPi)₂ which could substantiate the “trapping” hypothesis that it indeed might have something to do with DPP8/9 binding. More specific studies will be needed on the influence of DPP8 and DPP9 expression in CAFs on the cell in- and externalization behavior, for example, by comparing FAP-positive CAFs that are DPP8/9-positive to DPP8/9-negative CAFs.

4.2.8. Small Animal Studies

Small animal tumor models that are often used in preclinical investigations use tumor cells transfected artificially to express FAP. Except for sarcomas, where FAP is found on tumor cells directly, this does not mirror the situation of a real tumor lesions with tumor cells and complex CAF-containing TME. This very basic yet essential problem is a key challenge in *in vivo* tumor models targeting the TME. The results have to be interpreted cautiously and the prediction value for actual behavior in patients is limited.

Therefore, small animal studies were deemed less important in the context of this work.

Nevertheless, work on optimizing tumor models is constantly ongoing. For example, several new models utilized co-culturing different tumor cell types with CAFs or stromal fibroblasts [90–94].

The group of GOURNI again compared [¹⁷⁷Lu]Lu-DOTA.SA.FAPi and [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂, in a PC3 xenografts, a prostate cancer tumor model, to investigate biodistribution of monomer vs. dimer via SPECT imaging [69]. The tumor uptake at one-hour post-injection was 8–9 %IA/g for both radiotracers. However, significant differences were observed three days p.i. with 1.1 ± 0.3 %IA/g and 3.9 ± 0.5 %IA/g for monomer and dimer, respectively, demonstrating prolonged retention in small animals [69]. Analogous investigations are planned for [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ and [¹⁷⁷Lu]Lu-DO3A.Glu.(FAPi)₂ in the near future. Furthermore, therapeutic anti-tumor efficacy studies of the different compounds are planned to be investigated in small animals in the near future.

Several of the homo- and heterodimeric compounds were investigated in preclinical murine studies. The preclinical state of research regarding all those compounds was already presented in chapter 4.1.

4.2.9. Patient Studies

First clinical data on the therapeutic use of ^{90}Y - and ^{177}Lu -labeled FAPI-04 and FAPI-46 were published [95–99]. There are also data of [^{177}Lu]Lu-FAP-2286 and first preliminary results of TANDEM-3BP-3940-PTRT labeled with various radiometals (^{90}Y , ^{177}Lu and ^{225}Ac) as already presented in chapter 4.1. [64,66].

Clinical studies on FAP-RLT with numerous patients of the same cancer type are only available for [^{177}Lu]Lu-DOTAGA.(SA.FAPi)₂ in radioiodine-refractory differentiated thyroid cancer (RR-DTC) [32,100,101]. Table 5 summarizes the current state for each radiopharmaceutical on therapeutic clinical application only. The list does not include single cases but only contains publications in which treatment of multiple patients was reported.

First compassionate use of [^{177}Lu]Lu-FAPI-04 and [^{90}Y]Y-FAPI-46 of KUYUMCU et al [95]. and LINDNER et al. [96], respectively, did not monitor clinical responses but only looked at biodistribution. First dosimetry data were reported on [^{177}Lu]Lu-FAPI-04 with low absorbed dose in the tumor lesions of various cancer types (cf. Table 5). The highest deposition was observed in bone metastases (< 1 Gy/GBq) [95].

Two studies with [^{90}Y]Y-FAPI-46 were reported on larger cohorts (21 and 10 patients with various cancers) where clinical response was monitored systematically [97,98]. Stable disease was observed in 38% (including one patient with partial response) and 50% but went along with severe grade III or IV toxicities (e.g. hematological) in 38% and 40% of the patients in the two cohorts, respectively [97,98]. The usage of relatively high doses for a ^{90}Y -radiopharmaceutical (> 7 GBq in some cases) might have been one of the reasons for the severe side effects. Furthermore, FERDINANDUS et al. [97] published data on the median effective half-life of [^{90}Y]Y-FAPI-46 in the tumor lesions that was found to be 8.7 hours (range: 5.5 – 18 h).

[^{177}Lu]Lu-FAPI-46 was administered in lower activity amounts that resulted in fewer and less severe side effects (hematotoxicity grade I – III in 1/10 patients) [99]. No treatment response, but dosimetry to healthy organs was investigated. A high range in absorbed doses to the kidneys (0.89 Gy/GBq, range: 0.08 – 1.39) was observed.

Table 5: State of research on clinical applications of radioligand therapy with FAP-targeting radiopharmaceuticals. The clinical response was determined via RECIST criteria.

Radiopharmaceutical	Patients	Response	Side Effects	Dosimetry [Gy/GBq] (Lesion)	Dosimetry [Gy/GBq] (Organ)
Activity/cycle (no. cycles)	No. (Cancer Type)				
[¹⁷⁷ Lu]Lu-FAPI-04 [95]	4 (various: breast, thyroid, thymus, ovarian)	—	—	0.62 ± 0.55 (bone) 0.38 ± 0.22 (lymph node) etc.	0.04 (bone marrow) 0.25 (kidney) 0.11 (liver)
[⁹⁰ Y]Y-FAPI-46 [96]	2 (pancreatic, ovarian)	—	—	—	—
[⁹⁰ Y]Y-FAPI-46 [97] 3.8 – 7.5 GBq (1 – 3)	10 (pancreatic, ovarian)	SD (4/8) <i>t</i> _{1/2, eff} = 8.7 h	New Grade III/IV toxicity (4/10)	1.28 (IQR: 0.87 – 1.71)	0.04 (bone marrow) 0.52 (kidney) < 0.26 (liver)
[⁹⁰ Y]Y-FAPI-46 [98] 4.2 ± 1.5 GBq (1 – 4)	21 (pancreatic, gastric, prostate, sarcoma)	PR (1/21) SD (7/21)	Grade III/IV toxicity (8/21)	2.81 ± 1.25	0.04 (bone marrow) 0.53 (kidney) < 0.11 (liver)
[¹⁷⁷ Lu]Lu-FAPI-46 [99] 1.85 – 4.44 GBq (median: 2)	18 (various: breast, colon, ovarian etc.)	—	Grade I-III hematotoxicity 1/10	—	0.03 (whole-body) 0.89 (kidney) 0.14 (liver)
[¹⁷⁷ Lu]Lu-FAP-2286 [64] 5.8 ± 2.0 GBq (median: 2)	11 (various: pancreas, breast, ovarian etc.)	SD (2/11) Pain relief (3/11)	Grade III toxicity (3/11)	3.0 ± 2.7 (bone)	0.07 (whole-body) 0.05 (bone marrow) 1.0 (kidney)
⁹⁰ Y/ ¹⁷⁷ Lu/ ²²⁶ Ac-TANDEM 3BP-3940 [66] (various)	10 (various)	PR (1/10) SD?	Hematotoxicity grade ≤ II	—	—
[¹⁷⁷ Lu]Lu-DOTAGA.(SA.FAPI) ₂ [100] 0.6 – 1.5 GBq (1)	7 (thyroid, breast, paraganglioma)	<i>t</i> _{1/2, eff} = 86.6 h (IQR: 34 – 95)	—	6.7 ± 2.7	0.02 (whole-body) 0.02 (bone marrow) 0.37 (kidney)
[¹⁷⁷ Lu]Lu-DOTAGA.(SA.FAPI) ₂ [32] 8.2 ± 2.7 GBq*1 (2 – 4)	15 (thyroid)	PR (4/15) SD (3/15) <i>t</i> _{1/2, eff} = 88 h (IQR: 86 – 99)	No grade III/IV	10.8 (IQR: 4.2 – 89)	0.02 (whole-body) 0.026 (bone marrow) 0.30 (kidney) 2.0 (left colon)

*1 cumulative administered activity, not per cycle

A comparative study of the linear SA-based homodimer [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$ and the corresponding FAPi monomer [^{177}Lu]Lu-DOTA.SA.FAPi was conducted in 7 patients with multiple cancer types [100]. The dosimetry clearly showed superiority of the FAPi dimer as the tumor dose was 10-fold higher with a ca. 6-fold longer effective half-life (14 vs. 87 h). The absorbed doses were comparable (bone marrow) and even lower (whole-body, kidney) than of the abovementioned radiopharmaceuticals or of approved drugs like [^{177}Lu]Lu-PSMA-617.

BALLAL and co-workers [32] further investigated dosimetry and treatment response in 15 radioiodine-refractory differentiated thyroid cancer (RR-DTC) patients. A total of 45 cycles of [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$ was administered with a cumulative activity of 8.7 ± 2.7 GBq (range: 5.5 – 14.0 GBq) in an median of 3 cycles (range: 2 – 4 cycles). The absorbed tumor dose was even higher than in the previous work with 10.8 Gy/GBq (range: 4.2 – 89 Gy/GBq) vs. 6.7 ± 2.7 Gy/GBq and the calculated effective half-life was very similar (88 vs. 87 h) [32,100]. The organ dosimetry of the previous work [100] could be confirmed. The left colon was identified as the critical organ (2.0 ± 0.2 Gy/GBq) but it is known that the colon usually tolerates radiation well as can be seen in the high maximum of 38 Gy as the limit for external beam radiation therapy [32]. Grade I diarrhea occurred in 1/15 patients only, and no severe grade III/IV adverse events were registered at all. A total of 47% responded well to the treatment and showed stable disease (3/15) or even partial response (4/15) referring to molecular response, meaning decreased thyroglobulin levels [32].

The new FAPi homodimer DOTAGA.Glu.(FAPi) $_2$, synthesized within project 1 of this work, was chosen as a proof-of-concept patient study due to the improved *in vitro* binding affinities, radiolabeling properties and the higher hydrophilicity. [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ was investigated in comparison to [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$. First administrations with lower “scouting” doses of [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ indicated promising *in vivo* properties of the new radiopharmaceutical. Thus, a direct comparison in a medullary thyroid cancer patient was performed [68]. Pre-therapy PET/CT scans with [^{18}F]F-FDG (Figure 8a) and [^{68}Ga]Ga-DOTA.SA.FAPi (Figure 8b) both showed intense uptake in the primary lesion and metastases (e.g. in the liver). The patient was treated with two cycles of [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$ (5.5 GBq each). Figure 8c shows whole-body scans 24 and 48 hours p.i. during the second treatment cycle. Liver uptake 24 h p.i. and colon uptake 48 h p.i. was observed for [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$ indicating a rather slow hepatobiliary excretion. In contrast, very low uptake was registered in both organs 24 h p.i. for [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ (5.5 GBq) indicating a faster excretion that will eventually lead to minimized absorbed radiation doses to the liver and the colon in particular. At the same time, the tumor residence time was still long and showed the typical behavior of FAPi homodimers. This first comparison revealed a favorable pharmacological profile for [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ and encouraged further investigations of this new therapeutic FAPi dimer.

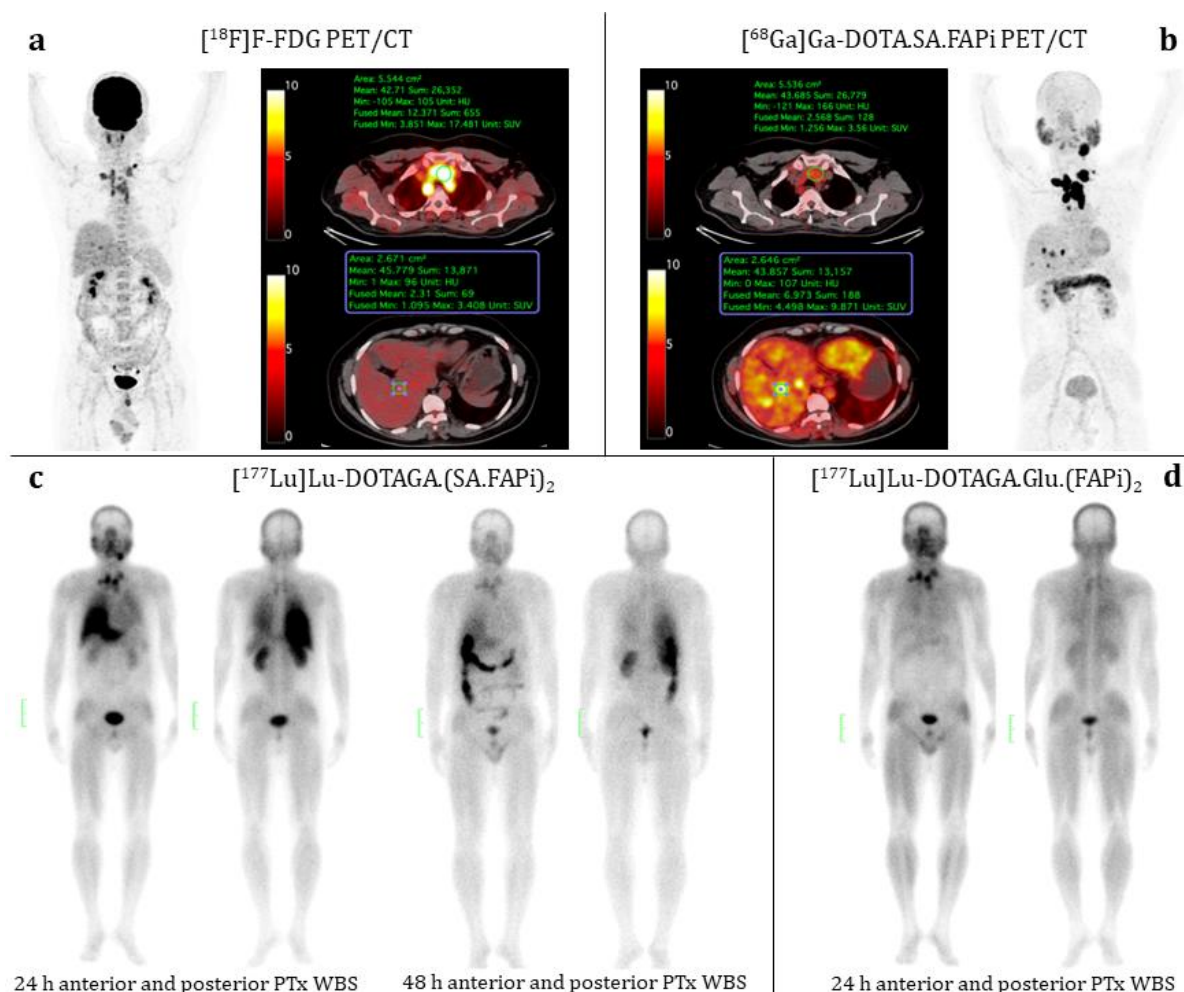


Figure 8: A medullary thyroid cancer patient underwent $[^{18}\text{F}]\text{F-FDG PET/CT}$ scan (a) and $[^{68}\text{Ga}]\text{Ga-DOTA.SA.FAPi PET/CT}$ scan (b). Subsequently, the patient was treated with two cycles of $[^{177}\text{Lu}]\text{Lu-DOTAGA.(SA.FAPi)}_2$ (5.5 GBq each) (c). “The whole-body scan during treatment at 24 h p.i. (c, left) reveals intense uptake in the neck and mediastinal metastases but a simultaneous intense radiotracer accumulation in the liver. In addition, 48 h p.i. shows the hepatobiliary excretion (c, right). Subsequent treatment in the same patient with 5.5 GBq of the new dimer $[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu.(FAPi)}_2$ showed only uptake in the neck lesions on whole-body scan at 24 h p.i. (d) and negligible uptake in the liver.” This figure was originally published in *Cancers* by MARTIN et al. Novel Generation of FAP Inhibitor-Based Homodimers for Improved Application in Radiotheranostics. *Cancers* **2023**, 15(6), 1889 [68]. © 2023 MDPI

$[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu.(FAPi)}_2$ was further applied systematically in more thyroid cancer patients and additionally in other cancer types, such as glioblastoma, breast and lung cancer.

For example, a case report on a 52-year-old glioblastoma patient was submitted to *Nuclear Medicine and Molecular Imaging* (on 7 April 2023) recently [102]. The patient showed intense uptake in FAPi PET/CT with $[^{68}\text{Ga}]\text{Ga-DOTA.SA.FAPi}$ in the primary brain tumor lesion (Figure 9a – c). Two cycles of $[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu.(FAPi)}_2$ RLT (7.4 GBq each) were administered within 8 weeks and planar whole-body scans during treatment showed significant tumor uptake 24 p.i. in both treatment cycles. Interim $[^{68}\text{Ga}]\text{Ga-DOTA.SA.FAPi PET/CT}$ scan (Figure 9d – f) demonstrated significantly reduced radiotracer uptake and additional control via MRI revealed a substantially decreased lesion size. No adverse events occurred during the 8-month follow-up.

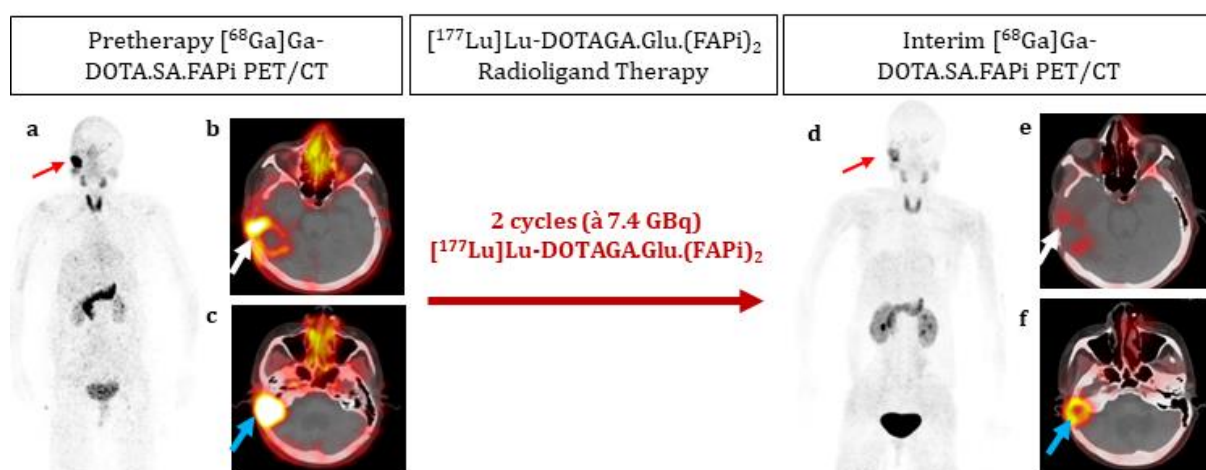


Figure 9: 52-year-old glioblastoma patient and a) the baseline whole-body PET with [^{68}Ga]Ga-DOTA.SA.FAPi and axial PET/CT images that show the high intensity uptake in the right parieto-temporo-occipital region (b, white arrow) and the right cerebellar region (c, blue arrow). The patient underwent two cycles of [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ radioligand therapy (7.4 GBq each) and d) interim [^{68}Ga]Ga-DOTA.SA.FAPi PET/CT after the two cycles showed significantly reduction in uptake and size of the brain tumor lesion in the whole-body PET d) and the axial PET/CT images e) and f).
Submitted to Nucl. Med. Mol. Imaging on April 07, 2023 [102].

Treatment of the first patients with [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$ was started around 3 years ago on the basis of compassionate use since the patients, mostly RR-DTC patients, had exhausted all established treatment options.

The first patients were treated with the new FAPi dimer [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ a little over one year ago. From that point on, all patients – even those who were treated with [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$ before – were treated with [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ mainly because of the reduced dose to the colon and liver.

At the moment, the largest cohort (39 patients) are thyroid cancer patients, followed by breast cancer patients (19 patients) as well as smaller numbers on other cancer types including lung cancer and glioblastoma with a total of more than 70 patients.

Until now, a minimum of 126 cycles (median: 5.5 GBq, range: 2 – 9.3 GBq) were administered to the 39 thyroid cancer patients alone (median: 3 cycles, range: 2 – 8 cycles). The current status of the thyroid cancer patients treated with ^{177}Lu -FAPi dimer RLT is as follows: (i) 51.2% (20/39) biochemical response manifested by decreased thyroglobulin levels, (ii) only 24% (7/39) show progressive disease and (iii) 6/39 patients died within a median follow-up of 20 months (range: 4 – 33 months). Observed adverse events are as follows: (i) grade III anemia in 5.1% (2/39), (ii) grade III thrombocytopenia in 7.6% (3/39), (iii) grade II diarrhea in 10.2% (4/39). No grade III renal or hepatotoxicity occurred.

Preliminary KAPLAN-MEIER curves of progression-free survival (PFS) and overall survival (OS) of the metastasized RR-DTC patients are shown in Figure 10a and b, respectively. For the PFS curve a follow-up time of 25 months is depicted with all 39 patients considered for this. A 33-month follow-up of 13 patients is considered for the OS curve.

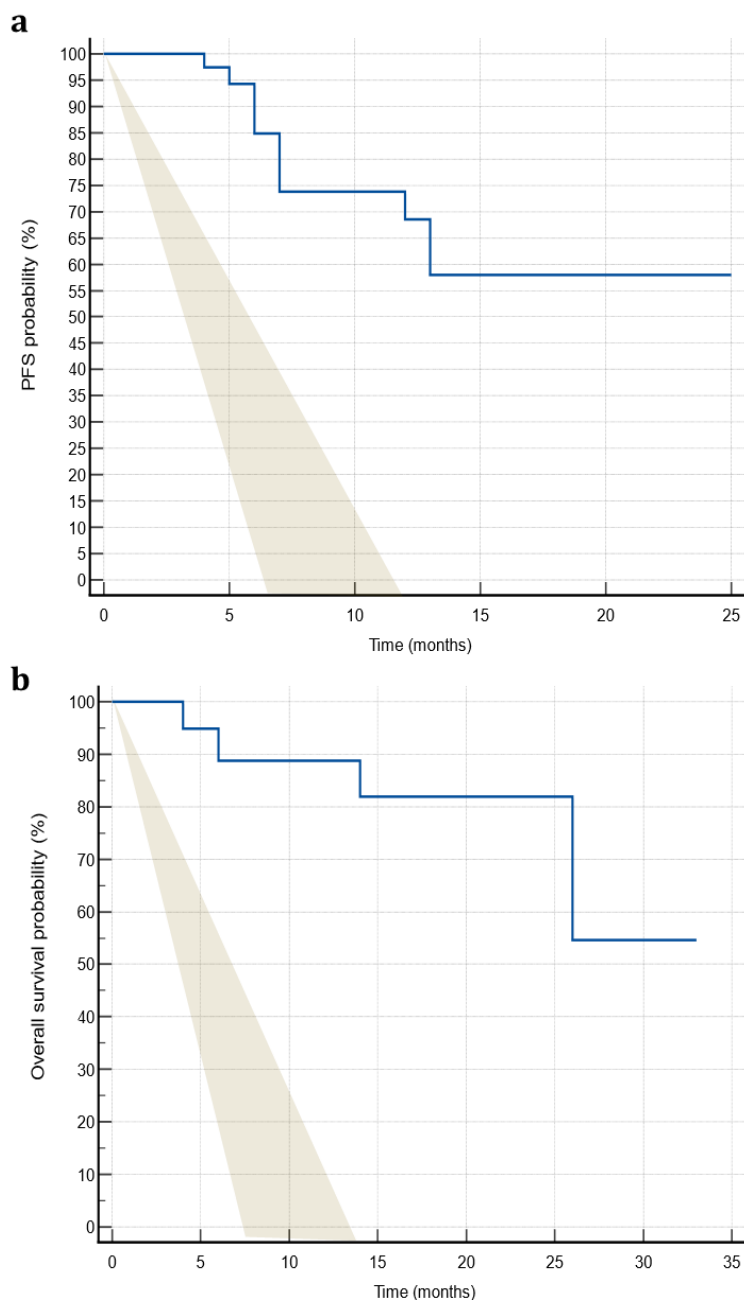


Figure 10: Preliminary KAPLAN-MEIER curves of a) progression-free survival (PFS) and b) overall survival regarding of metastatic radioiodine-refractory differentiated thyroid cancer (RR-DTC) treated with ^{177}Lu -FAPi dimer RLT (data unpublished). The grey-brown area indicates the survival curve of metastatic RR-DTC patients without treatment.

As described above, these data combine ^{177}Lu -FAPi dimer RLT with $[^{177}\text{Lu}]\text{Lu-DOTAGA.Glu.}(\text{FAPi})_2$ and $[^{177}\text{Lu}]\text{Lu-DOTAGA.}(\text{SA.FAPi})_2$ as the treatment switched seamlessly from the linear homodimer to the new FAPi homodimer. The median OS and PFS is not attained

yet. But from these data a prognosis of 12-month OS probability of 68.5% can be calculated. It cannot be compared to an untreated control group directly, but the grey-brown area indicates the average survival of untreated metastatic RR-DTC that is usually between 6 and 8 months (SANJANA BALLAL, personal communication, December 2022).

In a similar study, 19 breast cancer patients have been treated with ^{177}Lu -FAPi dimer RLT over almost 2 years. Figure 11 shows a striking case of a patient with infiltrating ductal carcinoma with extensive bone and liver metastases in the pre-therapy PET scans with ^{18}F -FDG (Figure 11a) and ^{68}Ga -Ga-DOTA.SA.FAPi (Figure 11b). After FAPi-dimer RLT with $4 \times 7.4 \text{ GBq}$ ^{177}Lu -Lu-DOTAGA.Glu.(FAPi) $_2$, the metastases have disappeared almost completely, e.g. the big lesion on the right shoulder, the pelvic/femur bone lesions and the liver metastases. Both ^{18}F -FDG (Figure 11 c) and ^{68}Ga -Ga-DOTA.SA.FAPi (Figure 11d) post-therapy PET scans show confirming results.

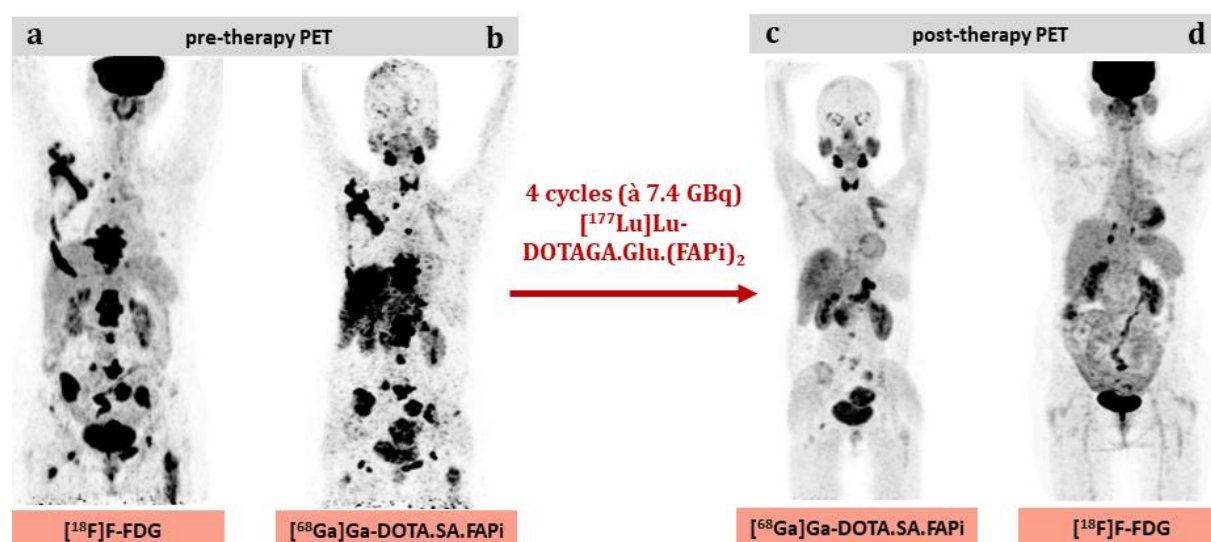


Figure 11: Breast cancer patient (infiltrating ductal carcinoma) with liver metastases and extensive bone metastases in pre-therapy PET scan with ^{18}F -FDG (a) and ^{68}Ga -Ga-DOTA.SA.FAPi (b). The patient was treated with four cycles of ^{177}Lu -Lu-DOTAGA.Glu.(FAPi) $_2$ radioligand therapy (7.4 GBq each). PET scans after the treatment showed significant decrease in the uptake and size of the bone and liver metastases with concordant results in c) ^{68}Ga -Ga-DOTA.SA.FAPi and d) ^{18}F -FDG PET scans (data unpublished).

Of the 19 patients, 9 were diagnosed with infiltrative ductal carcinoma, 9 with invasive ductal carcinoma and 1 with invasive lobular carcinoma. All patients suffered from bone metastases and 5 and 7 patients had additional liver and brain metastases, respectively. The patients were heavily pre-treated with surgery (14/19) and/or different kinds of chemotherapy (17/19).

A significant clinical response rate of 84% (16/19) was observed according to VAS criteria which assess pain intensity with the following details: (i) complete response (5/19, 26.3%), (ii) partial response (3/19, 15.7%), (iii) minimal response (8/19, 42%). Furthermore, two patients had stable disease (2/19, 11%) and only patient did not respond.

From the 9 patients still alive after the median follow-up of 14 month, 4/9 showed partial remission and 1/9 stable disease in post-therapy PET scans while 2/9 showed progressive disease and 2/9 have a pending post-therapy PET scan.

The preliminary KAPLAN-MEIER curves regarding PFS (Figure 12a) and OS (Figure 12b) show the 14-month and 22-month median follow-up, respectively. The median OS is 12 months (95% confidence interval (CI): 8 – 14 months) and the median PFS is 8.5 months (95% CI: 7.5 – 11.5 months). PFS/OS of an untreated control group was not available until now.

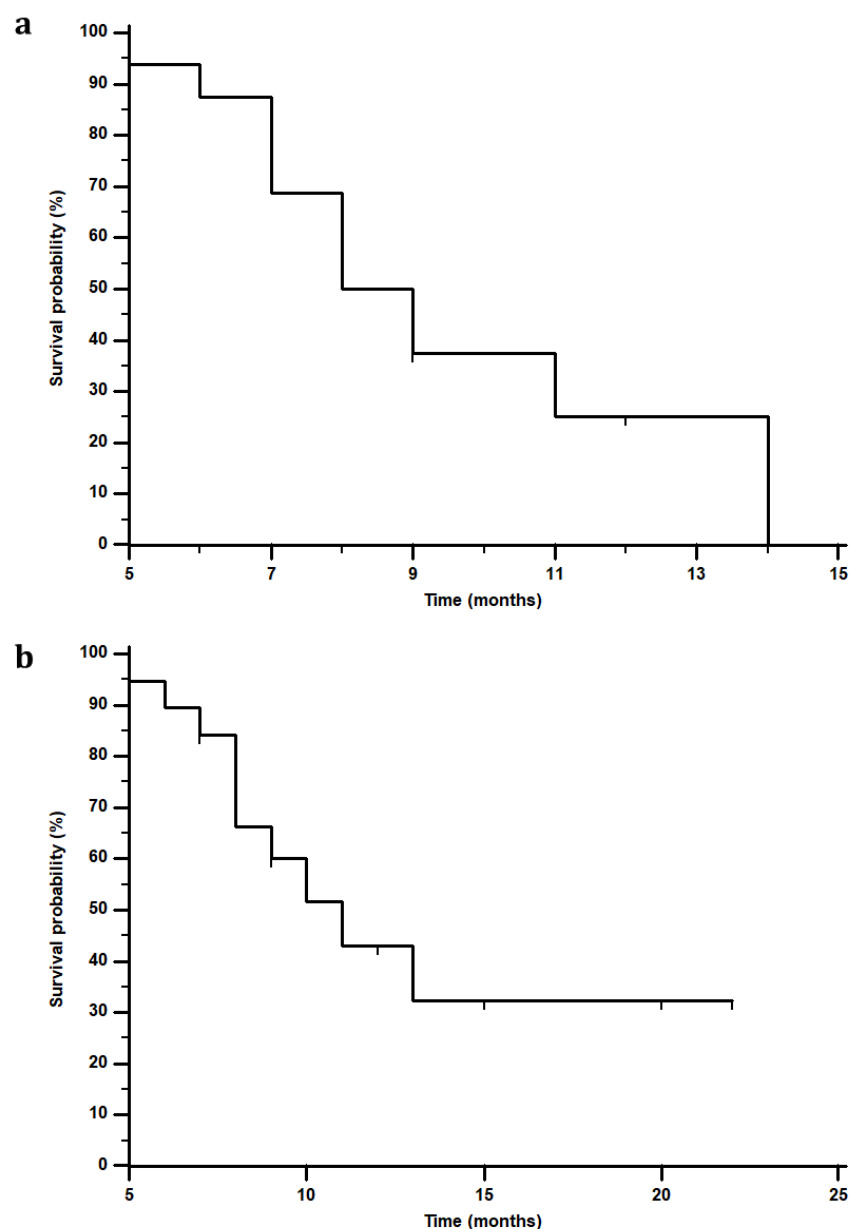


Figure 12: Preliminary KAPLAN-MEIER curves of a) progression-free survival (PFS) and b) overall survival regarding infiltrating or invasive breast cancer treated with ^{177}Lu -FAPi dimer RLT (data unpublished).

All these data on thyroid and breast cancer patients will be shortly published by our cooperation partners from All India Institute of Medical Sciences (AIIMS, New Delhi, India). Another publication on lung cancer is also planned.

Furthermore, first administrations of [^{225}Ac]Ac-DOTAGA.Glu.(FAPi) $_2$ were performed based on the high RCC and RCY that can be achieved in ^{225}Ac -labeling of DOTAGA.Glu.(FAPi) $_2$. FAP-TAT was mainly considered as a further treatment option for patients with progressive disease after ^{177}Lu -FAPi dimer RLT. Unfortunately, systematic applications of [^{225}Ac]Ac-DOTAGA.Glu.(FAPi) $_2$ are delayed because of the currently limited ^{225}Ac -availability.

4.2.10. Outlook

Despite the further research on development of new FAPi homodimers for theranostic application, the impressive clinical results of [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ deserve more attention by performing more and systematic studies.

[^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ will be further investigated in clinical studies addressing various cancer types. At first, detailed studies on biodistribution, dosimetry and effective half-life are very important to quantify the differences to [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$ regarding absorbed dose to tumor and healthy organs (colon, liver).

The clinical studies should be expanded in all directions. This includes larger cohorts of the cancer types that already have been investigated (thyroid, breast, lung cancer and glioblastoma) in comparison to non-treated control groups to more precisely determine OS and PFS. Ideally, this will further expand to bigger prospective multi-center studies.

Beyond that, the efficacy in other epithelial cancers (e.g. prostate, gastric, colon, liver, pancreatic cancer etc.) and in sarcomas is interesting as other publications showed intense uptake with FAPi radiotracers and sarcomas directly expressing FAP [5,103,104].

For the FAPi homodimers of project 2, the next step will be the evaluation *in vivo*. Most of them showed similar *in vitro* properties compared to DOTAGA.Glu.(FAPi) $_2$. DO3A.NPyr.(FAPi) $_2$ exhibited improved PREP and DPP4 selectivity. DOTAGA.NPyr.(FAPi) $_2$ and DOTAGA.PEG $_2$.Glu.(FAPi) $_2$ showed higher DPP4 selectivity and the latter additionally showed improved radiolabeling flexibility. These might be the first candidates for *in vivo* translation of the ^{177}Lu -labeled radiopharmaceuticals that should be performed in direct comparison to [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$.

One very general, broad and central aspect regarding FAP-RLT is the almost unstudied field of combinatorial therapies. Therapeutic studies on mice that systematically investigate different combinations would lead to a much better understanding on the direction of how CAF-addressing treatment can be optimized.

When taking a homodimeric FAPi radiopharmaceutical as a starting point, the radionuclide and the type of radiation will affect anti-tumor efficacy. A comparative investigation of ^{90}Y -, ^{177}Lu - and

^{225}Ac -labeled FAPI homodimer, e.g. DOTAGA.Glu.(FAPi) $_2$, is very interesting in this regard. It has to be kept in mind that addressing CAFs in the TME might lead to different therapeutic responses compared to addressing tumor cells directly.

Figure 13 illustrates a simplified tumor lesion of 2 mm diameter with a supposed 1:1 ratio of tumor-to-tumor stroma volume and a clear separation between the inner tumor cells and the outer tumor stroma. It visualizes the range of the three most relevant trivalent radiometals under this simplified assumption.

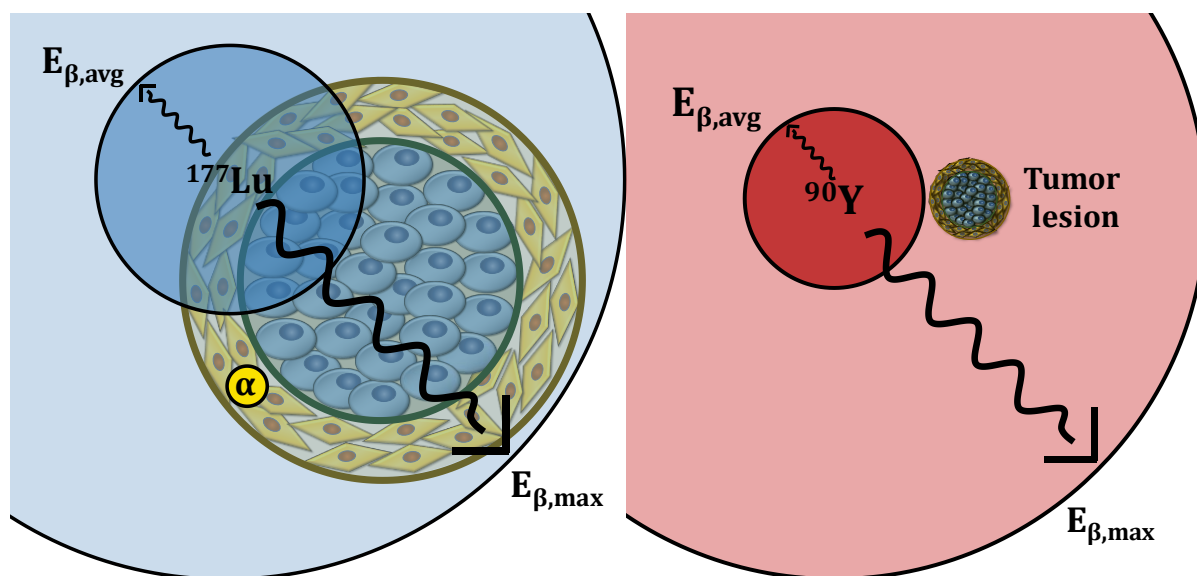


Figure 13: Tumor lesion (2 mm diameter) consisting of tumor cells in the center (blue) and an outer tumor stroma containing CAFs (yellow) in comparison with the range of different radiometals. A tumor-to-tumor stroma volume ratio 1:1 is assumed (ca. 200 – 300 μm thick tumor stroma). The range of an α particle from actinium-225 is max. 100 μm (left, yellow). Assuming an accumulation in the middle of the tumor stroma the range is not high enough to reach the tumor cells. The average range of lutetium-177 according to $E_{\beta,\text{avg}}$ is around 700 μm (dark blue) whereas the maximum range is ca. 2 mm (light blue) and just enough to potentially damage all cells of the tumor lesion. The average range of yttrium-90 (2.4 mm, dark red on the right) is already larger than the tumor lesion. The relatively high maximum range is ca. 12 mm and might lead to damage of the surrounding healthy tissue. [105–108].

This illustration clarifies that α emitters will more or less only damage the TME. But due to the higher LET, α emitters are able to severely damage the tumor stroma. The role of the TME and CAFs as a physical barrier has been discussed in chapter 1.2.3. This seems to lead to increased resistance to therapy because the tumor cells are protected by the TME which prevents the drug to get to the tumor cells where it can unfold its anti-tumor potency. Therefore, an interesting combination with α -emitting radiopharmaceuticals might be agents that address the tumor cells directly, e.g. a conventional chemotherapeutic agent, PARP or (tyrosine) kinase inhibitor. Similarly, a PD-1 inhibitor demonstrated higher anti-tumor efficacy in a combinatorial treatment with [^{177}Lu]Lu-FAP-2287 (a derivative of FAP-2286) in MCA205-mFAP xenografts [109]. Other possible approaches include drug delivery system targeting the tumor cells, immunotherapy like

immune checkpoint inhibitor or (Uni) CAR T cell therapy, etc. After the tumor stroma is damaged by the α emitter, a higher percentage of the second agent can reach the tumor cells.

The better penetration of radiation-damaged tumor stroma even applies when β^- emitter like lutetium-177 are used. [^{177}Lu]Lu-DOTAGA.(SA.FAPi) $_2$ was investigated in a combination therapy with the PARP inhibitor olaparib that showed synergistic effects in 4T1 murine triple-negative breast cancer xenografts [110].

Similarly, the simultaneous administration of the same compound labeled with two different radionuclides (TANDEM) approach might lead to synergistic effects. The ^{225}Ac -labeled FAPi homodimer predominantly damages the TME whereas the β^- emitter additionally damages the tumor cells due to crossfire effect.

Therefore, the further exploration of [^{225}Ac]Ac-DOTAGA.Glu.(FAPi) $_2$ and its therapeutic efficacy as a sole therapeutic agent and in combination with other cytostatic drugs is promising and intriguing and deserves further attention.

Overall, the question which treatment strategy is optimal for FAP-targeted radiopharmaceuticals is still unanswered because there are many variables. Beyond using different radionuclides and maybe using them simultaneously in a TANDEM approach, the combination of FAP ligand, radionuclide and potentially combining it with other treatments are just some of the variation possibilities.

A broad range of structures are possible within the “trislanker” concept with the new-generation of branched FAPi homodimers with central linker unit. The general molecular design was part of a patent that was drafted within the context of project 1 and 2 (cf. chapter 2). Beyond the homodimers described and discussed in this work, it is possible to synthesize many more FAPi homodimers. The structure variations of project 2 did not substantially alter the properties of the compounds, e.g. the *in vitro* binding affinities, but in some cases such as DOTAGA.PEG $_2$.Glu.(FAPi) $_2$ the radiolabeling seems to be more flexible regarding the reaction conditions indicating the positive effect of the larger distance and higher flexibility of the PEG $_2$ spacer that might have resulted in minimized steric hindrance.

Figure 14 shows some exemplary structures that could have promising properties. Beside the chelator DOTAGA, AAZTA ^{5} might also be an interesting option as the four carboxylic acid groups analogous to DOTAGA will enhance hydrophilicity and enable radiolabeling of radiometals like lutetium-177, scandium-44, etc.

Compounds with the 3-aminopyrrolidine (NPyr) derived central linker (CL) unit can be derivatized by introducing different spacer units like PEG $_n$ either between chelator and CL (Figure 14a), CL and FAPi TV (Figure 14c) or both (Figure 14b).

Principally, spacer units between CL and FAPi are possible with Glu as CL (Figure 14e and f). Here, it would be especially interesting to utilize NPyr as a spacer unit as in Figure 14f because of the reminiscence of the position of the heterocyclic linker in FAPi-04 and -46. The synthesis of such compounds was tried but proved to be synthetically challenging.

Furthermore, the combination with three spacer units at every position is possible (Figure 14b, g and h).

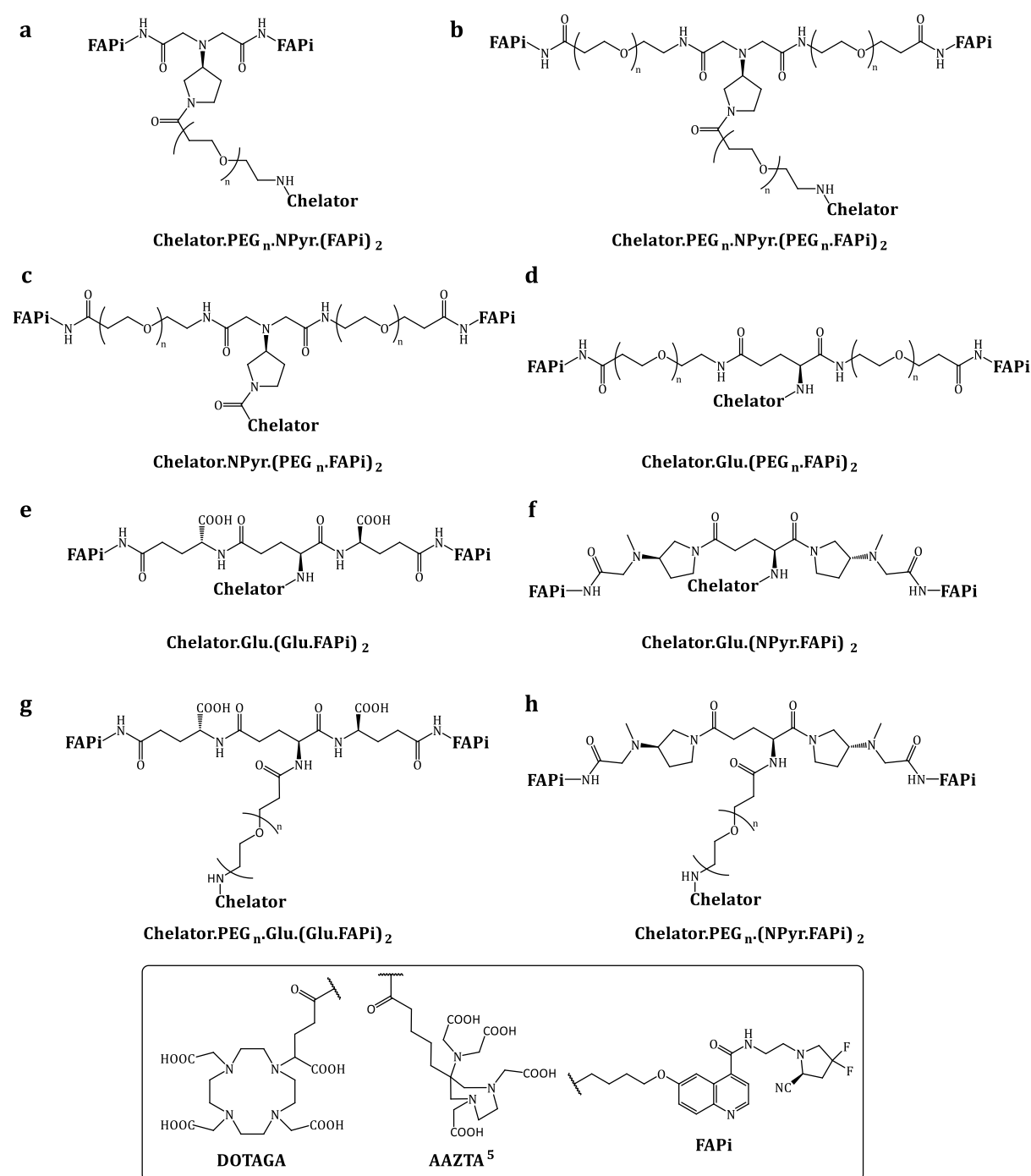


Figure 14: Further possible FAPi homodimer structures.

4.3. Novel Monomeric FAPi-Based Radiotracers – Results of Project 3 and 4 and Comparison with Literature

The primary interest of the present work was to develop new, improved FAPi homodimers for RLT. One of the basic principles of radiotheranostics is that the diagnostic and therapeutic molecules are structurally as similar as possible. The FAPi homodimers themselves are optimized for long tumor retention time and do not possess the perfect pharmacokinetic profile for ^{68}Ga -PET. This is why a monomer is used for ^{68}Ga -PET/CT guided ^{177}Lu -homodimer RLT. The currently used ^{68}Ga -DOTA.SA.FAPi is also not optimal because it is structurally quite different from the homodimers as it contains a squaramide linker and no central linker unit.

Figure 14a illustrates the concept of this project and why the molecules were chosen

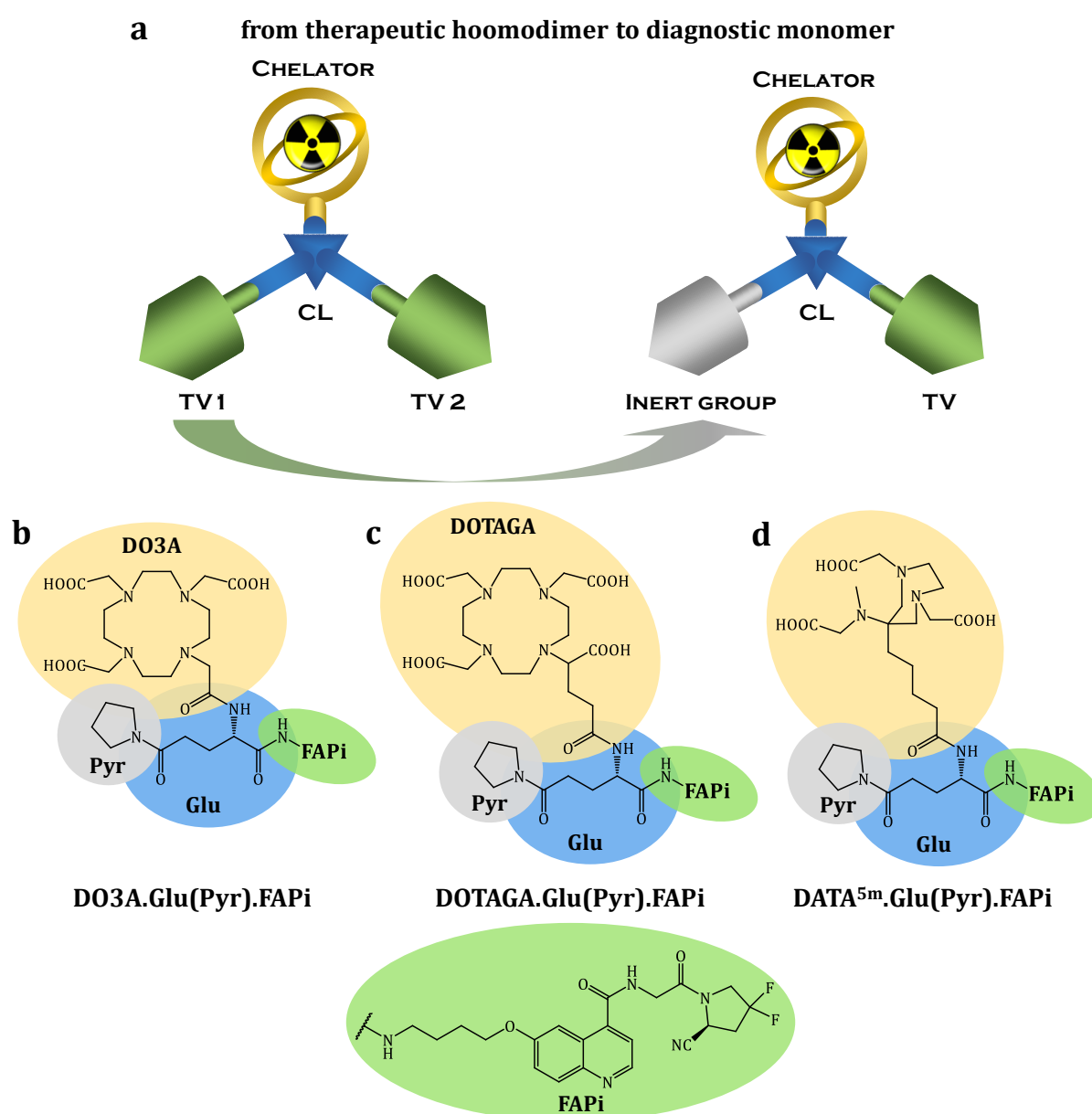


Figure 14: a) Structural transition from therapeutic homodimer to diagnostic monomer with the monomers b) DOTAGA.Glu(Pyr).FAPi, c) DO3A.Glu(Pyr).FAPi and d) DATA^{5m}.Glu(Pyr).FAPi.

The rationale behind this work was to provide novel FAPi-based monomers that could function as diagnostic counterparts to ^{177}Lu -homodimer RLT. Therefore, branched monomers with central linker unit similar to DOTAGA.Glu.(FAPi)₂ were designed, prepared and studied in project 3. The same glutamate (Glu) central linker unit was used but instead of a second FAPi TV, a pyrrolidine group was used as a “inert” group. The chelators DO3A and DOTAGA were used in reminiscence of the dimers of project 1 and 2. In addition, the hybrid chelator DATA^{5m} was used as it enables kit-type labeling at room temperature. The exact structures of DOTAGA.Glu(Pyr).FAPi, DO3A.Glu(Pyr).FAPi and DATA^{5m}.Glu(Pyr).FAPi are shown in Figure 14b – d.

Besides PET imaging, there is also a big demand for pan-tumor $^{99\text{m}}\text{Tc}$ -radiotracers applied in SPECT imaging. $^{99\text{m}}\text{Tc}$ -SPECT is the workhorse of nuclear medicine and the capacities enable very broad application especially with FAPi-based SPECT radiotracers that can be used in many different cancer types. Therefore, project 4 focused on novel $^{99\text{m}}\text{Tc}$ -FAPi radiotracers for application in oncological SPECT imaging. The final structures were designed to be similar to the successful ^{68}Ga -precursors DOTA.SA.FAPi and DATA^{5m}.SA.FAPi as illustrated by the general structure in Figure 15a. The macrocyclic/hybrid chelator was substituted with the suitable chelators N4 and MAG3 for $^{99\text{m}}\text{Tc}$ -radiolabeling. The precursor structures N4.S.FAPi and S-Bz-MAG3.SA.FAPi are presented in Figure 15b and c.

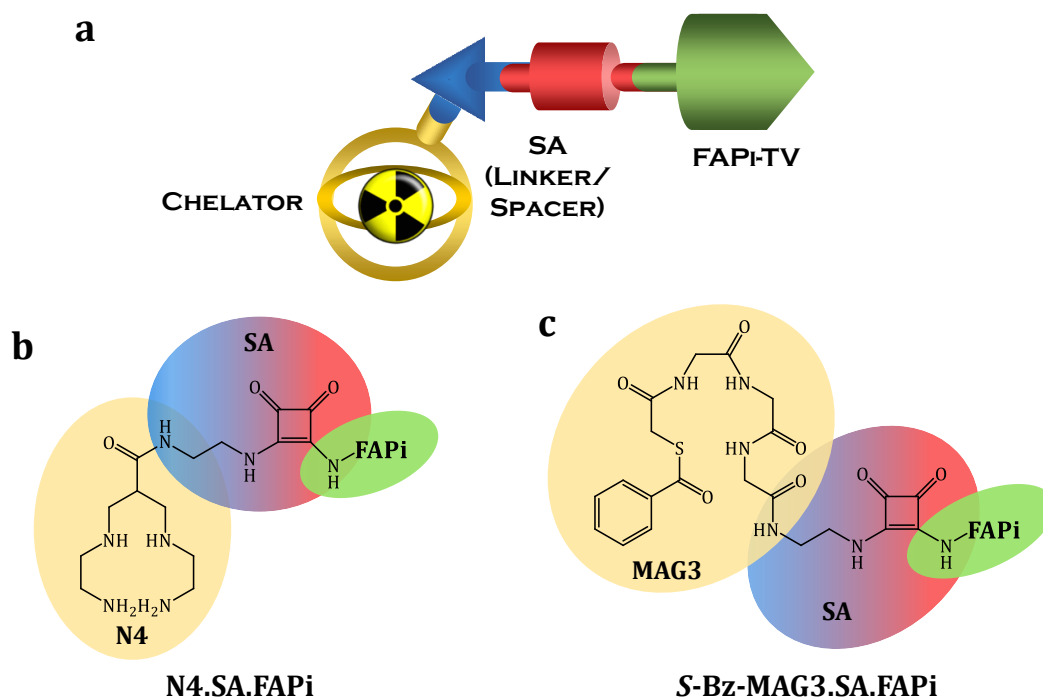


Figure 15: a) General structure of the $^{99\text{m}}\text{Tc}$ -monomers b) N4.SA.FAPi and c) S-Bz-MAG3.SA.FAPi.

Both projects comprise of organic synthesis, radiolabeling investigations and *in vitro* affinity and selectivity results. Furthermore, project 3 includes first proof-of-concept patient studies.

4.3.1. Organic synthesis

The intermediate Glu(Pyr).FAPi was synthesized in four steps with a yield of 86% and the chelators were coupled and then deprotected in the subsequent last two steps. The 6-step overall yield of the precursors DO3A.Glu(Pyr).FAPi, DOTAGA.Glu(Pyr).FAPi and DATA^{5m}.Glu(Pyr).FAPi was 31–33%. The ^{nat}Ga-complexes were synthesized with 63–67% yield.

S-Bz-MAG3.SA.FAPi and N4.SA.FAPi were prepared in a total of 9 and 6 steps, respectively. The first three steps, the synthesis of en.SA.FAPi (SA-linker/spacer + TV), was the same for both compounds. The synthesis of the N4 chelator was optimized (70% vs. 2% when synthesized according to ABIRAJ et al. [111]) and a totally different synthesis route was created that led to the successful synthesis of *S*-Bz-MAG3.SA.FAPi which was not successful in previous attempts in our group (data unpublished).

4.3.2. Radiolabeling

Reaction kinetics of ⁶⁸Ga-radiolabeling were investigated in three buffer systems for all three compounds. RCC > 97% was achieved in 1 M HEPES (pH = 5.5), 1 M AmOAc and NaOAc (pH = 4.5, respectively) after 5 – 10 min (10 nmol precursor, 50 MBq ⁶⁸Ga). Kinetics were slightly faster in HEPES. RCC seemed to be slightly higher in AmOAc than NaOAc. Therefore, the precursor amount was optimized in HEPES and AmOAc buffer. 5 nmol was still enough to reach RCC > 95% with 50 MBq ⁶⁸Ga. Slight formation of colloidal gallium-68 was observed for [⁶⁸Ga]Ga-DATA^{5m}.Glu(Pyr).FAPi with the AmOAc/MeOH radio-TLC system. RCP, determined via radio-HPLC, was already above 96% before purification and over 98% after purification via C18 cartridge for all compounds.

The results were similar to the clinically used [⁶⁸Ga]Ga-DOTA.SA.FAPi where the reaction kinetics were observed in 1 M AmOAc (pH = 5.5).

[^{99m}Tc]Tc-MAG3.SA.FAPi can be prepared at room temperature and 90 °C where 100 nmol and 20 nmol were needed to label 1 GBq ^{99m}Tc efficiently. High RCC of ca. 93 – 94% were achieved and RCP was also high (> 90%) at RT and when the reaction was not heated too long at 90 °C. Side-product formation seem to increasingly appear at longer heating times. N_xS_y chelators like MAG3 form two stereoisomers (*syn/anti*-isomer) and it is possible that one isomer is the kinetic and other isomer the thermodynamic product [112,113]. The additional peak might be corresponding to the slow formation of the thermodynamic product which would fit the observation that the additional peak was more prominent at elevated temperatures.

The radiolabeling results are summarized in Table 6.

Table 6: Radiolabeling results of the FAPi monomers investigated in this work compared to the reference radiotracers [⁶⁸Ga]Ga-DOTA.SA.FAPi, [⁶⁸Ga]Ga- and [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂.

Complex	RCC/RCY [%] (reaction time [min] and temperature)	RCP *2 [%]	logD _{7.4}	Complex Stability	
				[%] *3	
				HS	PBS
[⁶⁸ Ga]Ga- DO3A.Glu(Pyr).FAPi	> 97% *1 (5 – 10 min, 95 °C)	99.1 (98.7)	-2.40 ± 0.09	95.8 ± 0.2	97.4 ± 0.7
[⁶⁸ Ga]Ga- DOTAGA.Glu(Pyr).FAPi	> 98% *1 (5 – 10 min, 95 °C)	98.8 (96.5)	-2.45 ± 0.07	94.2 ± 0.7	95.7 ± 1.0
[⁶⁸ Ga]Ga- DATA ^{5m} .Glu(Pyr).FAPi	> 98% *1/*4 (5 – 10 min, RT)	99.2 (98.1)	-2.29 ± 0.07	85.0 ± 2.0	90.1 ± 0.7
[^{99m} Tc]Tc-MAG3.SA.FAPi	> 94 % (15 min, RT)	95.7 (RT)	—	75 – 80	70 – 80
	> 93% (5 min, 90 °C)	85 – 92 (90 °C)			(65 – 75) *5
[⁶⁸ Ga]Ga- DOTA.SA.FAPi [3]	> 97% (15 min, 95 °C)	—	-2.68 ± 0.06	> 98%	> 98% *6
[⁶⁸ Ga]Ga- DOTAGA.Glu.(FAPi) ₂	95.7 ± 0.1 (5 min, 95 °C)	99.1 (95.2)	-2.48 ± 0.05	96.5 ± 0.6	95.9 ± 0.1
[¹⁷⁷ Lu]Lu- DOTAGA.Glu.(FAPi) ₂	99.8 ± 0.1 (10 min, 95 °C)	99.8	-2.77 ± 0.10	99.4 ± 0.1	99.1 ± 0.1

*1 The RCC values include radiolabeling in 1 M HEPES (pH = 5.5) and in 1 M AmOAc (pH = 4.5).

*2 RCP of ⁶⁸Ga-labeled products (via radio-HPLC) before C18 cartridge purification is given in brackets.

*3 After incubation at 37 °C for 120 min (⁶⁸Ga) or 24 hours (^{99m}Tc).

*4 DATA^{5m}.Glu(Pyr).FAPi: AmOAc/MeOH radio-TLC gave slightly lower RCC = 90 – 95%.

*5 Stability of [^{99m}Tc]Tc-MAG3.SA.FAPi in pure formulation after 24 hours at RT in brackets.

*6 Stability of [⁶⁸Ga]Ga-DOTA.SA.FAPi was measured in saline rather than PBS.

4.3.3. Complex Stability

[⁶⁸Ga]Ga-DO3A.Glu(Pyr).FAPi and [⁶⁸Ga]Ga-DOTAGA.Glu(Pyr).FAPi showed to be almost completely stable with ca. 95% intact complex in HS and PBS after 120 min at 37 °C, respectively. The approximate radiotracer concentration was ca. 20 MBq/mL. The reference ⁶⁸Ga-FAPi radiotracers [⁶⁸Ga]Ga-DOTA.SA.FAPi, [⁶⁸Ga]Ga- and [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂ were also stable under these conditions (cf. Table 6).

[⁶⁸Ga]Ga-DATA^{5m}.Glu(Pyr).FAPi stability was high and around 85% in HS and 90% in PBS.

The complex [^{99m}Tc]Tc-MAG3.SA.FAPi was ca. 90% stable in saline and pure formulation after four hours at 37 °C and RT, respectively. Interestingly, no decomposition was observed in HS. After 24 hours which corresponds to four half-lives of ^{99m}Tc , still ca. 80% intact complex were still present in HS and saline after 24 hours. Around 75% intact complex were still measured via double radio-TLC system and radio-HPLC in the pure final formulation after one day.

4.3.4. Lipophilicity

All ^{68}Ga -radiotracers were hydrophilic and exhibited $\log D_{7.4}$ values between -2.3 and -2.5 (cf. Table 6). [^{68}Ga]Ga-DATA ^{5m} .Glu(Pyr).FAPi was the least hydrophilic ($\log D_{7.4} = -2.29 \pm 0.07$) of the new compounds. The branched monomer [^{68}Ga]Ga-DOTAGA.Glu(Pyr).FAPi showed comparable lipophilicity compared to its dimeric counterpart [^{68}Ga]Ga-DOTAGA.Glu.(FAPi) $_2$ within the margin of error ($\log D_{7.4}(\text{monomer}) = -2.45 \pm 0.07$ vs. $\log D_{7.4}(\text{dimer}) = -2.48 \pm 0.05$). Since [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ was more hydrophilic than the ^{68}Ga -labeled dimer, the same is true for the theranostic pair [^{68}Ga]Ga-DOTAGA.Glu(Pyr).FAPi and [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$.

4.3.5. FAP Affinity

The monomeric FAPi inhibitors that were synthesized were also investigated for their binding to FAP and the related proteases.

Subnanomolar $IC_{50}(\text{FAP})$ values were determined for all compounds. Table 7 gives an overview of the novel central linker-FAPi monomers (precursors and ^{nat}Ga -complexes) and ^{99m}Tc -precursors of project 3 and 4. In addition, reference compounds like the initial inhibitor UAMC-1110 and the FAPi monomers DOTA.SA.FAPi and DATA ^{5m} .SA.FAPi from earlier works of our group are listed, too.

The new “trislinker” monomers are all slightly more potent than the SA-based FAPi monomers with IC_{50} values in the range of 0.22 – 0.44 nM compared to 0.7 – 1.4 nM. Furthermore, they are as potent or even slightly more potent than UAMC-1110. Hence, the introduction of the Pyr group and the central linker Glu seems to have a positive effect.

In agreement with the observations of chapter 4.2.5., the ^{nat}Ga -complexes all have potency similar to its respective precursor. The IC_{50} values are either identical when taking the standard deviation into account (DATA ^{5m} compounds) or are only slightly higher (DOTA/DO3A and DOTAGA compounds).

Table 7: IC_{50} (FAP) value of all new monomeric FAP inhibitors of this work as well as other FAP inhibitors reference compounds from literature.

Compounds of this work	IC_{50} / nM FAP	Reference compounds	IC_{50} / nM FAP
DO3A.Glu.(Pyr).FAPi	0.22 ± 0.03	UAMC-1110 [31,70]	0.43 ± 0.02
[^{nat} Ga]Ga-DO3A.Glu.(Pyr).FAPi	0.34 ± 0.05	DOTA.SA.FAPi [3]	0.9 ± 0.1
DOTAGA.Glu.(Pyr).FAPi	0.35 ± 0.02	[^{nat} Ga]Ga-DOTA.SA.FAPi [3]	1.4 ± 0.2
[^{nat} Ga]Ga- DOTAGA.Glu.(Pyr).FAPi	0.44 ± 0.03	DATA ^{5m} .SA.FAPi [3]	0.8 ± 0.2
DATA ^{5m} .Glu.(Pyr).FAPi	0.42 ± 0.06	[^{nat} Ga]Ga-DATA ^{5m} .SA.FAPi [3]	0.7 ± 0.1
[^{nat} Ga]Ga- DATA ^{5m} .Glu.(Pyr).FAPi	0.41 ± 0.11		
N4.SA.FAPi (7)	0.67 ± 0.06		
S-Bz-MAG3.SA.FAPi (13)	0.46 ± 0.05		

The new SA-based precursors for application in ^{99m}Tc-SPECT are slightly more potent than its DOTA- and DATA^{5m}-counterparts for application ⁶⁸Ga-PET. The IC_{50} values (0.46 and 0.67 nM) are between the previously published SA-precursors and the new “trislinker” monomers.

4.3.6. FAP Selectivity

In addition to the high affinity, the new compounds are further characterized by extremely high DPP4 and PREP selectivity.

All compounds except S-Bz-MAG3.SA.FAPi had DPP4/FAP selectivity $> 10^4$. The selectivity is (^{nat}Ga)-DO3A.Glu(Pyr).FAPi and DOTAGA.Glu(Pyr).FAPi is even $> 2 \cdot 10^4$ and in the same region as UAMC-1110.

Similarly, the PREP/FAP selectivity was $> 10^4$ for DO3A.Glu(Pyr).FAPi and DOTAGA.Glu(Pyr).FAPi as well as its ^{nat}Ga-complexes. The DATA^{5m} and ^{99m}Tc-chelator-based compounds exhibited slightly lower PREP selectivity, yet still around 5000 which is similar to the first-generation SA-based compounds (except DATA^{5m}.SA.FAPi). The selectivity of all new compounds is higher than UAMC-1110. The difference is especially significant for DO3A.Glu(Pyr).FAPi and DOTAGA.Glu(Pyr).FAPi precursors and ^{nat}Ga-complexes, respectively.

The DPP8 and DPP9 selectivity varied a lot more and even showed some differences between precursor and metal complex in some cases (DO3A and DOTAGA derivatives). *S*-Bz-MAG3.SA.FAPi showed low DPP8 and DPP9 selectivity with *SI* = 109 and 35, respectively.

All the *SI* values are compiled in Table 8.

Table 8: Selectivity index (SI) as the ratio of IC_{50} value of the related proteases PREP, DPP4, DPP8 and DPP9 to IC_{50} (FAP) including all monomeric FAP inhibitors of this work as well as other FAP inhibitors reference compounds from literature.

Compound	<i>SI</i>			
	<i>PREP/FAP</i>	<i>DPP4/FAP</i>	<i>DPP8/FAP</i>	<i>DPP9/FAP</i>
UAMC-1110 [31,70]	4186	23256	23256	10930
DOTA.SA.FAPi [3]	6000	—	—	—
[^{nat} Ga]Ga-DOTA.SA.FAPi [3]	6214	> 714	—	—
DATA ^{5m} .SA.FAPi [3]	2113	—	—	—
[^{nat} Ga]Ga-DATA ^{5m} .SA.FAPi [3]	6714	> 1429	—	—
DO3A.Glu.(Pyr).FAPi	19955	> 26023	3290	5406
[^{nat} Ga]Ga-DO3A.Glu.(Pyr).FAPi	13767	> 29354	> 29354	> 29354
DOTAGA.Glu.(Pyr).FAPi	16328	> 14254	330	3648
[^{nat} Ga]Ga-DOTAGA.Glu.(Pyr).FAPi	23719	> 22962	3572	5188
DATA ^{5m} .Glu.(Pyr).FAPi	5565	> 11838	10581	4669
[^{nat} Ga]Ga-DATA ^{5m} .Glu.(Pyr).FAPi	8868	> 12227	> 12227	5590
N4.SA.FAPi (7)	5478	11104	6433	6821
<i>S</i> -Bz-MAG3.SA.FAPi (13)	5957	2348	109	35

Figure 16 visualizes the binding properties. The FAP IC_{50} values are plotted against the selectivity index of PREP (Figure 15a) and DPP4 (Figure 15b) over FAP.

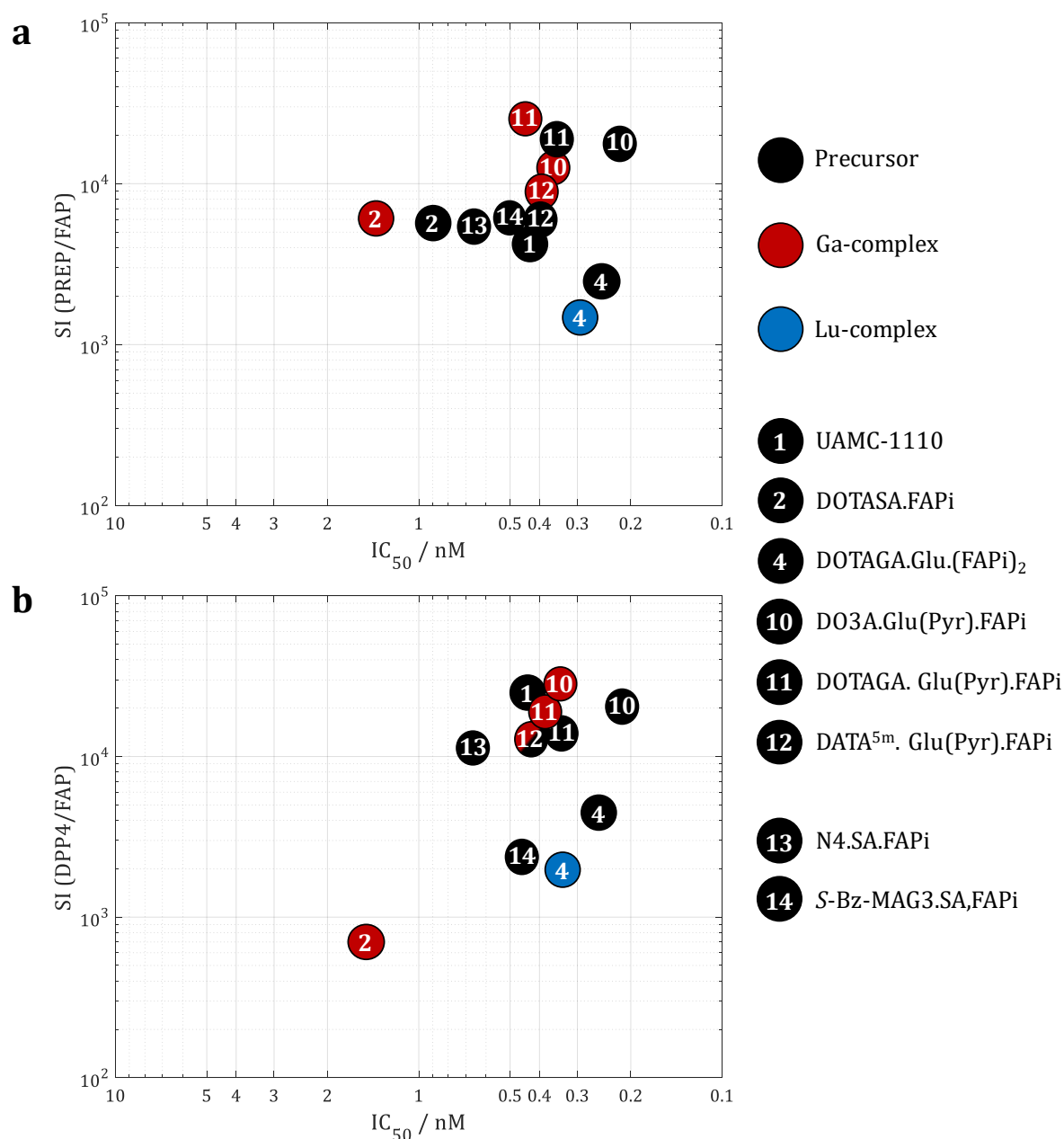


Figure 16: $IC_{50}(FAP)$ values of UAMC-1110 and all the FAPi monomers of this work plotted against a) the PREP/FAP selectivity index (SI) and b) the DPP4/FAP SI.

Overall, the novel-generation Ga-monomers showed improved affinity and selectivity and the binding properties of the new ^{99m}Tc -precursors were similar compared to DOTASA.FAPi and DATA^{5m}.SA.FAPi.

4.3.7. FAPi Monomers (Project 3) vs. FAPi Homodimers (Project 1)

The theranostic approach of this work was realized by combination of the diagnostic monomers of project 3 and the therapeutic homodimers of project 1. Table 9 compares the values for $\log D_{7.4}$ and *in vitro* binding studies of the ^{68}Ga -labeled monomers and the ^{177}Lu -labeled homodimers for the DOTAGA (left) and DO3A (right) derivatives, respectively.

Table 9: Comparison of the monomeric DO3A- and DOTAGA.Glu(Pyr).FAPi with its respective homodimeric counterpart DO3A- and DOTAGA.Glu.(FAPi)₂. The values in brackets are the values for the ^{68}Ga -labeled derivative ($\log D_{7.4}$) or the precursor.

	^{68}Ga -DOTAGA. Glu.(Pyr).FAPi	^{177}Lu -DOTAGA. Glu.(FAPi) ₂	^{68}Ga -DO3A. Glu.(Pyr).FAPi	^{177}Lu -DO3A. Glu.(FAPi) ₂
$\log D_{7.4}$	-2.45 ± 0.07	-2.77 ± 0.10 (-2.48 ± 0.05)	-2.40 ± 0.09	-1.77 ± 0.10 (-2.08 ± 0.07)
$IC_{50}(\text{FAP})$ [nM]	0.44 ± 0.03 (0.35 ± 0.02)	0.33 ± 0.02 (0.26 ± 0.04)	0.34 ± 0.05 (0.22 ± 0.03)	– (0.60 ± 0.04)
$SI(\text{PREP}/\text{FAP})$	23719 (16328)	1292 (2269)	13767 (19955)	– (1667)
$SI(\text{DPP4}/\text{FAP})$	> 22962 (> 14254)	1967 (4577)	> 29354 (> 26023)	– (900)
$SI(\text{DPP8}/\text{FAP})$	3572 (330)	661 (112)	> 29354 (3290)	– (1717)
$SI(\text{DPP9}/\text{FAP})$	5188 (3648)	557 (319)	> 29354 (5406)	– (1583)

The $\log D_{7.4}$ values of the theranostic pairs (^{68}Ga -monomer vs. ^{177}Lu -dimer) do not fully match. The values of the DOTAGA pair differ less than those of the DO3A pair. Interestingly, the ^{68}Ga -values of the DOTAGA homodimer fully matches the ^{68}Ga -monomer values. This indicates that the structures feature very similar properties. The substitution of the radiometal alters the lipophilicity slightly although no trend can be derived from the available data (cf. Table 2).

$IC_{50}(\text{FAP})$ potency matches quite well for the DOTAGA pair. For the DO3A pair only the precursor values can be compared and have a 2.7-fold difference (0.22 vs. 0.60 nM).

Significant differences can be seen in the selectivity data. The monomers are characterized by higher selectivity overall. Each compound, no matter if monomer or dimer, follows the trend that PREP and DPP4 selectivity (monomer: ca. 20 000 vs. dimer: 1000–2000) is higher compared o DPP8 and DPP9 selectivity (monomer: 3500–5500 vs. dimer: 500–700). The ratio of $SI(^{\text{nat}}\text{Ga}-$

monomer) to $SI(^{nat}\text{Lu-dimer})$ is somewhat similar for the proteases. For example, the SI ratio for DPP4 and DPP9 is around 10-fold (22 962 to 1967 and 5188 to 557). The SI ratio for PREP is a little higher (ca. 18-fold) and a little bit lower for DPP8 (ca. 6-fold). This at least shows a similar trend when comparing the binding properties to the different proteases.

Although the new FAPi monomers although the *in vitro* values do not fully match its homodimeric counterpart, they exhibit very high FAP potency and extremely high selectivity, significantly higher than the SA-based monomers used in ^{68}Ga -PET imaging until now. Hence, the novel FAPi monomers with DO3A and DOTAGA chelators are very promising candidates for improved ^{68}Ga -PET imaging and were still considered for human application.

4.3.8. ^{68}Ga -PET Patient Studies

The two compounds $[^{68}\text{Ga}]\text{Ga-DO3A.Glu(Pyr).FAPi}$ and $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu(Pyr).FAPi}$ were each investigated in a first proof-of-concept human PET study.

A 70-year women with breast cancer (invasive ductal carcinoma) underwent $[^{68}\text{Ga}]\text{Ga-DO3A.Glu(Pyr).FAPi}$ PET/CT imaging and Figure 17a shows the whole-body PET image one hour post-injection.

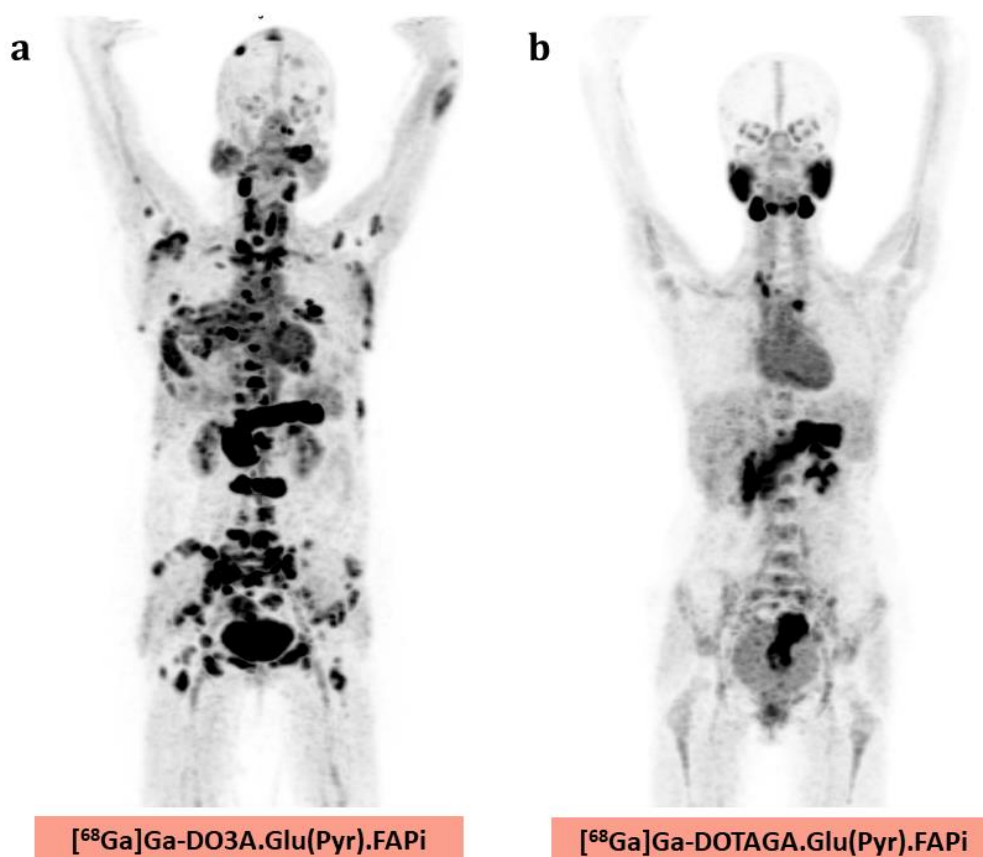


Figure 17: Whole-body PET scans (1 h p.i.) of a) invasive ductal carcinoma (breast cancer) patient using the radiotracer $[^{68}\text{Ga}]\text{Ga-DO3A.Glu(Pyr).FAPi}$ and b) a medullary thyroid cancer patient using $[^{68}\text{Ga}]\text{Ga-DOTAGA.Glu(Pyr).FAPi}$. Both radiotracers accumulate in the tumor lesions intensively.

The radiotracer showed intense accumulation in the many metastases (bone and mediastinal lymph nodes). It was cleared from the blood quickly, but showed some liver uptake. This might indicate a possible hepatobiliary excretion pathway. Besides this, physiological uptake in the pancreas is observed in accordance with previous observations with [^{68}Ga]Ga-DOTA.SA.FAPi [7,10,11].

[^{68}Ga]Ga-DOTAGA.Glu(Pyr).FAPi was investigated in a 19-year-old medullary thyroid cancer patient. The whole-body PET image in Figure 17b also shows high tumor uptake in the tumor lesions including liver, bone and lymph node metastases. The liver uptake is lower than for [^{68}Ga]Ga-DO3A.Glu(Pyr).FAPi whereas the blood pool activity is still higher at the one hour time point indicating a possibly slower (renal) clearance. Physiological pancreas uptake is comparable to [^{68}Ga]Ga-DOTA.SA.FAPi and [^{68}Ga]Ga-DO3A.Glu(Pyr).FAPi.

Overall, these results show that both ^{68}Ga -labeled radiotracer seem to work well in oncological PET/CT where many metastases could be detected in different organs because of high tumor uptake and good TBR. Furthermore, these first patient studies indicate the theranostic potential of the new ^{68}Ga -labeled radiotracers as a diagnostic counterpart to the therapeutic [^{177}Lu]Lu-DOTAGA.Glu.(FAPi)₂.

4.3.9. Outlook

Proof-of-concept PET/CT investigations with [^{68}Ga]Ga-DO3A.Glu(Pyr).FAPi and [^{68}Ga]Ga-DOTAGA.Glu(Pyr).FAPi in patients showed promising first results with high tumor uptake. Systematic biodistribution and dosimetry studies in direct comparison to [^{68}Ga]Ga-DOTA.SA.FAPi are required. [^{68}Ga]Ga-DOTAGA.Glu.(FAPi)₂ might also be involved in this study since the main aim is to obtain improved pre- and post-therapy PET scans to accompany RLT with [^{177}Lu]Lu-DOTAGA.Glu.(FAPi)₂. If one of the new monomeric FAPi radiotracers shows more similar biodistribution in comparison to DOTAGA.Glu.(FAPi)₂ than the comparison of [^{68}Ga]Ga-DOTA.SA.FAPi to DOTAGA.Glu.(FAPi)₂, this will lead to more precise pre- and post-therapy PET imaging and prediction on dosimetry of ^{177}Lu -FAPi dimer RLT.

Additionally, further studies will give a more complete picture on the slower clearance from blood (DOTAGA) and the liver uptake (DO3A) observed in first patients and if this is a regular feature of these radiotracers.

A comparison with [^{18}F]F-FDG for performance in tumor lesion detection rate will be interesting as well.

The precursors DO3A.Glu(Pyr).FAPi and DOTAGA.Glu(Pyr).FAPi can also form stable complexes with gadolinium. These might yield interesting new MRI contrast agents for improved MRI imaging of the tumor stroma/TME.

In case, new optimized FAPi homodimers should result out of project 2, the same approach can be applied as it was realized with DOTAGA.Glu.(FAPi)₂ vs. DOTAGA.Glu(Pyr).FAPi in project 1 and 3. The structure of the corresponding FAPi monomer can be modified analogously to provide a diagnostic counterpart for treatment assessment via ⁶⁸Ga-PET/CT.

[^{99m}Tc]Tc-MAG3.SA.FAPi was labeled successfully with high RCC. Since its *in vitro* properties are promising, it should be applied in SPECT imaging *in vivo*. This can be performed in FAP tumor-bearing mice that express FAP, ideally a tumor model with simultaneous presence of tumor cells and stromal cells (FAP-expressing CAFs). Alternatively, a sarcoma tumor model might also be interesting. Assuming that the ^{99m}Tc-FAPi radiotracer selectively accumulates in the tumor, it might further be translated into first clinical studies.

Besides small animal SPECT studies, first patient studies are planned in the near future, too. Here, biodistribution and pharmacological (PK/PD) characteristics will be investigated, ideally in various cancer types to demonstrate that the ^{99m}Tc-radiotracers are pan-tumor SPECT imaging agents.

N4.SA.FAPi was successfully synthesized and demonstrated a potent and selective FAP inhibitor. In the following, the radiolabeling has to be investigated and optimized to allow subsequent *in vivo* translation.

5. Conclusion

The tumor microenvironment (TME) is an essential part of epithelial tumors and contains a multitude of components. Among them, cancer-associated fibroblasts (CAFs) have been discovered as an oncological target. They are addressed via fibroblast activation protein alpha (FAP), a membrane-bound protease strongly expressed on CAFs in pretty much all epithelial cancers as well as sarcomas [103,104,114–116]. First radiotracers based on a highly potent and selective FAP inhibitor (FAPi) UAMC-1110 emerged in the last few years and have already demonstrated to be useful for molecular imaging with positron emission tomography (PET) [1–19]. The translation into effective therapeutic radiopharmaceuticals has enormous potential as FAP is a pan-tumor target with broad application range. However, effective radioligand therapy (RLT) targeting FAP remains challenging.

The aim of this work was to synthesize and investigate new FAPi-based radiopharmaceuticals for improved theranostic application with special focus on optimizing RLT with FAPi homodimers. The radiotracers are designed in a way that two FAPi targeting vectors and a bifunctional chelator for radiolabeling with a trivalent radiometal are coupled via a central linker unit (branched structure). The key homodimeric FAPi-based theranostic compounds were DO3A- and DOTAGA.Glu.(FAPi)₂, DO3A- and DOTAGA.NPyr.(FAPi)₂, DO3A- and DOTAGA.Glu₂.(FAPi)₂ and DOTAGA.PEG₂.Glu.(FAPi)₂.

Radiolabeling studies with ⁶⁸Ga, ¹¹⁷Lu, ²²⁵Ac, and ⁹⁰Y and *in vitro* binding affinity studies with FAP, PREP, DPP4, DPP8 and DPP9 of the compounds DOTAGA.Glu.(FAPi)₂ and DO3A.Glu.(FAPi)₂ (project 1) demonstrated high labeling yields, complex stability in different media, hydrophilicity, FAP affinity and selectivity over the related proteases. The improved properties compared to DOTAGA.(SA.FAPi)₂, a linear homodimer that showed promising therapeutic potential in previous works due to its prolonged tumor retention [31,32,100], led to the first human application of [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂.

A proof-of-concept study in a thyroid cancer patient demonstrated improved pharmacological properties while maintaining the long tumor retention of FAPi homodimers. These favorable properties *in vivo* include lower accumulation in liver and colon, most probably because of its increased hydrophilicity, and led to minimized radiation doses to these healthy organs that were critical organs in RLT with [¹⁷⁷Lu]Lu-DOTAGA.(SA.FAPi)₂. This increases tumor-to-organ ratio regarding absorbed dose [68].

Further systematic applications in patients who initially have been treated with ¹⁷⁷Lu-FAPi dimer RLT was continued with the new radiopharmaceutical [¹⁷⁷Lu]Lu-DOTAGA.Glu.(FAPi)₂. For

example, a glioblastoma patient showed partial response after two cycles of [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ FAP-RLT [102].

Systematic studies on clinical response of ^{177}Lu -FAPi dimer RLT in 39 thyroid cancer and 19 breast cancer patients demonstrated high response rates in both cases.

51.2% (20/39) biochemical response in thyroid cancer with few occurrences of grade II (10%) and III (13%) adverse events, respectively. Median overall survival and progression-free survival was not reached yet with a median follow-up of 33 and 25 months, respectively.

16/19 patients with breast cancer (84%) reported pain relief and at least 4 out of 9 patients that were still alive at 14-month follow-up showed partial remission in PET/CT scans.

Patients with other cancer types such as lung cancer have been treated with [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ and first applications of [^{225}Ac]Ac-DOTAGA.Glu.(FAPi) $_2$ have been performed already (data unpublished).

In total, more than 70 patients have already been treated with multiple cycles of with ^{177}Lu -FAPi dimer RLT. According to the clinical results of [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ in various cancer types, there is clear evidence that this “next-generation” FAPi homodimer is the most potent and efficacious therapeutic agent among the FAPi-based radiopharmaceuticals reported to date.

Five more FAPi homodimers with identical targeting vectors and chelators but different linker and spacer units were synthesized and investigated *in vitro* as well as in radiolabeling studies (^{68}Ga , ^{177}Lu). All compounds exhibited similar properties as DOTAGA.Glu.(FAPi) $_2$ and were superior to DOTAGA.(SA.FAPi) $_2$. DO3A.NPyr.(FAPi) $_2$ showed improved DPP4 and PREP selectivity and all the DOTAGA compounds DOTAGA.NPyr.(FAPi) $_2$, DOTAGA.PEG $_2$.Glu.(FAPi) $_2$ and DOTAGA.Glu $_2$.(FAPi) $_2$ are also interesting compounds for future therapeutic *in vivo* investigations.

New monomeric FAPi radiotracers were synthesized mainly for diagnostic purposes and PET imaging with ^{68}Ga . The aim was to develop an improved diagnostic counterpart to the therapeutic [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ for PET/CT scans pre- and post-FAPi dimer RLT. The radiotracers are reminiscent of the FAPi homodimers as one FAPi targeting vector is substituted by an inert pyrrolidine group.

Kit-type labeling at room temperature is possible with the hybrid chelator DATA ^{5m} that was used in addition to DO3A and DOTAGA. ^{68}Ga -radiolabeling showed excellent results in several buffer systems. Especially the ^{68}Ga -labeled DO3A- and DOTAGA-radiotracers showed high radiochemical conversion rates and purity, complex stability and hydrophilicity similar to [^{68}Ga]Ga-DOTAGA.Glu.(FAPi) $_2$. High binding affinity in combination with very high PREP and DPP4 selectivity superior to [^{68}Ga]Ga-DOTA.SA.FAPi was observed. First of proof-of-concept imaging was performed in one patient each. First impression seems to indicate similar performance than the routinely used [^{68}Ga]Ga-DOTA.SA.FAPi. Further studies are pending.

Besides the compounds that were synthesized for the application with trivalent radiometals, two precursors N4.SA.FAPi and S-Bz-MAG3.SA.FAPi were successfully synthesized in a 7 and 9 step organic synthesis. S-Bz-MAG3.SA.FAPi was subsequently radiolabeled with ^{99m}Tc at 90 °C and at room temperature for potential kit-type radiolabeling to give high radiochemical conversion after short reaction times (5 – 15 min). Complex stability was high for four hours (ca. 90%) and still 65 – 80% after 24 hours in different media. [^{99m}Tc]Tc-MAG3.SA.FAPi is an interesting candidate for *in vivo* ^{99m}Tc -SPECT studies and first applications in small animal studies and patients are in preparation.

Research on cancer therapy is constantly evolving to find more potent cytostatic agents to improve treatment response and more selective agents to minimize side effects. Smart Drug Delivery Systems (SDDS), also called Targeted Drug Delivery, are promising strategies to achieve superior accumulation in the region of interest compared to systemic chemotherapy. Hopefully, this will increasingly find its way into the clinics as the reduced side effects will improve quality of life for the patients besides prolonged survival.

The TME has been discovered as a key player in tumor growth and consequently as an oncological target in cancer treatment. Different markers like CAFs expressing FAP that can be addressed via FAP inhibitors vascular cells (endothelial cells) or macrophages expressing $\alpha_v\beta_3$ integrins addressed by RGD-containing peptides have been identified as interesting target for radiopharmaceuticals [1–3,29,75–77,117].

Modern radiopharmaceuticals work similar to SDDS in the sense that they contain a targeting vector to ensure a selective accumulation of the radiopharmaceutical in the tumor lesion. This will result in a high radiation dose to the tumors and low doses to healthy organs.

The field of radioligand therapy made a big step with the approval of the ^{177}Lu -labeled molecule radiopharmaceuticals Luthathera® in 2017/2018 for use in SSTR-positive neuroendocrine tumors and again with Pluvicto® in 2022 for PSMA-positive metastatic castration-resistant prostate cancer. This development encouraged to go in a similar therapeutic and theranostic direction by addressing other targets. Pan-tumor targets are particularly interesting as they have a broad application potential. This includes oncological targets like integrins ($\alpha_v\beta_3$ etc.), CXCR4 and FAP among others [75–77,117,118].

Monomeric FAP inhibitors are very suitable imaging agents already [1–3,5,10–12]. Investigations on FAP-targeting radioligand therapy on the other hand, are still in an early stage. Substantial improvements with regard to tumor retention and tumor-absorbed dose were made by introducing a second FAPi targeting vector (homodimers) [31,32,100]. Other strategies like

albumin binders, cyclic peptides or heterodimers (e.g. FAPi-RGD) are explored in parallel [44–46,52–54,62,64,66]. Investigating the effect of multimerization has started although first preliminary results did not show improved tumor retention or anti-tumor effect but only increased background [36]. The FAPi homodimer approach, including the results of this work, is the clinically most studied and has shown very promising results so far [31,32,100]. The quite different cyclic peptide approach might also be suited. As no direct comparative data are available for these different approaches yet, it is hard to predict what will turn out to be the most effective treatment option.

Besides that, it is still not entirely clear how effective RLT by targeting the TME actually is compared to addressing the tumor cells directly. Among other studies, this work and the currently ongoing systematic clinical studies using [^{177}Lu]Lu-DOTAGA.Glu.(FAPi) $_2$ will help to provide a clearer picture of treatment efficacy.

Current research is already heading towards addressing tumor lesions in its complex entirety rather than focusing solely on one target. Hence, just a finding a suitable TME/CAF-addressing cytostatic agent will probably not be the end of story. The other way around, radiopharmaceuticals targeting the TME will probably also benefit from a combination with another cytostatic agent to potentially achieve synergistic effects in order to optimize treatment efficacy and effectiveness. First studies have been performed by combining FAP-RLT with PARP inhibitors or PD-1 inhibitor for example [109,110]. But when taking a look at the countless possibilities, the research appears to be in a pretty early phase with a lot of ground still to cover in this area.

Assuming that the problems of ^{225}Ac -availability will be overcome in the next years, [^{225}Ac]Ac-DOTAGA.Glu.(FAPi) $_2$ or other ^{225}Ac -labeled FAP-targeting radiopharmaceuticals in general might be a game changer in the future. Actinium-225 in particular can deposit very high energy (up to 28 MeV per atom) in a small volume due to its complex decay chain. Hence, it is probably very efficient in destroying CAFs and the TME. In a combined strategy, FAP-targeting alpha-particle therapy (FAP-TAT) could severely damage the TME and break up the tumor stroma barrier for a second tumor cell-targeting agent that can subsequently reach the tumor cells more easily.

An exciting dual approach for the future might be the combination of FAP-TAT followed by Universal chimeric antigen receptor (Uni CAR) T cell therapy. Uni CAR T cells are modified T cells that are part of the immune system. CAR T cells are programmed to kill (tumor) cells. Uni CAR T cells are equipped with an additional “on/off-switch” mechanism because CAR T cells were found to be highly toxic [119]. For example, a peptide like E5B9 can bind to an epitope that triggers the activation of the Uni CAR T cell. If E5B9 is coupled with a tumor/TME-selective targeting vector Uni CAR T cells are activated only in (or in close proximity) to the tumor lesion [119,120].

Alternatively, a tumor-targeting SDDS with a cytostatic payload like doxorubicin or Vedotin (Val-Cit-MMAE), with Val-Cit linker being cleavable via Cathepsin B, is definitely an interesting option, too.

The TANDEM approach with a CAF-addressing FAPi-based precursor like DOTAGA.Glu.(FAPi)₂ that is both labeled with an α emitter (²²⁵Ac) and a β^- emitter (¹⁷⁷Lu, ⁹⁰Y) also seems worth trying. This will exploit the TME-damaging potential an α emitter together with the crossfire effect of a β^- emitter to also damage the DNA of tumor cells. If a PARP inhibitor would be additionally given as a third component, this would hamper the DNA-repairing enzymes so that the tumor cannot recover from the radiation damage in the TME and the tumor.

In a totally different approach, FAP can also be utilized as the oncological target of an SDDS. Several FAP substrates with a cytostatic payload have been developed over the last years [37,121–124]. However, most of the groups used unspecific targeting vectors. A Gly-Pro containing peptide sequence was used most of the times. The peptide is supposed to be cleaved at the post-proline amide bond to release the cytostatic. But none of these compounds were tested for selectivity over DPP4 and PREP in particular. Nevertheless, compound AVA6000 entered early phase I studies [123,125].

With a selective targeting vector this approach is even more promising. This was rationale behind utilizing the first synthetically produced highly PREP-selective FAP substrates with efficient cleavage rates evaluated by the group of VAN DER VEKEN [126]. They were originally designed as fluorogenic probes but were altered within project 5 to be coupled to a cytostatic drug with amino group to form the post-proline amide bond. Because of the micromolar affinity of the FAP substrates, the aim was to further couple it with the high-affinity FAP inhibitor UAMC-1110 to enhance accumulation in the TME. This project is described in the appendix (chapter 6.1.).

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6. Appendix

6.1. FAP-Targeted Drug Delivery with Different Cytostatic Prodrugs Based on a Selective Small-Molecule FAP Substrate Targeting Vector

Summary

Besides developing (radio)pharmaceuticals based on small molecule Fibroblast activation protein alpha (FAP) inhibitors, targeted drug delivery addressing cancer-associated fibroblasts (CAFs) has also been identified as a potentially promising way for cancer treatment. FAP is a pan-tumor target and expressed in the tumor microenvironment (TME) of most epithelial cancers [9]. CAF-targeted drug delivery tries to selectively accumulate the cytostatic payload in the tumor (microenvironment). Several small molecule cytostatic prodrugs have been published that are supposed to be cleaved by FAP [30–34]. Indeed, some anti-tumor efficacy has been reported in several studies the drug delivery systems are not optimal [33–36]. These early attempts did not report any proof that these prodrugs are selectively cleaved by FAP and not by closely related proteases like prolyl endopeptidase (PREP) or dipeptidyl peptidase 4 (DPP4) instead. Especially PREP has similar endopeptidase activity but is expressed more ubiquitously in the human body. It must be assumed that they had not FAP/PREP selectivity when comparing it to numerous studies on small molecule FAP inhibitors that demonstrated that selectivity is only achieved if specific groups are chosen [27,28]. Recent discovery of the first synthetically prepared small molecule FAP substrates opened up the possibility to utilize the FAP substrate targeting vector “FAPs” and modify it to act as a cytostatic prodrug.

In this work, the FAP substrate targeting moiety was synthesized from scratch first in 13 steps with an overall yield of 18%. The 4-aminobutoxy side chain was coupled to the quinoline moiety to yield the 6-(4-aminobutoxy)-4-quinolinoyl moiety. In parallel, the difluorinated Gly-Pro unit was synthesized in five steps and then coupled to the alkyl-quinoline moiety. After one more saponification step, Boc-FAPs-COOH was obtained that was subsequently coupled two three different cytostatic drugs: doxorubicin (Dox), monomethyl auristatin E (MMAE) and rucaparib. Final Boc-cleavage and purification via RP-HPLC resulted in the compounds FAPs.cytostatic that were subsequently analyzed *in vitro* for the cleavability by FAP through in-house developed method that combines ultra performance liquid chromatography in multiple reaction mode (UPLC-MRM) with mass spectrometry (MS). No cleaving products were observed for FAPs.Dox and FAPs.Rucaparib. Instead, they showed FAP inhibition properties. FAPs.MMAE was cleaved to a small percentage of ca. 1.5% after 24 hours by FAP and PREP, meaning a low efficiency without selectivity.

Accordingly, the original strategy was adapted (i) to achieve a very high and selective accumulation in the TME by using a very high-affinity FAP inhibitor and (ii) to achieve efficient cleavage by FAP of post-proline amide bond of FAPs that is coupled to the cytostatic with the self-immolative linker unit PABC (*p*-aminobenzyl carbamate) unit in between FAPs and the cytostatic payload. This modified second-generation drug delivery system with PABC was chosen because the aromatic structure of PABC is structurally similar to the selective and proficient FAP

substrates from DE DECKER et al. [29] that all incorporated aromatic amines at post-proline position. Furthermore, the FAPs.PABC.cytostatic unit is supposed to be coupled to FAPi via squaric acid (SA). When the compounds FAPi.SA.FAPs.PABC.cytostatic are cleaved by FAP, the cytostatic will be released in the same way as without PABC due to the 1,6-elimination of PABC and the cleavage of the carbamate linking group between PABC and the cytostatic.

Different synthesis routes were tried. Preferably, the synthesis route was designed to have a FAPi.SA.FAPs.PABC building block that can act as a platform where different cytostatics can be coupled to it in the last reaction step. Two different synthesis routes did not work because no reaction occurred in coupling of FAPi.SA to FAPs.PAB-OH or FAPs.PABC-PNP. The third synthesis route was performed in which FAPi.SA was coupled in the last step to FAPs.PABC.cytostatic. This appears to be less optimal because FAPi.SA coupling has to be done for cytostatic separately, but FAPs.PABC.cytostatic could be identified/synthesized for all three cytostatics. Final deprotection and FAPi.SA coupling did not work for the cytostatics Dox and MMAE. FAPi.SA.FAPs.PABC.Rucaprib could be identified. Further studies on optimizing reaction conditions are required but could not be finished due to time constraints.

Author Contributions

The project was conceptualized and the study was planned by MARCEL MARTIN, Frank Roesch, Pieter Van Der Veken and Ingrid De Meester. The organic synthesis and compound characterization the was carried out by MARCEL MARTIN with the support of Maike Elisabeth Schneider and Kevin Machel in the synthesis of Boc-FAPs-COOH, the synthetic platform of the FAP substrate targeting moiety. The *in vitro* studies were performed by Yentl Van Rymenant, Emile Verhulst and Sophie Lyssens and analyzed and interpreted together with MARCEL MARTIN.

MARCEL MARTIN drafted this version of the manuscript.

FAP-Targeted Drug Delivery with Different Cytostatic Prodrugs Based on a Selective Small-Molecule FAP Substrate Targeting Vector

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Abstract

Fibroblast activation protein alpha (FAP) has emerged as a promising pan-tumor target over the last years. Several approaches are being pursued to address it with a therapeutic agent including monoclonal antibodies, small molecule FAP inhibitors, FAP-targeting radiopharmaceuticals and FAP-targeted drug delivery of cytostatic prodrugs. These prodrugs are FAP substrates and cleaved by FAP. In this way, the cytostatic payload is released into the tumor microenvironment to exert its therapeutic effect. Previously published FAP-cleavable prodrugs had the major drawback of lacking selectivity to related proteases like DPP4 and PREP in particular. The recent evaluation of the first synthetic small molecules FAP substrates with high selectivity to DPP4 and PREP while simultaneously efficient FAP-cleaving was reported.

These newly developed FAP substrates were adopted as a targeting moiety and coupled with three different cytostatic drugs doxorubicin (Dox), monomethyl auristatin E (MMAE) and rucaparib. After successful organic synthesis of the substrate targeting moiety (FAPs) and subsequent coupling to the cytostatics the cleavability by FAP was analyzed *in vitro* via an in-house developed UPLC-MRM MS assay. FAPs.Dox and FAPs.Rucaparib did not show any FAP-cleavage but surprisingly FAP inhibition properties, whereas FAPs.MMAE did show low cleaving efficiency and lacked selectivity towards PREP.

A second generation of compounds with an additional self-immolative linker *p*-aminobenzyl carbamate (PABC) should improve cleaving properties because of the aromatic amines in post-proline position. Different synthesis routes were tried but the second amidation step of the asymmetrical coupling via squaric acid (SA) was the key problematic step in all of them. The compounds with all three cytostatics could be prepared up to the penultimate synthesis step. Product formation of FAPi.SA.FAPs.PABC.Rucaparib was observed. Further studies on different reaction conditions (or the alternative synthesis routes) are needed to complete the synthesis of the FAPi.SA.FAPs.cytostatic compounds and subsequently investigate cleavability by FAP *in vitro* again.

Keywords

Fibroblast activation protein alpha (FAP); FAP-targeted drug delivery; FAP inhibitors (FAPi); FAP substrates (FAPs); cytostatic prodrugs; small molecule drug delivery system; FAP-cleavable cytostatic prodrug; tumor microenvironment (TME); cancer-associated fibroblasts (CAFs)

1. Introduction

Cancer-associated fibroblasts (CAFs) are present in the tumor microenvironment (TME) of epithelial tumors. Other important components of the TME are inflammatory, immune, vascular cells and extracellular matrix (ECM). The TME is crucial for the tumor supply and therefore plays a supporting role in tumor growth (tumor cell proliferation, tumor invasion, metastasis, angiogenesis) and also acts as a physical barrier that increases tumor resistance, e.g. of chemotherapy [1–5]. The transforming growth factor TGF- β 1 is mainly responsible for activating fibroblasts in the TME by mesenchymal-mesenchymal transition (MMT) [4–8]. The CAFs, in turn, influence the cancer cells and enhance their proliferation directly and indirectly. CAFs promote epithelial-mesenchymal transition (EMT). On top, it produces many different cytokines, tumor growth factors and enzymes like matrix metalloproteinases (MMPs). This positive feedback loop between CAFs and cancer cells induces increased cancer cell proliferation, angiogenesis, strengthening of apoptosis resistance of tumor cells during invasion into new tissues (metastasis) and ECM remodeling which ultimately leads to tumor growth [4,7,8].

In most epithelial tumors ($\geq 90\%$), CAFs strongly express fibroblast activation protein alpha (FAP) [2,9]. FAP belongs to the S9b peptidase family and is closely related to the other post-proline selective serin protease dipeptidyl peptidase 4 (DPP4). It is a type II integral membrane glycoprotein with largely extracellular domain and is enzymatically active as a homodimer [10]. The ectopeptidase has a catalytic triad consisting of Ser⁶²⁴, Asp⁷⁰² and His⁷³⁴ and also possesses endopeptidase activity like prolyl endopeptidase (PREP), the only other prolyl endopeptidase in humans, in addition to the DPP activity [10]. FAP is a more efficient endopeptidase as DPP and cleaves peptides preferably after Gly-Pro units [11]. The *in vivo* role of FAP is not fully understood but several substrates have been identified. These are neuropeptides (neuropeptide Y, peptide YY, substance P etc.), type I collagen and α_2 -antiplasmin, which is a plasmin inhibitor where the FAP cleavage leads to α_2 -antiplasmin inhibition and therefore to reduced fibrinolysis and ultimately to scar formation [3,12–15]. Type I collagen is major component of the ECM, indicating an involvement of FAP in ECM remodeling that CAFs are known for [3,10,16,17]. Beyond that, it seems to also play a role in tumor growth (immunosuppression, angiogenesis, cell proliferation and invasion) [18–24]. FAP is absent in most adult healthy tissue which makes it an interesting pan-tumor target. It is only expressed by activated fibroblasts at sites of inflammation, during wound healing and in fibrosis (liver cirrhosis, arthritis etc.) in addition to CAFs [3,13,25,26].

FAP has been already identified as an interesting target for drug delivery due to its unique expression pattern several strategies by different groups. This includes protoxin peptides derived from the toxins thapsigargin [27], melittin [28] and a combination of promelittin loaded onto reduced graphene oxide nanosheets that contained the cytostatic doxorubicin (Dox) [29]. Dox is an DNA-intercalating agent that inhibits DNA repair by topoisomerase II.

First small molecules containing a Gly-Pro sequence coupled with Dox were synthesized that are cleaved by FAP and release free Dox in the TME (cf. Figure 1a and b) [30–32].

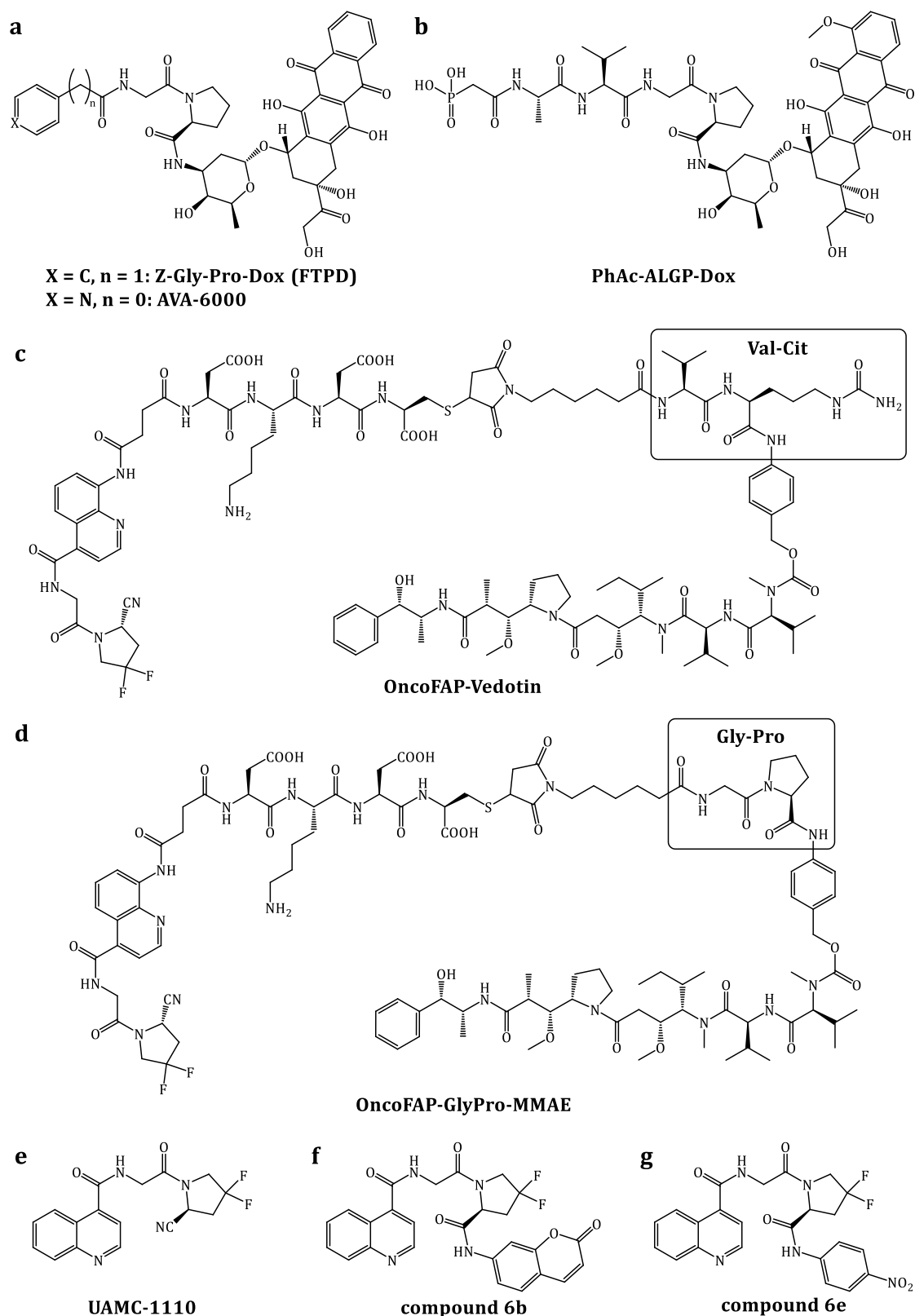


Figure 1: Structure of the a) FAP inhibitor UAMC-1110, b) and c) FAP substrates from De Decker et al. [33], FAP-prodrugs d) Z-Gly-Pro-Dox (FTPD, X = C, n = 1) and AVA-6000 (X = N, n = 0), e) PhAc-ALGP-Dox and f) OncoFAP-Vedotin with Cathepsin-cleavable Val-Cit linker and g) FAPi-prodrug OncoFAP-MMAE with FAP-cleavable Gly-Pro unit.

These works observed antitumor efficacy of FAP-targeted drug delivery and the prodrugs were well tolerated with reduced cardiotoxicity compared to free Dox. No selectivity data with respect to the related proteases (PREP, DPPs) were reported. In comparison with similar works on FAPi, it can be assumed that there was no or low selectivity at least for PREP. Nevertheless, the potential of FAP-cleavable prodrugs was demonstrated in first proof-of-concept studies.

OncoFAP-Vedotin (cf. Figure 1c) utilizes a FAP inhibitor OncoFAP, a Val-Cit linker coupled to the cytostatic monomethyl auristatin E (MMAE) via the self-immolative linker PABC (*p*-aminobenzyl carbamate). The Val-Cit linker is cleavable by Cathepsin B, an enzyme strongly expressed in many different cancer types. The antimetabolic agent MMAE is only used in conjugated prodrugs because systemic use of MMAE is very toxic due to its ability to efficiently inhibit tubulin polymerization. OncoFAP-Vedotin showed promising in human FAP⁺ SKRC52 xenograft studies in combination with the monoclonal antibody-cytokine fusion protein L19-IL2 as it was well tolerated and demonstrated high therapeutic effect [34].

Recently, the same group extended the approach where different dipeptide sequences like Gly-Pro instead of Val-Cit were introduced [35]. This was the first time a FAP inhibitor and FAP substrate moiety were combined in one molecule. OncoFAP-Glyro-MMAE (cf. Figure 1d) showed superior performance compared to OncoFAP-Vedotin [35].

Extensive structure-activity relationship (SAR) studies by JANSEN et al. [36,37] from the group of PIETER VAN DER VEKEN (University of Antwerp, Belgium) led to the discovery of UAMC-1110 (cf. Figure 1e) as a FAP inhibitor (FAPi) with high affinity and simultaneous high selectivity over PREP, DPP4, 8, and 9.

The carbonitrile warhead of UAMC-1110 was substituted by a FAP-cleavable amide bond by DE DECKER et al. [33]. It was coupled to different aromatic fluorophores to yield the first small-molecule FAP substrates with high FAP/PREP and FAP/DPPs selectivity while still maintaining good catalytic efficiency by FAP [33]. Difluorination of the proline ring at 4,4'-position proved to be superior to the non-fluorinated equivalent just like L-Gly at P2 position compared to D-Ala. The compounds with the aromatic residues 7-amino-4-methylcoumarin (AMC, compound **6b**, cf. Figure 1f) and *para*-nitroaniline (pNA, compound **6e**, cf. Figure 1g) were identified as the leading candidates.

In this study similar prodrugs were designed where the cytostatic was supposed to be cleaved by FAP itself. The difference to OncoFAP-GlyPro-MMAE (Figure 1d) by ZANA et al. [35] is that the selective FAP substrate targeting moiety in **6b** and **6e** (Figure 1f and g) was coupled to the cytostatic with the aim to have prodrug with high selectivity over PREP and the DPPs. The FAP affinity of **6b** and **6e** is only in the micromolar range in contrast to subnanomolar affinity of UAMC-1110. Therefore, the prodrugs were supposed to be conjugated with a FAPi to ensure high and

selective accumulation in the tumor stroma. The FAPi targeting vector will preferably bind to the protease due to its higher affinity, yet the inhibition is reversible since the imidate that is formed from the carbonitrile warhead with a serine residue (–OH group) of FAP can be hydrolyzed again. To summarize, FAP has a dual role in our approach. Firstly, it is the oncological target to ensure high and selective accumulation of the prodrug in the TME. Secondly, it represents the enzyme to cleave the cytostatic prodrug and to eventually release the free cytostatic in its active form.

In a first step, conjugates of the FAP substrate targeting vector FAPs with the cytostatic Dox, MMAE and Rucaparib were synthesized, respectively (general structure FAPs.cytostatic, shown in Figure 2a).

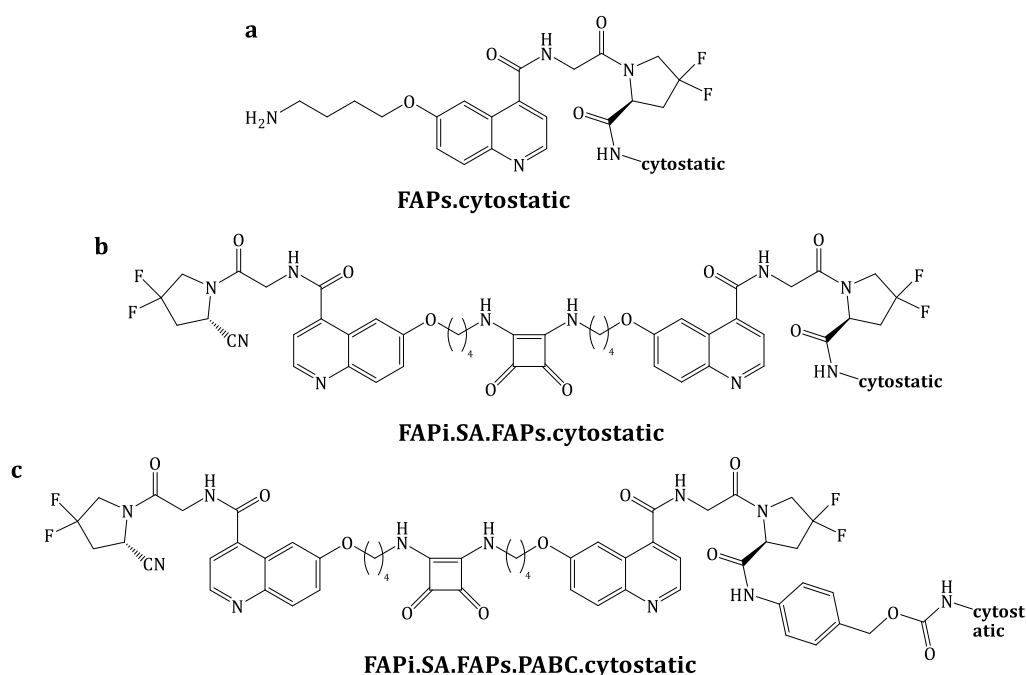


Figure 2: General structure of a) FAPs.cytostatic and b) FAPi.SA.FAPs.cytostatic with cytostatic = MMAE, Dox or Rucaparib.

The cleavage by FAP was investigated in *in vitro* assays similar to DE DECKER et al. [33]. To form the FAP-cleavable amide bond with FAPs, the cytostatic must have an amino group. Rucaparib is a poly(ADP-ribose) polymerase (PARP) inhibitor that prevents DNA repair and is often used in a combination treatment with other cytostatics. In the case of FAP, Rucaparib or a Rucaparib conjugate could be used in combination with FAP-targeted radioligand therapy (RLT) where different studies already indicated the synergistic effect of PARP inhibitors and RLT [38–40].

In a second step, the FAPs.cytostatic building blocks were supposed to be coupled with FAPi targeting vector via squaramide (SA) as shown in Figure 2b. It is also an option to introduce an additional self-immolative linker unit like PABC (cf. Figure 2c) between FAPs and the cytostatic. Potential cleavage of the post-proline amide bond will trigger the 1,6-elimination of PABC which results in the release of the cytostatic drug in the same form as without the self-immolative linker.

2. Results and Discussion

2.1. Organic Synthesis of FAPs.cytostatic

The FAP substrate building block Boc-FAPs-COOH **16** was synthesized according to Figure 3.

16 can be divided into three subunits: the synthesis of the 4-aminobutoxy alkyl chain, the quinoline and the Gly-Pro subunit. Several reactions steps were carried out with each of the subunits before they were coupled in the convergent synthesis.

For the alkyl subunit, *tert*-butyl (4-bromobutyl)carbamate **3** was obtained from 4-aminobutanol **1** over 2 steps with 63%. At first the hydroxyl group of **1** was substituted with bromine (S_N1) by heating **1** in 47% hydrobromic acid (HBr) and afterwards the Boc-protecting group was introduced with di-*tert*-butyl decarbonate (Boc_2O) and triethylamine (TEA).

For the quinoline subunit, 6-Methoxyquinoline-4-carboxylic acid **4** was quantitatively transferred into 6-hydroxyquinoline-4-carboxylic acid methyl ester **6** by demethylating with 47% HBr and then selectively methylating the carboxylic acid group with an excess of thionyl chloride and methanol (*in situ* formation of methyl chloride).

3 and **6** were coupled with the mild base cesium carbonate in DMF. **3** had to be used in slight excess to reach high conversion of **6**. The methyl ester was introduced to prevent a second substitution of **3** at the carboxylic acid ("dimer"). Although only a mild base was used, saponification was observed to some extent and therefore also a little bit of dimer formation was observed. The reaction time (3 h) and temperature (70 °C) was kept as short and as low as possible to minimize saponification, respectively, but the reaction did not occur below 60 °C. Potassium carbonate was also tried as an alternative but was found to be inferior (lower conversion and/or higher temperatures/longer reaction times needed). Subsequent saponification of **7** with 1 M LiOH yielded Boc-Chino-COOH **8** with 34% over 2 steps. In other smaller scale saponification steps the yields were higher (80–90%, data not shown).

The Gly-Pro subunit had to be built in 5 steps. *N*-Boc-*trans*-4-hydroxy-L-proline methyl ester **9** was oxidized to the respective ketone with TCCA and the catalyst TEMPO in the first step. Difluorination with diethylaminosulfur trifluoride (DAST), followed by *N*-Boc deprotection with DCM/TFA (3:1) was carried out. Boc-Gly-OH was coupled to Pro-COOMe **12** with HBTU and HOBT in DMF. After final Boc-cleavage Gly-Pro-COOMe **14** was obtained with a yield of 48% over 5 steps.

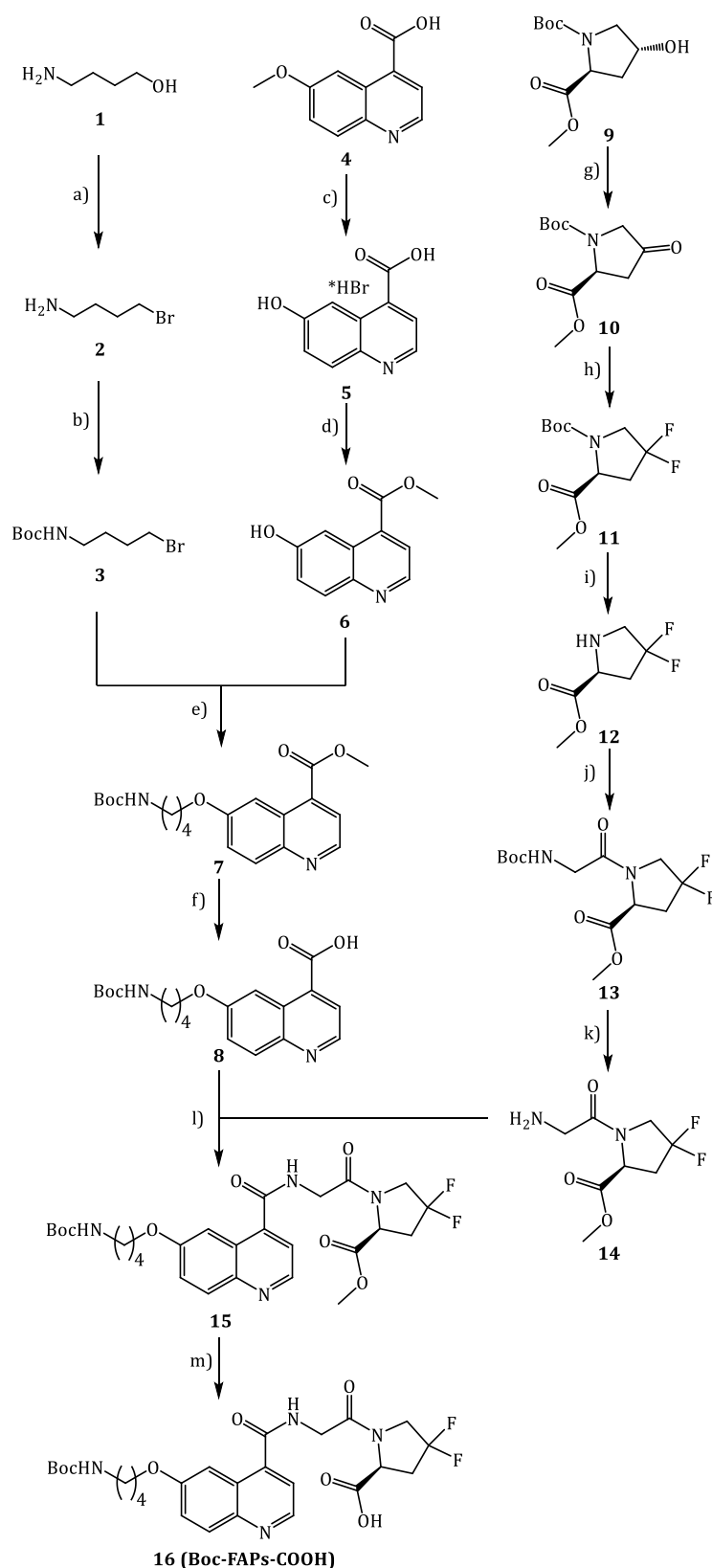


Figure 3: Synthesis of Boc-FAPs **16:** (a) HBr (47%), 126 °C, 4 h, 96%; (b) Boc₂O, TEA, THF, RT, 19 h, 66%; (c) HBr (47%), 126 °C, 1 d, 100%; (d) SOCl₂, MeOH, 0 °C–RT, 2 d, 100%; (e) Cs₂CO₃, DMF, 70 °C, 1 d, 59%; (f) 1 M LiOH, 1,4-dioxane, RT, 4 h, 57%; (g) N-Boc-trans-4-hydroxy-L-proline methyl ester, TCCA, TEMPO, DCM, 0 °C–RT, 1 h, 87%; (h) DAST, DCM, RT, 2 d, 81%; (i) DCM/TFA (3:1), RT, 4 h, 100%; (j) 1. Boc-Gly-OH, HBTU, HOBT, DIPEA, DCM/DMF (1:2), RT, 1 h; 2. **12**, DIPEA, DCM/DMF (1:2), RT, 17 h, 68%; (k) DCM/TFA (3:1), RT, 5 h, 100%; (l) 1. **8**, HATU, DIPEA, DMF, RT, 90 min; 2. **14**, DIPEA, DMF, RT, 2 h, 45%; (m) 1,4-dioxane/1 M LiOH (4:1), RT, 3 h, 85%.

The coupling of Gly-Pro-COOMe **14** with Boc-Chino-COOH **8** was performed with HATU as a coupling reagent. Because of this coupling, appropriate protecting groups were chosen (methyl ester for **14** and Boc-protecting group at the alkyl chain of **8**). Boc-FAPs-COOMe **15** was obtained with 45%. In a smaller scale coupling (1 mmol vs. 4 mmol) a higher yield of 64% could be achieved (data not shown). Boc-FAPs-COOH **16** was obtained after final saponification which was again done with 1 M LiOH (85% yield). The overall yield was 18% (13 steps).

Boc-FAPs-COOH **16** was deprotected to obtain FAPs-COOH **17**. This was used for further synthesis but also used as a reference compound for the quantification of the FAP cleavage in the following *in vitro* experiments. Figure 4 also shows the synthesis of the FAPs.cytostatic conjugates.

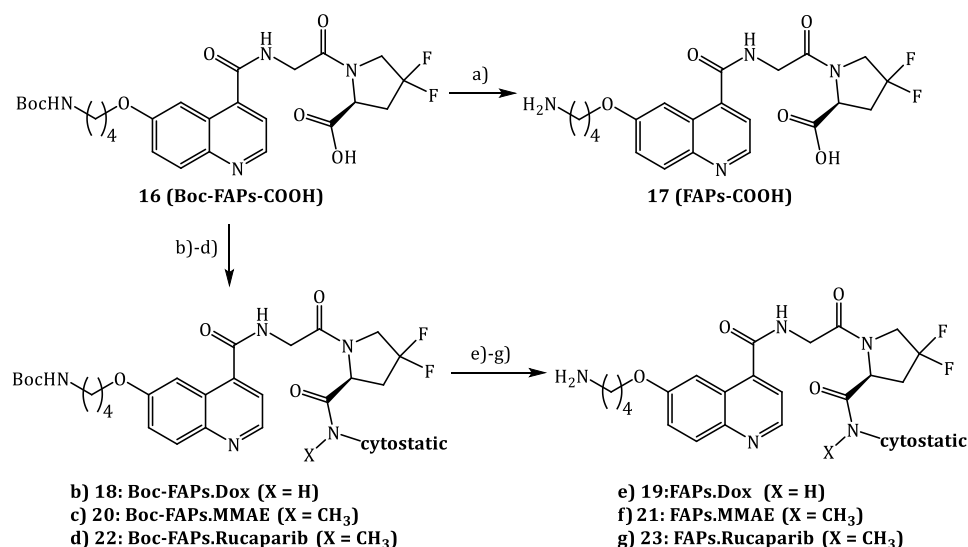


Figure 4: Synthesis of the conjugates FAPs.cytostatic: a) TFA/TIPS/H₂O (95:2.5:2.5), RT, 45 min, 76%; b) 1. HATU, DIPEA, DMF, RT, 2 h; 2. Dox*HCl, DIPEA, DMF, RT, 2 d, 95%; c) 1. HATU, DIPEA, DMF, RT, 90 min; 2. MMAE, DIPEA, DMF, RT-40 °C, 5 d, 42%; d) 1. HATU, DIPEA, DMF, RT, 60 min; 2. Rucaparib*H₃PO₄, DIPEA, DMF, RT, 1 d, 54%; e) DCM/TFA (4:1), RT, 6 h, 5%; f) DCM/TFA (4:1), RT, 1 h, 66%; g) DCM/TFA (3:1), RT, 3 h, 41%.

The cytostatics doxorubicin (Dox), monomethyl auristatin E (MMAE) and Rucaparib were coupled to **16** with HATU. Subsequently, the amino group was deprotected with DCM/TFA (3:1–4:1) and the final products were obtained after semipreparative RP-HPLC. The yields over 2 steps were 5% (FAPs.Dox), 28% (FAPs.MMAE) and 22% (FAPs.Rucaparib), respectively. The synthesis with Dox were carried out to avoid decomposition since Dox is light-sensitive. Since some steps like purification (e.g. column chromatography/RP-HPLC) could not be carried out completely in the dark this might explain the rather low yield. For MMAE it was observed that no complete conversion in the coupling with **16** could be reached even when more HATU was added and the reaction temperature was increased. MMAE is a rather big molecule and a secondary amine which can lead to steric hindrance in the coupling reaction. Therefore, more reactive coupling conditions should be tried, for example transferring **16** into a mixed anhydride with ethyl chloroformate (ECF) as it was done for the synthesis of **26** (cf. Figure 11).

2.2. In Vitro Studies

2.2.1. Cleavage of FAPs.cytostatic by FAP and PREP (Short Incubation)

The cleavage products of FAPs.cytostatic are FAPs-COOH **17** and the respective free cytostatic-NHX (X = H, CH₃). The linear calibration curves of **17** in the FAP and PREP assay buffer is shown in Figure 5 and enables to quantify cleavage efficiency of the samples.

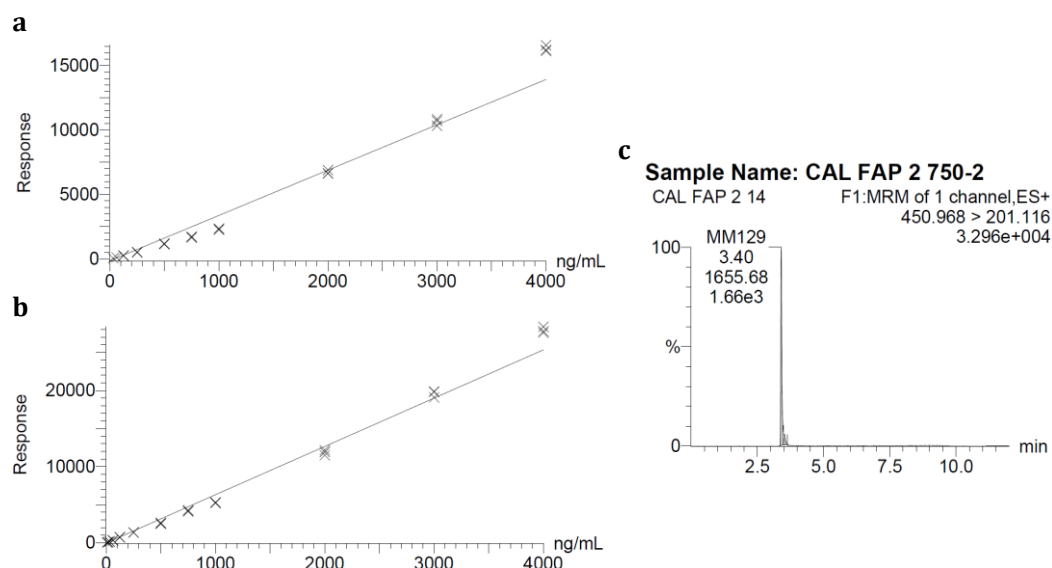


Figure 5: Calibration curves of FAPs-COOH **17** in a) FAP assay buffer, b) PREP assay buffer and c) exemplary UPLC/MS (ESI⁺) chromatogram with t_R (**17**) = 3.4 min.

The FAPs.cytostatic compounds were used in a 10 μ M final concentration. The enzyme was added and incubation at 37 $^{\circ}$ C was carried out for 30 minutes where samples at different time points were taken for analysis.

In the measurements with the compounds FAPs.Dox **19** and FAPs.Rucaparib **23** no peak at the retention time t_R = 3.4 min could be observed at none of the time points, indicating the compounds are probably not cleaved by FAP (cf. Figure 6).

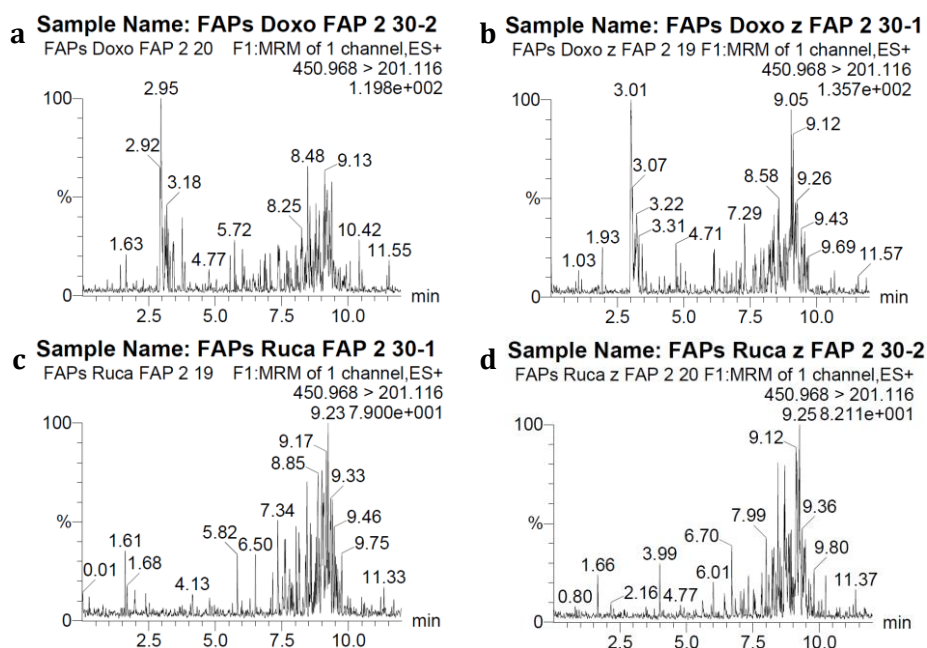


Figure 6: Exemplary UPLC/MS chromatograms after 30 minutes incubation at 37 °C of a) FAPs. Dox **19** with FAP, b) FAPs. Dox **19** in FAP assay buffer, c) FAPs. Rucaparib **23** with FAP and d) FAPs. Rucaparib **23** in FAP assay buffer only. No cleavage product FAPs-COOH **17** (t_R **17** = 3.4 min) was found. The intensities were low for all measurements.

Investigation with PREP (and PREP assay buffer only) showed similar results (data not shown). Moreover, it is important to mention that all the measurements are characterized by a very low intensity (cf. Figure 6), especially compared to the calibration curve (cf. Figure 5c) and the visible peaks are probably just background noise.

Regarding FAPs.MMAE **21**, a small peak was observed at t_R = 3.4 min after the longer incubation periods (25 and 30 min). The intensity of this small peak is very low. Nevertheless, it seems to increase with incubation time. The intensity of the peak could not be calculated by the software since the concentrations are lower than of the calibration curve. In contrast to the incubation with FAP as exemplary shown in Figure 7a, no cleavage product **17** was detected in the control incubation with the FAP assay buffer only (see Figure 7b).

The peak at t_R = 6.4 min most likely results from in-source fragmentation of the intact substrate **21** that is also present in the analyzed mixture. Hence, it can be assumed that FAPs.MMAE compound is slowly cleavable by FAP, but not as efficient as compared to previous FAP substrates such as compound **6b** or **6e** (cf. Figure 1f and g) studied by DE DECKER et al. [33].

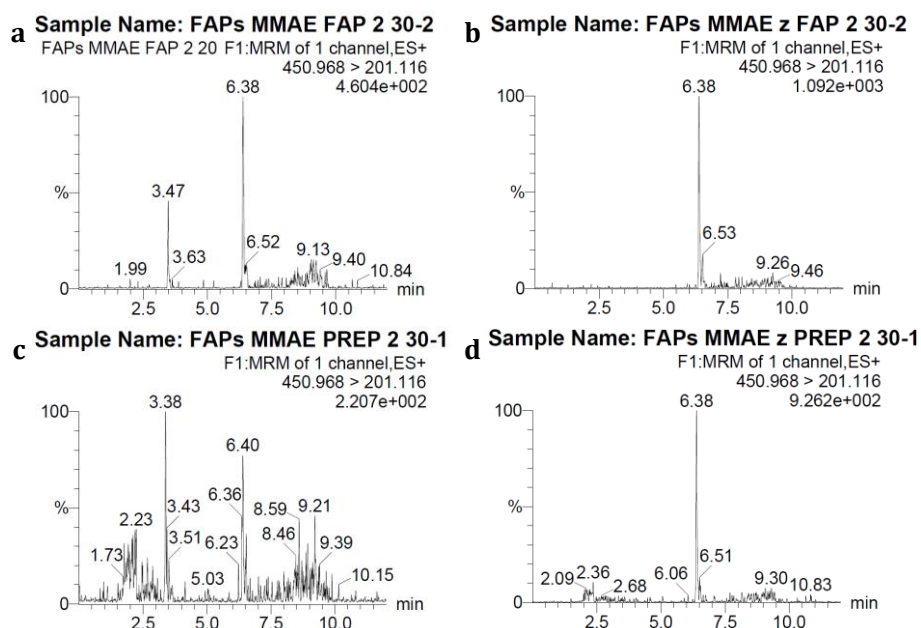


Figure 7: Exemplary UPLC/MS chromatograms after 30 minutes incubation at 37 °C of FAPs.MMAE a) with FAP, b) in FAP assay buffer only, c) with PREP and d) in PREP assay buffer only. A small peak (low intensities) of FAPs-COOH **17** (t_R **17** = 3.4 min) was found in a) and c) but not for b) and d).

Similar results were obtained with PREP, detection of small amounts of **17** after longer incubation times with PREP (cf. Figure 7c) and no detection in the PREP buffer only (cf. Figure 7d). **21** seems to not be selectively cleaved by FAP only.

Given these intriguing und unexpected results, two more experiments were performed.

2.2.2. FAP Inhibition Properties of FAPs.cytostatic

Since low cleavage (FAPs.MMAE **21**) or no cleavage (FAPs.Dox **19** and FAPs.Rucaparib **23**) was observed in the UPLC/MS experiments mentioned above, it was investigated whether the compounds inhibit FAP. Therefore, the remaining enzymatic activity of FAP was investigated with two different compound concentrations (80 and 8000 nM) at two different time points (15 min and 24 hours), respectively. The results are summarized in Table 1 where V_i/V_0 represents the ratio of the enzymatic activity in the presence of the respective sample/potential inhibitor V_i and in the absence of the sample V_0 .

Table 1: Ratio of enzymatic activity of FAP after incubation at 37 °C with (V_i) and without (V_0) the compounds FAPs.Dox **19**, FAP.s MMAE **21** and FAPs.Rucaparib **23** at different concentrations for 15 minutes and 24 hours, respectively.

c / nM	V_i/V_0 (15 min) / %			V_i/V_0 (24 h) / %		
	19	21	23	19	21	23
80	82	93	80	83	87	72
8000	1	94	2	1	80	0

A very low V_i/V_o ratio and therefore strong inhibition (>95%) of FAP was measured with the compounds **19** and **23** after 15 minutes and 24 hours of incubation at 37 °C and higher concentration (8 μ M). Compound **21** has only a small effect on the FAP activity and at 80 nM concentration, none of the compounds causes significant FAP inhibition.

These results are compatible with the observations from the first cleavage experiments in the previous section where cleavage by FAP was found for FAPs.MMAE **21**, but not for FAPs.Dox **19** and FAPs.Rucaparib **23**.

2.2.3. Cleavage of FAPs.cytostatic by FAP and PREP (Long Incubation)

Longer incubation times of up to 24 hours were investigated along with different concentrations (10 μ M and 100 nM) in a second set of cleavage assays.

Calibration curves were determined again in FAP and PREP assay buffer (Figure 8a and b, respectively) and were both linear and a retention time $t_R = 3.3$ min for **17** was observed (see Figure 8c).

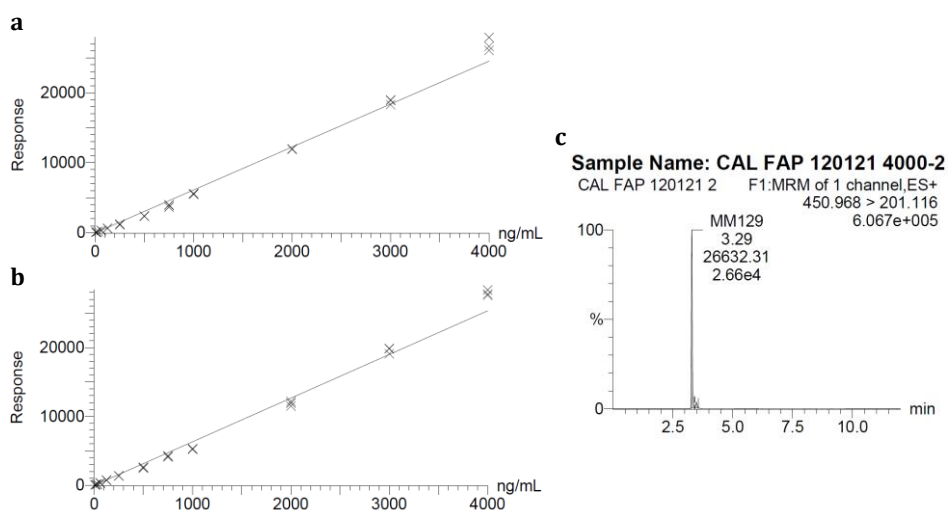


Figure 8: Calibration curves of FAPs-COOH **17** in a) FAP assay buffer, b) PREP assay buffer and c) exemplary UPLC/MS (ESI⁺) chromatogram with t_R (**17**) = 3.3 min.

In contrast to the first experiments, FAPs.Dox **19** showed a cleavage peak with FAP (see Figure 9a), but also in the FAP assay buffer incubation without any enzyme (see Figure 9b) after longer incubation times (24 hours). Hence, the formation of **17** is most likely due to decomposition/autohydrolysis of the compound and not induced by selective enzymatic cleavage. The intensities were again too low compared to the calibration curve to allow quantification. For **23**, no peak was observed at any time point in any assay setup (with and without FAP or PREP) as some examples are shown in Figure 9c and d, indicating that **23** is whether cleaved by FAP nor by PREP even after longer incubation periods.

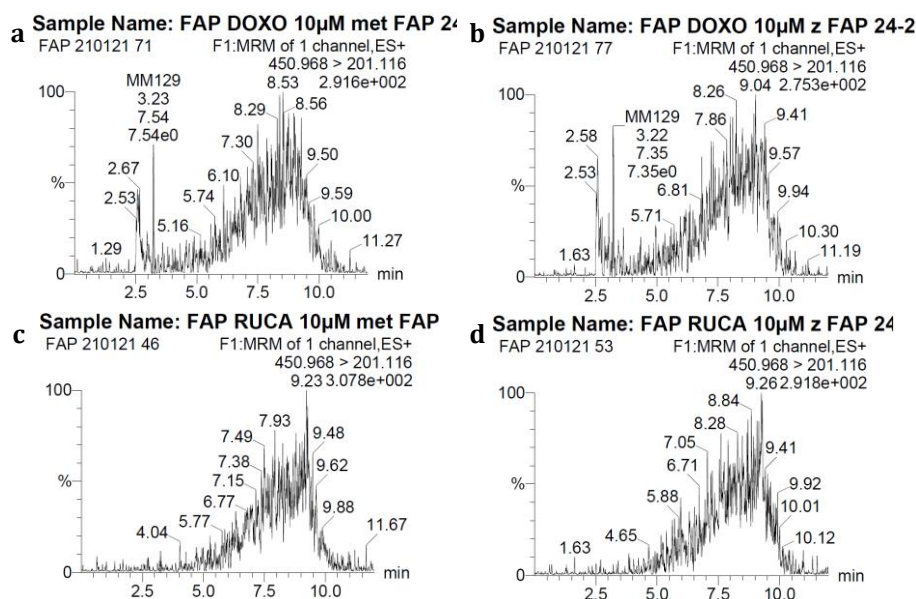


Figure 9: Exemplary UPLC/MS chromatograms after 24 hours incubation at 37 °C of a) FAPs. Dox **19** with FAP, b) FAPs. Dox **19** in FAP assay buffer, c) FAPs. Rucaparib **23** with FAP and d) FAPs. Rucaparib **23** in FAP assay buffer only. Small amounts of cleavage product FAPs-COOH **17** were found with **19** with and without FAP (a and b). No cleavage product **17** was found for **23** (c and d).

Regarding FAPs.MMAE **21**, a cleavage peak was visible again in all the samples incubated for 1, 4, 8 and 24 hours with FAP (see Figure 10a). In contrast, it was not visible in the samples with only FAP assay buffer after 0, 1, 4, and 8 hours. After 24 hours, a very small peak was observed, implicating that minimal autohydrolysis occurs with long incubation (see Figure 10b). When comparing the samples with and without FAP (as shown exemplary in Figure 10a and b), the intensity of the cleavage product peak is much higher with FAP than without. This again suggests that **21** is cleaved by FAP.

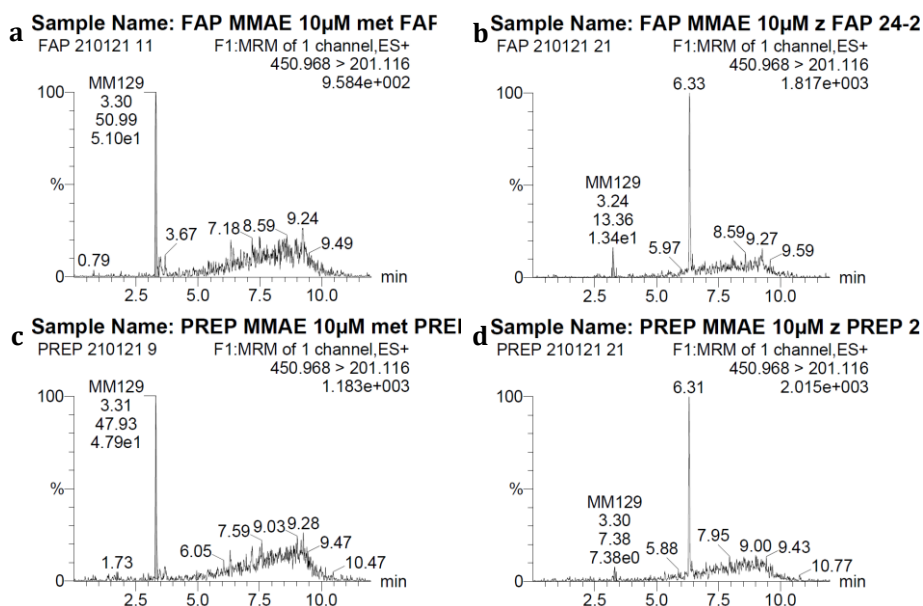


Figure 10: Exemplary UPLC/MS chromatograms after 24 hours incubation at 37 °C of FAPs.MMAE **21** (10 µmol) a) with FAP, b) in FAP assay buffer only, c) with PREP and d) in PREP assay buffer only. A relatively high peak of FAPs-COOH **17** (t_R **17** = 3.4 min) was found in a) and c) but only a small peak in b) and d).

However, when comparing the calibration curve with the concentrations of **17** (14.9 nM) of the FAPs.MMAE **21** samples incubated for 24 hours, this lies within the lower range of the calibration curve. When 100% cleavage efficiency by FAP would be achieved, a concentration of 1000 nM would be measured. Hence, 14.9 nM of formed product **17** corresponds to $\approx$ 1.5% formation of cleavage product **17**. Therefore, it can be concluded that the cleavage **21** by FAP is very limited even at prolonged incubation times (24 h at 37 °C).

Similar results were obtained for PREP, indicating that the compound is also cleavable by PREP (cf. Figure 10c and d). The concentration of product **17** (13.85 nM) is very similar to the product formation with FAP (14.9 nM). The similar cleavage efficiencies show that **21** is not selective for FAP with respect to PREP.

No cleavage product could be observed with 100 nM at any time point. So, measuring with lower concentrations only causes more problems by increased noise in the chromatogram.

The results are in agreement with the FAP inhibition screening experiments and the FAP inhibiting properties of FAPs.Dox **19** and FAPs.Rucaparib **23** may contribute to the understanding of the results. Only FAPs.MMAE **21** showed cleavability by FAP although be it not very efficient and selective when compared to cleavage by PREP.

2.3. Organic Synthesis of FAPi.SA.FAPs.PABC.cytostatic

None of the original compounds FAPs.cytostatic did show efficient cleavability by FAP and selectivity over PREP. The highly selective FAP substrates found by DE DECKER et al. [33] that were cleaved by FAP efficiently all had an aromatic amine unit at the P1' position (cf. Figure 1f and g). Therefore, the original molecular design was altered and *para*-aminobenzyl carbamate (PABC) was introduced as a self-immolative linker unit between FAPs and the cytostatic. When the amide bond is cleaved by FAP, PABC undergoes 1,6-elimination and CO₂ is formed alongside with *p*-xylylene and the free cytostatic-NH₂ can be released in the same way than without the self-immolative linker. Secondly, the FAP inhibitor structure FAPi should also be coupled to the molecule via squaramide (SA). Coupling via squaric acid-based allows asymmetric diamidation under normally mild conditions via pH control in aqueous buffer solution, but is also possible in organic solvents [41–43].

The order of coupling the different units is principally interchangeable. On the other hand, it is desirable to introduce the cytostatic as late as possible in the synthesis. This simplifies the organic synthesis because every step before the cytostatic is introduced only needs to be done once whereas every step after cytostatic coupling has to be carried out for each conjugate separately. On the other hand, the cytostatic (Dox in particular) might not be completely unstable and decomposition of the molecule might occur in one of the following reaction steps. Figure 11 gives an overview over the different possible synthesis routes for FAPi.SA.FAPs.cytostatic.

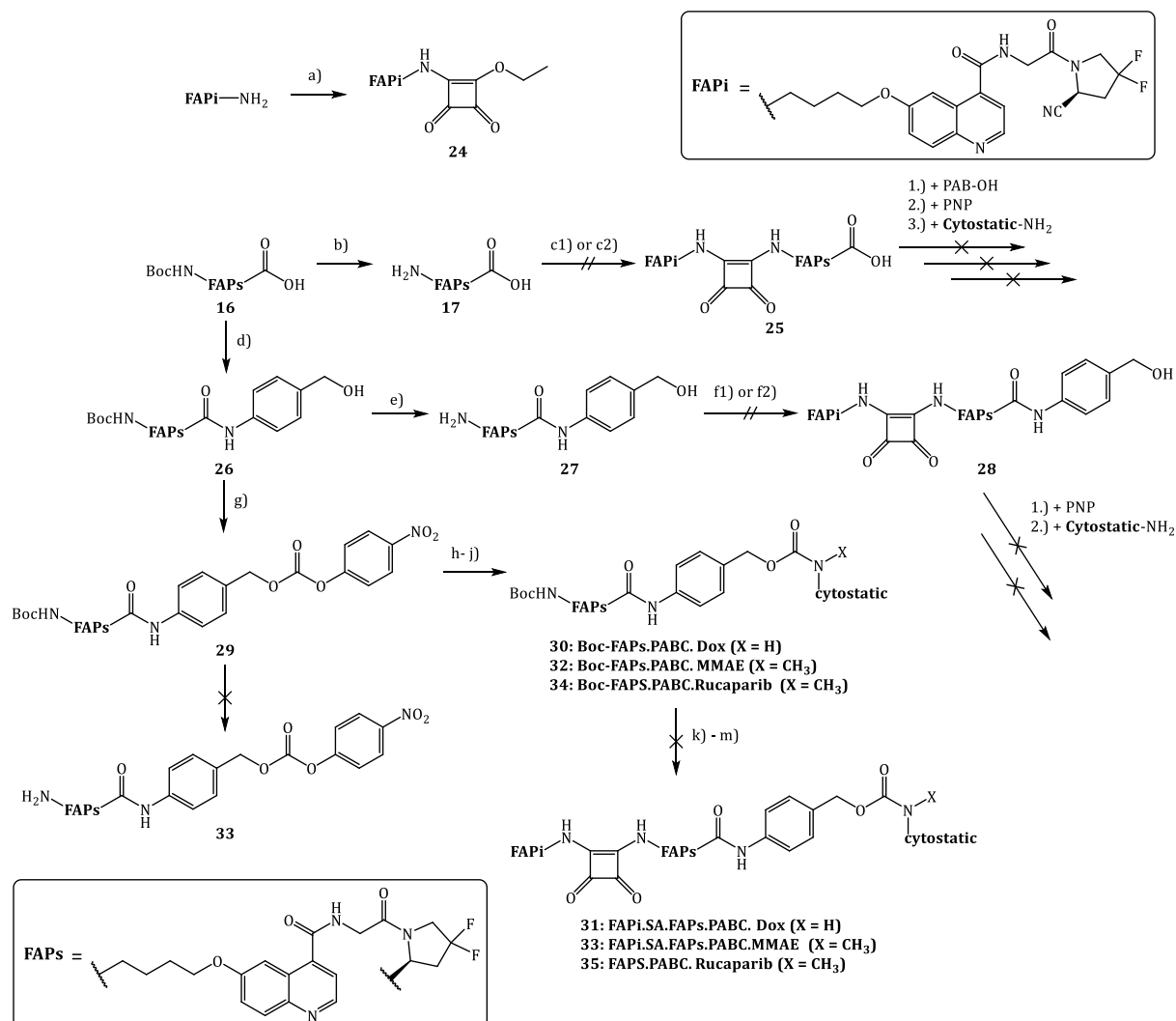


Figure 11: Synthesis of the conjugates FAPi.SA.FAPs.PABC.cytostatic: a) SA-diethylester, TEA, EtOH, RT, 2 d, 88%; b) TFA/TIPS/H₂O (95:2.5:2.5), RT, 45 min, 76%; c1) FAPi.SA, EtOH/MeOH (1:1) or MeCN/MeOH (1:1), TEA, RT–70 °C, 4 d, or c2) FAPi.SA, 0.5 M phosphate buffer (pH = 10), 40 °C, 7 d; d) 1. ECF, TEA, DCM/DMF (3:1), 0 °C, 1 h; 2. PAB-OH, TEA, DCM/DMF (3:1), 0 °C–RT, 1 d, 99%; e) DCM/TFA (3:1), RT, 1 h; f1) FAPi.SA, DMF, TEA, RT–80 °C, 8 d, f2) FAPi.SA, 0.5 M phosphate buffer (pH = 10), 40 °C, 3 d; g) Bis(4-nitrophenyl) carbonate, DIPEA, DMF, RT, 1 d, 65%; h) Dox*HCl, DIPEA, DMF, RT, 4 d; i) MMAE, DIPEA, DMF, 40 – 50 °C, 5 d; j) Rucaparib*H₃PO₄, DIPEA, DMF, RT, 1 d; k) 1. TFA/TIPS/H₂O (92.5:5:2.5), RT, 1 h; 2. FAPi.SA **24**, TEA, DMF, 40 °C, 2 d; l) 1. TFA/TIPS/H₂O (92.5:5:2.5), RT, 1 h; 2. FAPi.SA **24**, TEA, DMF, 50 – 60 °C, 3 d; m) 1. MeCN, 4 M HCl in 1,4-dioxane, RT, 4 h; 2. FAPi.SA **24**, TEA, DMF/MeOH (2:1), 40 °C, 4d.

For the first amidation step, FAPi-NH₂ was coupled to squaric acid diethyl ester in ethanol with small amounts of triethylamine (TEA) to obtain FAPi.SA **24**.

Subsequently, it was tried to couple FAPi.SA **24** to FAPs-COOH **17** to synthesize FAPi.SA.FAPs **25**. The coupling was performed in aqueous conditions (0.5 M phosphate buffer (pH = 10)) and in organic solvents with excess of TEA but no product formation could be observed via TLC and LC/MS. No substantial conversion of the reactants seemed to take place even though the reaction conditions were already comparably harsh compared to usual reaction conditions of the second amidation step (pH = 10 instead of 9 and elevated temperatures instead of room temperature).

The temperature was gradually increased. Until 40 °C, the reactants were still present in the reaction mixture in high amounts, indicating that they did not react. When the temperature was further increased up to 70 °C and more TEA was added, decomposition of the reactants occurred as they could not be identified in the following TLC and LC/MS measurements anymore.

Alternatively, *para*-aminobenzyl alcohol (PAB-OH) was coupled to Boc-FAPs-COOH **16** with ethyl chloroformate (ECF) as the coupling reagent which was found to have superior conversion rates compared to HATU and NHS with EDC*HCl in small scale test reactions. Boc-FAPs.PAB-OH **26** was obtained in quantitative yield (99%). After subsequent Boc-cleavage SA-coupling of **18** should yield FAPi.SA.FAPs.PAB-OH **28**. Again, neither in phosphate buffer (pH = 10) nor in organic solvent (DMF + TEA) any product could be detected even when the temperature was increased.

These two routes would have allowed to couple the respective cytostatic to the building block in the final step but were both not successful.

As a consequence, another synthetic pathway was elaborated. The hydroxyl group of PAB-OH was activated and transferred into a (4-nitrophenyl)carbonate (PNP) group and the compound Boc-FAPs.PABC.PNP **29** was synthesized successfully (65% yield). This was followed by coupling of the cytostatic-NH₂ with DIPEA in DMF. For all three cytostatics product formation FAPs.PABC.cytostatic could be observed via LC/MS. Subsequently, the Boc-protecting group was removed before FAPi.SA **24** can be finally coupled to FAPs.PABC.cytostatic in the second amidation step. TEA in excess and elevated temperatures were used for the final coupling to FAPi.SA **24**. Some product FAPi.SA.FAPs.PABC.Rucaparib **35** was identified in a small test reaction once but no product could be isolated in subsequent RP-HPLC purification. Unfortunately, this could not be reproduced in a bigger scale. Although TLC did show that a reaction occurred in the synthesis of FAPi.SA.FAPs.PABC.Dox **31** and FAPi.SA.FAPs.PABC.MMAE **33**, no product could be identified via LC/MS as well as RP-HPLC in combination with LC/MS.

3. Conclusion and Outlook

This study describes the synthesis and *in vitro* analysis of new cytostatic prodrugs for FAP-targeted drug delivery. FAP is utilized in two distinctive ways where it simultaneously serves as (i) an oncological target and (ii) a cleaving enzyme. Firstly, the compounds include a high-affinity FAP inhibitor, coupled via squaric acid (SA), to achieve high and selective accumulation in the TME. Secondly, the new compounds are designed to further include a highly-selective FAP substrate targeting vector FAPs coupled to a cytostatic drug via a post-proline amide bond that is cleavable by FAP.

At first, the Boc-FAPs-COOH **16** building block was synthesized successfully in a 13-step synthesis with an overall yield of 18%. Subsequently, it was coupled to the cytostatic drugs doxorubicin (Dox), monomethyl auristatin E (MMAE) and rucaparib to yield the first-generation compounds FAPs.cytostatic.

These compounds were evaluated *in vitro* to determine FAP-cleaving efficiency. FAPs.Dox **19** and FAPs.Rucaparib **23** did not show any cleavability. FAPs.MMAE **21** showed low formation of the cleavage product FAPs-COOH **17** by FAP and PREP indicating low efficiency and selectivity. Longer incubation times and different concentrations were tested in a second series of experiments. FAPs.Dox **19** showed some formation of **17** although really low concentrations that might result from compound instability. Surprisingly, FAPs.Dox **19** and FAPs.Rucaparib **23** did show significant FAP inhibition. Although FAPs.MMAE **21** has no strong FAP inhibition properties, it is still not efficiently cleaved since the maximal product formation ("substrate conversion") by FAP is only 1.5%.

Within the combinatorial FAPi/FAPs approach, the self-immolative linker PABC (*p*-aminobenzyl carbamate) was introduced between FAPs and the cytostatic since the originally found FAP substrates by DE DECKER et al. [33] all had aromatic amines at the post-proline position. The synthesis was performed utilizing different synthesis routes. FAPi.SA.FAPs.PABC.Rucaparib **35** was identified but no product could be isolated from this small scale reaction. Organic synthesis (reaction conditions and upscale) still needs further optimization.

Mild Boc-cleavage conditions like with 4 M HCl in 1,4-dioxane seem to be better for the penultimate reaction step than harsh conditions with TFA for further synthesis in the future. Systematic test reactions to find the best suited base (perhaps a more reactive base than TEA) in combination with a suitable reaction temperature are needed for the final SA coupling reaction step. From past experience and literature, the SA coupling usually works quite well although the second step might require more harsh conditions than the usually proposed reaction conditions

(e.g. pH = 9). However, elevated temperatures and a large excess of TEA was not sufficient but rather led to decomposition of the reactants.

Theoretically, **18** could also be coupled to **29** (basic conditions pH > 9 or TEA) after Boc-cleavage under acidic conditions (HCl or TFA) and before introducing the cytostatic unit (basic conditions with DIPEA). But activated PNP-carbonate esters are probably not stable.

The new approach to selective FAP-targeted drug delivery is very promising and future attempts should further evaluate different synthesis routes to obtain FAPi.SA.FAPs.PABC.cytostatic compounds. After that, the compounds are supposed to be investigated *in vitro* again to check cleavability. If selective cleavage is observed, further murine *in vivo* studies of FAP-positive tumors, or even better of tumor cells co-cultured with FAP-positive cancer-associated fibroblasts, should investigate anti-tumor efficacy and tolerability of treatment.

Alternatively, a coupling to a chelator might also be totally new approach to combine FAP-targeted drug delivery and FAP-targeted radionuclide therapy in one molecule.

4. Materials and Methods

4.1. Chemicals and Equipment

All basic chemicals were purchased from Sigma-Aldrich and Merck (Darmstadt, Germany), TCI (Eschborn, Germany), ABCR (Karlsruhe, Germany), Alfa Aesar by Thermo Fisher Scientific (Kandel, Germany), Acros Organics (Schwerte, Germany) and VWR (Bruchsal, Germany). Dry solvents were bought from Acros Organics, Alfa Aesar by Thermo Fischer Scientific and Merck and deuterated solvents from Deutero (Kastellaun, Germany). The cytostatics monomethyl auristatin E (MMAE) and doxorubicin hydrochloride (Dox*HCl) were purchased from Hycultec (Beutelsbach, Germany). Rucaparib*H₃PO₄ was kindly provided from the research group of Prof. Tanja Schirmeister (JGU Mainz, Germany). (*S*)-6-(4-aminobutoxy)-*N*-(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide (FAPi-NH₂*TFA) was purchased from KE Biochem Co. (Shanghai, China).

Thin-layer chromatography (TLC) plates coated with silica gel 60 F₂₅₄ from Merck were used. For analysis they were investigated via UV lamp (λ = 254 and 366 nm) as well as staining with potassium permanganate. Silica 60 (0.040-0.063 mm particle size) was used for column chromatography.

Nuclear magnetic resonance (NMR) spectra were recorded in deuterated solvents on an Avance II 400 spectrometer (400 MHz, 5 mm BBFO sample head with z-gradient and ATM, SampleXPress 60 sample changer) or an Avance III 300 spectrometer (300 MHz, 5 mm BBFO sample head with z-gradient and ATM, BACS 60 sample changer) from Bruker (Rheinstätten, Germany) were used. MestReNova 14.2.0 software from Mestrelab Research (Santiago de Compostela, Spain) was used to analyze the spectra.

Liquid chromatography coupled with electrospray ionization mass spectrometry (ESI-LC/MS) mass spectra were measured using Agilent Technologies (Santa Clara, USA) 1220 Infinity LC coupled to an Agilent Technologies 6130B Single Quadrupole LC/MS system with an Agilent Zorbax SB-C18 column (21x50 mm, 1.8 μ m) with a linear gradient of acetonitrile (MeCN, HPLC grade)/H₂O (Milli-Q®) + 0.05% formic acid (HFO) and a flow rate of 0.5 mL/min.

Semi-preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) was performed using LaChrom HPLC 7000 series (Merck Hitachi) with L-7100 pump, L-7400 UV detector (λ = 254 nm), a D-7000 interface and autosampler. Separation was usually performed using a Phenomenex (Aschaffenburg, Germany) Synergi Max-RP C18 column (250x10 mm, 4 μ m). In cases where a Licrospher® 100 RP18 EC column (250x10 mm, 5 μ m) from Merck was used this is remarked specifically. A linear gradient of MeCN/H₂O + 0.1% trifluoacetic acid (TFA) and a flow rate of 5 mL/min was used for all RP-HPLC runs.

4.2. Organic Synthesis

4.2.1. Synthesis of FAPs

4-Bromobutylamine (**2**)

47% hydrobromic acid (HBr, 70 mL) was slowly added to 4-aminobutanol **1** (5.39 g, 60.5 mmol, 1.00 eq) and then heated for 4 hours under reflux. The reaction mixture was concentrated *in vacuo* and 4-bromobutylamine **2** was obtained as a colorless solid (13.5 g, 58.0 mmol, 96%). It was used directly in the next step without further purification. ¹H-NMR (400 MHz, MeOD): δ [ppm] = 3.51 (t, J = 6.4 Hz, 2H), 2.98 (t, J = 7.6 Hz, 2H), 2.02 – 1.73 (m, 4H). MS (ESI⁺): m/z (%) = 152.0 (100, [M+H]⁺), 154.0 (98, [M+H]⁺), calculated for C₄H₁₀BrN: 151.00 [M].

tert-Butyl (4-bromobutyl)carbamate (**3**)

4-Bromobutylamine **2** (7.01 g, 30.1 mmol, 1.00 eq) and di-*tert*-butyl dicarbonate (Boc₂O, 7.34 g, 33.6 mmol, 1.12 eq) were dissolved in dry tetrahydrofuran (THF, 34 mL) under argon atmosphere. Triethylamine (TEA, 4.6 mL, 36.1 mmol, 1.20 eq) was then added, followed by MeOH (36 mL) to turn the suspension into a clear solution again. The solution was stirred overnight at room temperature (RT). The solvent was removed *in vacuo* and diluted HBr was added to the residue until pH = 2.5. The aqueous solution was extracted with diethyl ether (Et₂O, 5×80 mL) and the combined organic phases were washed once with saturated NaHCO₃ (10 mL) and Brine (10 mL) and then dried over Na₂SO₄. The solvent was removed under reduced pressure and after column chromatography (cyclohexane/ethyl acetate (CH/EA), 5:1) product **3** was obtained as a colorless solid (5.08 g, 20.2 mmol, 66%). ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 3.36 - 3.21 (m, 4H), 1.86 - 1.76 (m, 4H), 1.41 (s, 9H). MS (ESI⁺): m/z (%) = 196.0 (100, [M-^tBu+H]⁺), 198.0 (100, [M-^tBu+H]⁺), calculated for C₉H₁₈BrNO₂: 251.05 [M].

6-Hydroxyquinoline-4-carboxylic acid hydrobromide (**5**)

6-Methoxyquinoline-4-carboxylic acid **4** (2.46 g, 12.1 mmol, 1.00 eq) was dissolved in 47% HBr (28.2 mL, 242.4 mmol, 20 eq) and heated for one day under reflux. After cooling to RT, the hydrobromic acid was partially removed *in vacuo* and the precipitate was then filtered off and washed first with cold EA (20 mL) and then with cold EA/MeOH (9:1, 10 mL). Product **5** was obtained as a yellow solid (3.25 g, 12.1 mmol, 100%). ¹H-NMR (400 MHz, MeOD): δ [ppm] = 9.05 (d, J = 5.6 Hz, 1H), 8.41 (d, J = 5.6 Hz, 1H), 8.33 (d, J = 2.6 Hz, 1H), 8.19 (d, J = 9.3 Hz, 1H), 7.77 (dd, J = 9.3, 2.6 Hz, 1H). MS (ESI⁺): m/z (%) = 190.0 (100, [M+H]⁺), 191.0 (12, [M+H]⁺), calculated for C₁₀H₈BrNO₃: 189.04 [M].

6-Hydroxyquinoline-4-carboxylic acid methyl ester (6)

Dry MeOH (20 mL) was cooled to 0°C under argon atmosphere and SOCl₂ (4.43 mL, 61.1 mmol, 5.05 eq) was added dropwise. 6-Hydroxyquinoline-4-carboxylic acid hydrobromide **5** (3.25 g, 12.1 mmol, 1.00 eq) was dissolved in dry MeOH (20 mL) and also cooled to 0°C under argon atmosphere. Then, the SOCl₂-MeOH solution was added dropwise to **5**. At first, the reaction solution was allowed to warm to RT and heated under reflux for one day afterwards. SOCl₂ (2.91 g, 24.4 mmol, 2.02 eq) and MeOH (20 mL) were again combined at 0 °C and added to the reaction mixture at RT. The solution was heated at reflux for an additional day. The addition of the SOCl₂-MeOH solution was repeated one more time and after another 4 hours of heating under reflux, the solvent was removed under reduced pressure. Product **6** was obtained as a yellow solid (2.46 g, 12.1 mmol, 100%) which was used in the next step without any further purification. ¹H-NMR (400 MHz, MeOD): δ [ppm] = 9.02 (d, *J* = 5.5 Hz, 1H), 8.38 (d, *J* = 5.5 Hz, 1H), 8.24 (d, *J* = 2.6 Hz, 1H), 8.17 (d, *J* = 9.3 Hz, 1H), 7.75 (dd, *J* = 9.3, 2.6 Hz, 1H), 4.09 (s, 3H). MS (ESI⁺): *m/z* (%) = 204.0 (100, [M+H]⁺), 205.1 (12, [M+H]⁺), calculated for C₁₁H₉NO₃: 203.06 [M].

Methyl 6-(4-((tert-butoxycarbonyl)amino)butoxy)quinoline-4-carboxylate (7, Boc-Chino-COOMe)

6-Hydroxyquinoline-4-carboxylic acid methyl ester **6** (2.46 g, 12.1 mmol, 1.00 eq) and Cs₂CO₃ (4.37 g, 13.4 mmol, 1.25 eq) were suspended in dry *N,N*-dimethylformamide (DMF, 55 mL). The reaction solution was heated to 70°C. Then, *tert*-butyl (4-bromobutyl)carbamate **3** (3.76 g, 14.9 mmol, 1.22 eq), dissolved in dry DMF (80 mL), was dropped into the reaction mixture. The solution was stirred for 3 hours at 70°C before some more **3** (1.23 g, 4.88 mmol, 0.4 eq., again dissolved in dry DMF (20 mL), was added. It was stirred overnight at 70°C. After another addition of **3** (308 mg, 1.22 mmol, 0.1 eq) and another 3 hours at 70°C, the solvent was removed *in vacuo* and the residue was taken up in diluted HBr (150 mL, pH = 2.6). It was extracted with EA (5×80 mL), and the organic phase was washed with Brine (20 mL) and dried over Na₂SO₄. The solvent was removed under reduced pressure and Boc-Chino-COOMe **7** was purified via column chromatography (CHCl₃/MeOH, 100:1) to give a yellowish solid (2.68 g, 7.17 mmol, 59%). ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 8.84 (d, *J* = 4.5 Hz, 1H), 8.21 (d, *J* = 2.8 Hz, 1H), 8.07 (d, *J* = 9.2 Hz, 1H), 7.92 (d, *J* = 4.5 Hz, 1H), 7.41 (dd, *J* = 9.2, 2.8 Hz, 1H), 4.66 (s, 1H), 4.15 (t, *J* = 6.2 Hz, 2H), 4.02 (s, 3H), 3.23 (q, *J* = 6.7 Hz, 2H), 1.90 (tt, *J* = 8.6, 6.0 Hz, 2H), 1.77 – 1.68 (m, 2H), 1.44 (s, 9H). MS (ESI⁺): *m/z* (%) = 375.2 (100, [M+H]⁺), 376.2 (23, [M+H]⁺), calculated for C₂₀H₂₆N₂O₅: 374.18 [M].

6-(4-((tert-Butoxycarbonyl)amino)butoxy)quinoline-4-carboxylic acid (8, Boc-Chino-COOH)

Boc-Chino-COOMe **7** (3.34 g, 8.92 mmol, 1.00 eq) was dissolved in 1,4-dioxane (40 mL) and 1 M LiOH (17.8 mL, 17.8 mmol, 2.00 eq) was added and stirred for 4 hours at RT. The organic solvent was removed *in vacuo* and the solution was adjusted to pH = 3.5 with 1 M HCl. The aqueous solution was extracted with EA (8×80 mL), the organic phase was dried over Na₂SO₄ and the

solvent was removed under reduced pressure. Boc-Chino-COOH **8** (1.82 g, 5.05 mmol, 57%) was obtained as a yellowish solid. $^1\text{H-NMR}$ (400 MHz, DMSO- d_6): δ [ppm] = 8.86 (d, J = 4.5 Hz, 1H), 8.15 (d, J = 2.8 Hz, 1H), 8.02 (d, J = 9.3 Hz, 1H), 7.92 (d, J = 4.4 Hz, 1H), 7.49 (dd, J = 9.2 Hz, 2.8 Hz, 1H), 6.87 (t, J = 5.8 Hz, 1H), 4.10 (t, J = 6.3 Hz, 2H), 3.00 (q, J = 6.6 Hz, 2H), 1.78 (q, J = 11.8, 6.5 Hz, 2H), 1.62 - 1.51 (m, 2H), 1.37 (s, 9H). MS (ESI $^+$): m/z (%) = 261.1 (20, [M-Boc+H] $^+$), 361.2 (100, [M+H] $^+$), 362.2 (22, [M+H] $^+$), calculated for $\text{C}_{19}\text{H}_{24}\text{N}_2\text{O}_5$: 360.17 [M].

1-(tert-Butyl) 2-methyl (S)-4-oxopyrrolidine-1,2-dicarboxylate (10, Boc-ProO-COOMe)

N-Boc-*trans*-4-hydroxy-L-proline methyl ester **9** (5.00 g, 20.4 mmol, 1.00 eq) was dissolved in dry dichloromethane (DCM, 20 mL) under argon atmosphere. At first trichloroisocyanuric acid (TCCA, 5.00 g, 21.5 mmol, 1.05 eq) and then (2,2,6,6-tetramethylpiperidin-1-yl)oxyl (TEMPO, 32.0 mg, 205 μmol , 0.01 eq) was added at 0 $^\circ\text{C}$. After 15 minutes at 0 $^\circ\text{C}$ it was allowed to warm to RT and was stirred at RT for another 45 minutes before it was filtered over Celite $^\circ$. The filtrate was washed with saturated Na_2CO_3 (10 mL). The aqueous phase was extracted with DCM (2x20 mL). The combined organic phases were washed with brine (20 mL) and dried over Na_2SO_4 before the solvent was removed *in vacuo*. Column chromatography (CH/EA, 5:1–1:1) yielded Boc-ProO-COOMe **10** (4.30 g, 17.7 mmol, 87%) as a yellowish oil. $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ [ppm] = 4.75 (dd, J = 39.3, 10.5 Hz, 1H), 3.91 – 3.84 (m, 2H), 3.75 (s, 3H), 2.90 (dd, J = 17.7, 10.7 Hz, 1H), 2.57 (dd, J = 18.8, 2.7 Hz, 1H), 1.45 (d, J = 7.7 Hz, 9H). MS (ESI $^+$): m/z (%) = 188.1 (100, [M- ^tBu +H] $^+$), 189.1 (7, [M- ^tBu +H] $^+$), calculated for $\text{C}_{11}\text{H}_{19}\text{NO}_5$: 243.11 [M].

1-(tert-butyl) 2-methyl (S)-4,4-difluoropyrrolidine-1,2-dicarboxylate (11, Boc-Pro-COOMe)

Boc-ProO-COOMe **10** (2.10 g, 8.63 mmol, 1.00 eq) was dissolved in dry DCM (10 mL) under argon atmosphere. Diethylaminosulfur trifluoride (DAST, 1.71 mL, 13.0 mmol, 1.50 eq) dissolved in dry DCM (10 mL) was added and the reaction solution was stirred at room temperature for two days. More DAST (0.57 mL, 4.32 mmol, 0.50 eq) was added after one day. The solution was then washed with 1 M Na_2CO_3 (25 mL). The organic phase was separated and the aqueous phase was extracted with DCM (5x 20 mL). The combined organic phases were washed with a brine (15 mL), dried over Na_2SO_4 and then completely concentrated under reduced pressure. After subsequent purification via column chromatography (CH/EA, 10:1) Boc-Pro-COOMe **11** was obtained as a yellow oil (1.85 g, 6.96 mmol, 81%). $^1\text{H-NMR}$ (400 MHz, CDCl_3): δ [ppm] = 4.49 (ddd, J = 39.8, 9.1, 5.3 Hz, 1H), 3.95 – 3.72 (m, 5H), 2.79 – 2.59 (m, 1H), 2.53 – 2.37 (m, 1H), 1.49 – 1.35 (m, 9H). $^{19}\text{F-NMR}$ (400 MHz, CDCl_3 , no ^1H -decoupling): δ [ppm] = -99.90 (p, J = 13.3 Hz, 1F), -100.07 (p, J = 14.5 Hz, 0.2 F), -100.70 (p, J = 14.3 Hz, 0.4F), -101.37 (p, J = 12.1 Hz, 0.4F), -101.99 (p, J = 12.2 Hz, 0.2F). MS (ESI $^+$): m/z (%) = 166.1 (100, [M-Boc+H] $^+$), 167.0 (6, [M-Boc+H] $^+$), 210.1 (75, [M- ^tBu +H] $^+$), 211.0 (6, [M- ^tBu +H] $^+$), calculated for $\text{C}_{11}\text{H}_{17}\text{F}_2\text{NO}_4$: 265.11 [M].

Methyl (S)-4,4-difluoropyrrolidine-2-carboxylate (12, Pro-COOMe)

Boc-Pro-COOMe **11** (1.82 g, 6.86 mmol, 1.00 eq) was dissolved in DCM (12 mL) and TFA (4 mL). The solution was stirred for 4 hours at RT. The solvents were removed via co-distillation with DCM (3x 20 mL) and MeOH (3x 15 mL). The obtained yellow oil was used for the next step without further purification. ¹H-NMR (400 MHz, MeOD): δ [ppm] = 4.82 (t, *J* = 8.7 Hz, 1H), 3.89 (s, 3H), 3.87 – 3.78 (m, 2H), 3.05 – 2.91 (m, 1H), 2.86 – 2.71 (m, 1H). MS (ESI⁺): *m/z* (%) = 166.1 (100, [M+H]⁺), 167.1 (6, [M+H]⁺), calculated for C₆H₉F₂NO₂: 165.06 [M].

Methyl (S)-1-((tert-butoxycarbonyl)glycyl)-4,4-difluoropyrrolidine-2-carboxylate (13, Boc-Gly-Pro-COOMe)

Boc-Gly-OH (1.45 g, 8.30 mmol, 1.11 eq), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 3.50 g, 9.23 mmol, 1.23 eq), and 1-hydroxybenzotriazole (HOBt, 1.25 g, 9.23 mmol, 1.23 eq) and *N,N*-diisopropylethylamine (DIPEA, 1.53 mL, 1.16 g, 8.99 mmol, 1.20 eq) were dissolved in dry DCM (15 mL) and dry DMF (30 mL) under argon atmosphere. The solution was stirred for one hour at RT. A solution of Boc-Pro-COOMe **12** (6.86 mmol, 1.00 eq) and DIPEA (3.18 mL, 2.42 g, 18.72 mmol, 2.50 eq) in dry DCM (3 mL) and dry DMF (6 mL) was added in portions over 30 minutes. After 17 hours at RT, the solvents were removed under reduced pressure and the residue was taken up in water (20 mL). The aqueous phase was extracted with dichloromethane (3x 20 mL). The organic phase was washed with brine (10 mL) and dried over Na₂SO₄ and DCM was removed under reduced pressure. Since the first column chromatography purification with DCM/MeOH (100:1–2) did not yield a clean product, a second column chromatography with *n*-hexane/EA (3:2) was carried out. Boc-Gly-Pro-COOMe **13** (1.55 g, 4.68 mmol, 68%) was obtained as a colorless oil. ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 5.36 (s, 1H), 4.79 – 4.65 (m, 1H), 4.09 – 3.67 (m, 7H), 2.93 – 2.43 (m, 2H), 1.44 (s, 9H). MS (ESI⁺): *m/z* (%) = 223.1 (38, [M–Boc+H]⁺), 267.1 (100, [M–^tBu+H]⁺), 268.1 (11, [M–^tBu+H]⁺), calculated for C₁₃H₂₀F₂N₂O₅: 322.13[M].

Methyl (S)-4,4-difluoro-1-glycylpyrrolidine-2-carboxylate (14, Gly-Pro-COOMe)

Boc-Gly-Pro-COOMe **13** (1.29 g, 4.05 mmol, 1.00 eq) was dissolved in DCM (6 mL) and TFA (2 mL) and stirred at RT for 4 hours. More TFA (0.6 mL) was added and after one more hour the solvents were completely removed *in vacuo* via co-distillation with DCM (3x 20 mL) and MeOH (2x 20 mL). The obtained yellow oil was used for the next step without further purification. ¹H NMR (400 MHz, MeOD) δ = 5.03 – 4.92 (m, 0.3H), 4.81 (dd, *J* = 9.7, 4.4 Hz, 0.7H), 4.09 – 3.92 (m, 4H), 3.83 – 3.72 (m, 3H), 3.01 – 2.51 (m, 2H). MS (ESI⁺): *m/z* (%) = 223.1 (100, [M+H]⁺), 224.1 (10, [M+H]⁺), calculated for C₈H₁₂F₂N₂O₃: 222.08 [M].

Methyl (S)-1-((6-(4-((tert-butoxycarbonyl)amino)butoxy)quinoline-4-carbonyl)glycyl)-4,4-difluoropyrrolidine-2-carboxylate (15, Boc-FAPs-COOMe)

Boc-Chino-COOH **8** (1.46 g, 4.05 mmol, 1.00 eq) and 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxide hexafluorophosphate (HATU, 1.85 g, 4.86 mmol, 1.20 eq) were dissolved in dry DMF (20 mL) and DIPEA (867 μ L, 5.10 mmol, 1.26 eq) was added. The solution was stirred for 90 minutes under argon atmosphere at RT. A solution of Gly-Pro-COOMe **14** (4.05 mmol, 1.00 eq) and DIPEA (1.40 mL 8.23 mmol, 2.03 eq) in DMF (8 mL) was added and stirred for another two hours. DMF was removed *in vacuo* and the residue was taken up in EA (75 mL). The organic phase was washed 1 M citric acid (15 mL) and brine (15 mL). The aqueous phase was extracted with more EA (3x15 mL). The combined organic phases were then dried over Na₂SO₄ and after the solvent was evaporated under reduced pressure column chromatography (CHCl₃/MeOH, 100:2–5) yielded Boc-FAPs-COOMe **15** as a colorless solid (1.03 g, 1.82 mmol, 45%). ¹H NMR (400 MHz, MeOD) δ = 8.73 (d, *J* = 4.4 Hz, 1H), 7.96 (dd, *J* = 9.3, 2.3 Hz, 1H), 7.85 (dd, *J* = 4.6, 2.7 Hz, 1H), 7.57 (d, *J* = 4.5 Hz, 1H), 7.48 – 7.40 (m, 1H), 5.17 (dd, *J* = 9.7, 2.2 Hz, 0.3H), 4.82 (dd, *J* = 9.7, 4.6 Hz, 0.7H), 4.48 – 3.97 (m, 6H), 3.85 (s, 0.8H), 3.76 (s, 2.2H), 3.17 – 3.10 (m, 2H), 3.05 – 2.77 (m, 1H), 2.64 – 2.49 (m, 1H), 1.92 – 1.82 (m, 2H), 1.74 – 1.63 (m, 2H), 1.43 (s, 9H). MS (ESI⁺): *m/z* (%) = 565.3 (100, [M+H]⁺), 566.3 (28, [M+H]⁺), calculated for C₂₇H₃₄F₂N₄O₇: 564.24 [M].

(S)-1-((6-(4-((tert-Butoxycarbonyl)amino)butoxy)quinoline-4-carbonyl)glycyl)-4,4-difluoropyrrolidine-2-carboxylic acid (16, Boc-FAPs-COOH)

Boc-FAPs-COOMe **15** (1.03 g, 1.82 mmol, 1.00 eq) was dissolved in 1,4-dioxane (30 mL) and 1 M LiOH (7.20 mL, 7.20 mmol, 3.96 eq) and stirred at RT for 3 hours. 1,4-Dioxane was removed and water (10 mL) was added. The aqueous solution was adjusted to pH = 3.5 with 1 M HCl and extracted with EA (3x 15 mL). The organic phase was dried over Na₂SO₄ and EA was completely removed *in vacuo* to obtain Boc-FAPs-COOH **16** (853 mg, 1.55 mmol, 85%) as a yellowish solid. ¹H NMR (400 MHz, MeOD) δ = 8.74 (d, *J* = 4.5 Hz, 1H), 7.96 (dd, *J* = 9.3, 2.7 Hz, 1H), 7.86 (dd, *J* = 16.1, 2.8 Hz, 1H), 7.58 (dd, *J* = 4.5, 1.7 Hz, 1H), 7.46 (ddd, *J* = 9.3, 4.0, 2.7 Hz, 1H), 5.14 – 5.03 (m, 0H), 4.80 (dd, *J* = 9.8, 4.3 Hz, 1H), 4.52 – 3.78 (m, 6H), 3.18 – 3.10 (m, 2H), 3.07 – 2.51 (m, 2H), 1.93 – 1.81 (m, 2H), 1.75 – 1.62 (m, 2H), 1.43 (s, 9H). MS (ESI⁺): *m/z* (%) = 551.2 (100, [M+H]⁺), 552.2 (30, [M+H]⁺), calculated for C₂₆H₃₂F₂N₄O₇: 550.22 [M].

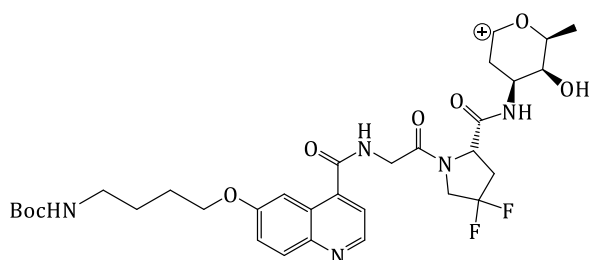
4.2.2. Synthesis of FAPs.cytostatic

(*S*)-1-((6-(4-((*tert*-Butoxycarbonyl)amino)butoxy)quinoline-4-carbonyl)glycyl)-4,4-difluoropyrrolidine-2-carboxylic acid (**17**, FAPs-COOH)

Boc-FAPs-COOH **16** (65.0 mg, 118 μ mol, 1.00 eq) was dissolved in TFA (760 μ L), triisopropyl silane (TIPS, 20 μ L) and MilliQ[®] (20 μ L) and stirred at RT for 45 minutes. The solvents were removed via co-distillation with MeOH (3x 10 mL). The crude product was purified via semipreparative RP-HPLC (10% MeCN isocratic, t_R = 15-16 min) and FAPs-COOH **17** (40.3 mg, 89.5 μ mol, 76%) was obtained as a yellow solid (99.5% purity: cf. analytical RP-HPLC in Figure S1). MS (ESI⁺): m/z (%) = 226.1 (23, [M+H]²⁺), 451.2 (100, [M+H]⁺), 452.2 (24, [M+H]⁺), 901.3 (58, [2M+H]⁺), 902.3 (27, [2M+H]⁺), calculated for C₂₆H₃₂F₂N₄O₇: 550.22 [M].

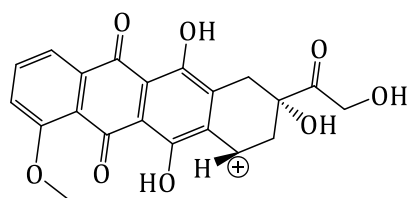
tert-Butyl 4-((4-((2-((*S*)-4,4-difluoro-2-(((2*S*,3*S*,4*S*,6*R*)-3-hydroxy-2-methyl-6-(((1*S*,3*S*)-3,5,12-trihydroxy-3-(2-hydroxyacetyl)-6,11-dioxo-1,2,3,4,6,11-hexahydrotetracen-1-yl)oxy)tetrahydro-2*H*-pyran-4-yl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)carbamate (**18**, Boc-FAPs.Dox)

Boc-FAPs-COOH **16** (46.5 mg, 84.5 μ mol, 1.00 eq), HATU (40.5 mg, 107 μ mol, 1.26 eq) and DIPEA (21.6 μ L, 127 μ mol, 1.50 eq) were dissolved in dry DMF (2 mL) and stirred for two hours at RT under argon atmosphere. A solution of Dox*HCl (55.0 mg, 94.8 μ mol, 1.12 eq) and DIPEA (40.5 μ L, 238 μ mol, 2.82 eq) in dry DMF (5 mL) was added. The reaction was stirred in the dark for a total of two days where additional HATU (33.0 mg, 86.7 μ mol, 1.03 eq) was added after one day and the temperature was slightly increased to 35 °C during at the second day. Purification via column chromatography (CHCl₃/MeOH, 10:1) yielded Boc-FAPs-Dox **18** (86.0 mg, 79.9 μ mol, 95%) as a red solid. LC-MS (ESI⁺): m/z (%) = 562.3 (85, [F1-Boc]⁺), 563.3 (26, [F1-Boc]⁺), 606.3 (14, [F1-*t*Bu]⁺), 662.3 (100, [F1]⁺), 663.3 (38, [F1]⁺), calculated for C₅₂H₅₇F₂N₅O₁₆: 1075.39 [M] and [C₃₂H₄₂F₂N₅O₈]⁺: 662.30 [F1]⁺ which refers to the following fragment [F1]⁺:



6-(4-Aminobutoxy)-N-(2-((S)-4,4-difluoro-2-(((2S,3S,4S,6R)-3-hydroxy-2-methyl-6-(((1S,3S)-3,5,12-trihydroxy-3-(2-hydroxyacetyl)-10-methoxy-6,11-dioxo-1,2,3,4,6,11-hexahydrotetracen-1-yl)oxy)tetrahydro-2H-pyran-4-yl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide (**19**, FAPs.Dox)

Boc-FAPs-Dox **18** (86.0 mg, 79.9 μmol , 1.00 eq) was dissolved in dry DCM (3 mL) and TFA (750 μL) and stirred at RT for six hours. After co-distillation with DCM (3x10 mL) and removal of the solvent, the crude product was purified via semipreparative RP-HPLC (31% MeCN isocratic, t_R = 12.0-12.5 min) and FAPs.Dox **19** (3.5 mg, 3.6 μmol , 5%) was obtained as a red solid (99.9% purity: cf. analytical RP-HPLC in Figure S2). LC-MS (ESI⁺): m/z (%) = 397.1 (100, [F2]⁺), 398.1 (21, [F2]⁺), calculated for C₄₉H₆₇F₂N₁₁O₁₄: 1071.48 [M] and [C₂₁H₁₇O₈]⁺: 397.09 [F2]⁺ which refers to the following fragment [F]⁺:



tert-Butyl 4-((4-((2-((S)-4,4-difluoro-2-(((S)-1-(((S)-1-(((2S,3S,5S)-1-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-2-yl)(methyl)amino)-3-methyl-1-oxobutan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)(methyl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)carbamate (**20**, Boc-FAPs.MMAE)

Boc-FAPs-COOH **16** (46.5 mg, 84.5 μmol , 1.00 eq), HATU (40.5 mg, 107 μmol , 1.26 eq) and DIPEA (21.6 μL , 127 μmol , 1.50 eq) were dissolved in dry DMF (2 mL) and stirred for 90 minutes at RT under argon atmosphere. A solution of MMAE (64.6 mg, 90.0 μmol , 1.07 eq) and DIPEA (38.2 μL , 225 μmol , 2.66 eq) in dry DMF (1.5 mL) was added. The reaction was stirred at RT for one day before additional HATU (33.0 mg, 86.7 μmol , 1.03 eq) was added and the stirring was continued at 40 °C for two more days. HATU (33.0 mg, 86.7 μmol , 1.03 eq) and DIPEA (14.8 μL , 87.0 μmol , 1.03 eq) were added and after two more days at 40 °C the solvents were removed *in vacuo*. After column chromatography (CHCl₃/MeOH, 100:3–6) the product Boc-FAPs.MMAE **20** (44.0 mg, 35.2 μmol , 42%) was obtained as an orange solid. LC-MS (ESI⁺): m/z (%) = 575.9 (100, [M–Boc+H]²⁺), 576.4 (70, [M–Boc+H]²⁺), 1250.6 (7, [M+H]⁺), 1251.6 (5, [M+H]⁺), calculated for C₆₅H₉₇F₂N₉O₁₃: 1249.72 [M].

6-(4-Aminobutoxy)-N-(2-((S)-4,4-difluoro-2-(((S)-1-(((S)-1-(((2S,3S,5S)-1-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-2-yl)(methyl)amino)-3-methyl-1-oxobutan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)(methyl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide (**21**, FAPs.MMAE)

Boc-FAPs.MMAE **20** (44.0 mg, 35.2 μ mol, 1.00 eq) was dissolved in dry DCM (2 mL) and TFA (500 μ L) and stirred at RT for one hour. After removal of the solvents and subsequent purification via semipreparative RP-HPLC (with Licrospher® 100 RP18 EC column, 38% MeCN for 8 min + 40% MeCN for 7 min, t_R = 10-11 min) and FAPs.MMAE **21** (26.6 mg, 23.1 μ mol, 66%) was obtained as a yellow solid (98.7% purity: cf. analytical RP-HPLC in Figure S3). LC-MS (ESI⁺): m/z (%) = 384.4 (24, [M+H]²⁺), 575.9 (100, [M+H]²⁺), 576.4 (64, [M+H]²⁺), 1150.6 (6, [M+H]⁺), 1150.6 (4, [M+H]⁺), calculated for C₄₉H₆₇F₂N₁₁O₁₄: 1071.48 [M].

tert-Butyl (S)-(4-((4-((2-((4,4-difluoro-2-((4-(8-fluoro-1-oxo-2,3,4,6-tetrahydro-1H-azepino[5,4,3-cd]indol-5-yl)benzyl)(methyl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)carbamate (**22**, Boc-FAPs.Rucaparib)

Boc-FAPs-COOH **16** (30.0 mg, 54.5 μ mol, 1.00 eq), HATU (25.5 mg, 67.1 μ mol, 1.23 eq) and DIPEA (12.2 μ L, 71.7 μ mol, 1.32 eq) were dissolved in dry DMF (1 mL) and stirred for 60 minutes at RT under argon atmosphere. A solution of Rucaparib*H₃PO₄ (20.0 mg, 47.5 μ mol, 0.87 eq) and DIPEA (19.0 μ L, 112 μ mol, 2.06 eq) in dry DMF (2 mL) was added. The reaction was stirred at RT overnight and more HATU (25.0 mg, 65.7 μ mol, 1.21 eq) was added. After another three hours at RT the solvents were removed under reduced pressure. After column chromatography (DCM/MeOH, 100:5–10) the product Boc-FAPs.Rucaparib **22** (25.0 mg, 29.2 μ mol, 54%) was obtained as a yellow solid. LC-MS (ESI⁺): m/z (%) = 856.3 (100, [M+H]⁺), 857.3 (50, [M+H]⁺), 858.3 (15, [M+H]⁺), calculated for C₄₅H₄₈F₂N₇O₇: 855.36 [M]

(S)-6-(4-Aminobutoxy)-N-(2-(4,4-difluoro-2-((4-(8-fluoro-1-oxo-2,3,4,6-tetrahydro-1H-azepino[5,4,3-cd]indol-5-yl)benzyl)(methyl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide (**23**, FAPs.Rucaparib)

Boc-FAPs.Rucaparib **22** (25.0 mg, 29.2 μ mol, 1.00 eq) was dissolved in DCM (3 mL) and TFA (1 mL) and stirred for three hours at RT. The solvents were removed via co-distillation with DCM (5x 10 mL) and MeOH (5x 10 mL) The crude product was purified via semipreparative RP-HPLC (26% MeCN isocratic, t_R = 13 min) FAPs.Rucaparib **23** (9.0 mg, 11.9 μ mol, 41%) was obtained as a yellow-orange solid after lyophilization (98.7% purity: cf. analytical RP-HPLC in Figure S4). LC-MS (ESI⁺): m/z (%) = 378.7 (100, [M+H]²⁺), 756.3 (23, [M+H]⁺), calculated for C₄₀H₄₀F₂N₇O₅: 755.30 [M].

4.2.3. Synthesis of FAPi.SA.FAPs.PABC.cytostatic

(S)-N-(2-(2-Cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)-6-(4-((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)amino)butoxy)quinoline-4-carboxamide (24, FAPi.SA)

FAPi-NH₂*TFA (131 mg, 241 μmol, 1.50 eq) was dissolved in ethanol (EtOH, 5 mL) and both 3,4-diethoxy-3-cyclobutene-1,2-dione (SA-diethylester, 37.0 mg, 32.2 μL, 217 μmol, 0.90 eq) and triethylamine (TEA, 50.0 μL, 362 μmol, 1.50 eq) was added. After 5 hours, more TEA (30.0 μL, 216 μmol, 0.90 eq) was added. It was stirred at RT overnight, TEA (20.0 μL, 144 μmol, 0.60 eq) was added once more and after another day at RT the solvents were removed under reduced pressure. FAPi.SA **24** was obtained after column chromatography (DCM/MeOH, 100:5–7) as a yellowish solid (118 mg, 212 μmol, 88%). ¹H-NMR (400 MHz, DMSO-d₆): δ [ppm] = 9.10 (t, *J* = 6.0 Hz, 1H), 8.83 (d, *J* = 4.4 Hz, 1H), 8.00 (d, *J* = 9.2 Hz, 1H), 7.89 (d, *J* = 2.8 Hz, 1H), 7.54 (d, *J* = 4.4 Hz, 1H), 7.47 (dd, *J* = 9.2, 2.7 Hz, 1H), 5.13 (d, *J* = 9.2 Hz, 1H), 4.63 (p, *J* = 6.8 Hz, 2H), 4.39–4.06 (m, 6H), 3.57 (q, *J* = 6.6 Hz, 1H), 3.39 (q, *J* = 6.5 Hz, 1H), 3.02–2.76 (m, 2H), 1.89–1.67 (m, 4H), 1.40–1.27 (m, 3H). MS (ESI⁺): *m/z* (%) = 556.2 (100, [M+H]⁺), 557.2 (31, [M+H]⁺), calculated for C₂₇H₂₇F₂N₅O₆: 555.19 [M].

(S)-1-((6-(4-((2-((4-((2-((S)-2-Cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)butoxy)quinoline-4-carbonyl)glycyl)-4,4-difluoropyrrolidine-2-carboxylic acid (25, FAPi.SA.FAPs-COOH)

c1) FAPs-COOH **17** (91.9 mg, 204 μmol, 1.00 eq) was taken up in ethanol (EtOH, 1 mL) and added to FAPi.SA (118 mg, 212 μmol, 1.04 eq) which was suspended in MeOH (2 mL) and EtOH (2 mL) and TEA (100 μL, 721 μmol, 3.53 eq). The reaction was stirred at RT overnight. The solvents were removed and taken up in MeCN (4 mL), MeOH (4 mL) and TEA (200 μL, 1.44 mmol, 7.06 eq) and heated at 40 °C for one day. No product could be observed at any point but the reactants were still detectable via TLC and LC/MS. More TEA (1 mL, 7.21 mmol, 35.3 eq) was added and the reaction was heated at 70 °C for 2 days. Neither the reactants nor any product could be observed at this point and the reaction was discontinued.

c2) FAPs-COOH **17** (9.0 mg, 20.0 μmol, 1.00 eq) was dissolved 0.5 M phosphate buffer (pH = 10.0) and FAPi.SA (11.1 mg, 20.0 μmol, 1.00 eq) was added. The reaction was shaken at 40 °C for 7 days and was discontinued because no product was detectable via TLC and LC/MS.

tert-Butyl (S)-4-((4-((2-(4,4-difluoro-2-((4-(hydroxymethyl)phenyl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)carbamate (26, Boc-FAPs.PAB-OH)

Boc-FAPs-COOH **16** (110.0 mg, 200 μmol, 1.00 eq) and TEA (41.6 μL, 300 μmol, 1.50 eq) was dissolved in dry DCM (3mL) and dry DMF (1 mL) under argon atmosphere. At 0 °C, ethyl chloroformate (ECF, 28.7 μL, 300 μmol, 1.50 eq) was added. After one hour a solution of *para*-

aminobenzyl alcohol (PAB-OH, 38.0 mg, 309 μ mol, 1.55 eq) and TEA (41.6 μ L, 300 μ mol, 1.50 eq) in dry DCM/DMF (3:1, 2 mL) was added. The reaction was allowed to slowly warm to RT and stirring continued overnight. The addition of ECF (9.6 μ L, 100 μ mol, 0.50 eq), TEA (13.9 μ L, 100 μ mol, 0.50 eq) and PAB-OH (12.3 mg, 100 μ mol, 0.50 eq) with more TEA (13.9 μ L, 100 μ mol, 0.50 eq) one hour later was repeated. After another 5 hours at RT the solvents were removed under reduced pressure and subsequent column chromatography (CHCl₃/MeOH, 100:5–7.5) yielded Boc-FAPs.PAB-OH **26** (130 mg, 198 μ mol, 99%) as a yellow solid. ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 9.27 (s, 1H), 8.74 (d, *J* = 4.4 Hz, 1H), 8.00 (d, *J* = 9.2 Hz, 1H), 7.62 (s, 1H), 7.57 – 7.27 (m, 5H), 7.25 – 7.12 (m, 2H), 4.99 – 4.91 (m, 1H), 4.79 – 4.69 (m, 1H), 4.59 (s, 2H), 4.53 – 4.43 (m, 1H), 4.25 – 3.96 (m, 5H), 3.23 – 2.64 (m, 4H), 1.79 – 1.74 (m, 2H), 1.65 – 1.58 (m, 2H), 1.45 (s, 9H), 1.25 (s, 1H). MS (ESI⁺): *m/z* (%) = 556.3 (15, [M–Boc+H]⁺), 600.3 (15, [M–*t*Bu+H]⁺), 656.3 (100, [M+H]⁺), 657.2 (14, [M+H]⁺), calculated for C₃₃H₃₉F₂N₅O₇: 655.28 [M].

(S)-6-(4-Aminobutoxy)-*N*-(2-(4,4-difluoro-2-((4-(hydroxymethyl)phenyl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide (**27**, FAPs.PAB-OH)

Boc-FAPs.PAB-OH **26** (13.1 mg, 20.0 μ mol, 1.00 eq) was dissolved in dry DCM (1.5 mL) and TFA (500 μ L) and stirred at RT for 90 minutes. The solvents were removed *in vacuo* and the yellow oil was used without further purification. MS (ESI⁺): *m/z* (%) = 278.5 (100, [M+H]²⁺), 556.2 (34, [M+H]⁺), calculated for C₂₈H₃₁F₂N₅O₅: 555.23 [M].

N-(2-((*S*)-2-Cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)-6-(4-((2-((4-((2-((*S*)-4,4-difluoro-2-((4-(hydroxymethyl)phenyl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)butoxy)quinoline-4-carboxamide (**28**, FAPi.SA.FAPs.PAB-OH)

f1) FAPs.PAB-OH **27** (20.0 μ mol, 1.00 eq), FAPi.SA **24** (11.1 mg, 20.0 μ mol, 1.00 eq) which was and TEA (50 μ L, 360 μ mol, 18.0 eq) was dissolved in dry DMF (1 mL) and the solution was heated at 40 °C under argon atmosphere for on days. The temperature was increased in 10 °C steps every day until 80 °C were reached after 5 days and more TEA (50 μ L, 360 μ mol, 18.0 eq) was added after two days. The heating at continued for another 3 days and the reaction was discontinued.

f2) FAPs.PAB-OH **27** (20.0 μ mol, 1.00 eq), FAPi.SA (11.1 mg, 20.0 μ mol, 1.00 eq) were suspended in 0.5 M phosphate buffer (pH = 10.0). EA (500 μ L) and DMSO (500 μ L) was added to improve solubility but still the reaction mixture was not a clear solution. The reaction was stirred at 40 °C for 3 days and was discontinued after no product was detectable via TLC and LC/MS.

tert-Butyl (S)-4-((4-((2-((4,4-difluoro-2-((4-(((4-nitrophenoxy)carbonyl)oxy)methyl)phenyl)carbamoyl)pyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)carbamate (**29**, Boc-FAPs.PABC-PNP)

Boc-FAPs.PAB-OH **26** (130 mg, 198 μ mol, 1.00 eq), Bis(4-nitrophenyl) carbonate (268 mg, 880 μ mol, 4.44 eq) and DIPEA (112 μ L, 660 μ mol, 3.33 eq) were dissolved in dry DMF (3 mL) and stirred for one day at RT. Bis(4-nitrophenyl) carbonate (134 mg, 440 μ mol, 2.22 eq) and DIPEA (56.1 μ L, 330 μ mol, 1.67 eq) was added again and after another 4 hours at RT the solvent was removed *in vacuo*. First column chromatography (CHCl₃/MeOH, 100:5) did not yield clean product. After a second column chromatography (CHCl₃/MeOH, 100:4–5, longer column) Boc-FAPs.PABC-PNP **29** was obtained as a yellow solid (105 mg, 128 μ mol, 65%). ¹H-NMR (400 MHz, CDCl₃): δ [ppm] = 9.46 (s, 1H), 8.78 (d, *J* = 4.5 Hz, 1H), 8.29 – 8.21 (m, 2H), 8.04 – 7.97 (m, 1H), 7.65 – 7.46 (m, 4H), 7.38 – 7.31 (m, 4H), 7.04 (s, 1H), 5.21 (s, 2H), 5.07 – 4.92 (m, 1H), 4.80 – 4.67 (m, 1H), 4.58 – 4.40 (m, 1H), 4.31 – 3.93 (m, 5H), 3.30 – 3.04 (m, 2H), 2.94 – 2.62 (m, 2H), 1.85 – 1.59 (m, 4H), 1.45 (s, 9H), 1.25 (s, 1H). MS (ESI⁺): *m/z* (%) = 721.3 (11, [M–Boc+H]⁺), 821.3 (100, [M+H]⁺), 822.3 (16, [M+H]⁺), calculated for C₄₀H₄₂F₂N₆O₁₁: 820.29 [M].

4-((S)-1-((6-(4-((*tert*-Butoxycarbonyl)amino)butoxy)quinoline-4-carbonyl)glycyl)-4,4-difluoropyrrolidine-2-carboxamido)benzyl ((2*S*,3*S*,4*S*,6*R*)-3-hydroxy-2-methyl-6-(((1*S*,3*S*)-3,5,12-trihydroxy-3-(2-hydroxyacetyl)-10-methoxy-6,11-dioxo-1,2,3,4,6,11-hexahydrotetracen-1-yl)oxy)tetrahydro-2*H*-pyran-4-yl)carbamate (**30**, Boc-FAPs.PABC.Dox)

Boc-FAPs.PABC-PNP **29** (12.3 mg, 15.0 μ mol, 1.00 eq), Dox*HCl (8.7 mg, 15.0 μ mol, 1.00 eq) and DIPEA (5.1 μ L, 30.0 μ mol, 2.00 eq) was dissolved in dry DMF (1 mL) stirred under argon atmosphere for 4 days at RT. The solvent was removed under reduced pressure and the red precipitate was used without further purification. MS (ESI⁺): *m/z* (%) = 1225.9 (100, [M+H]⁺), 1226.3 (17, [M+H]⁺), calculated for C₆₁H₆₆F₂N₆O₁₉: 1224.44 [M].

4-((S)-1-((6-(4-((2-((4-((2-((S)-2-Cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)butoxy)quinoline-4-carbonyl)glycyl)-4,4-difluoropyrrolidine-2-carboxamido)benzyl ((2*S*,3*S*,4*S*,6*R*)-3-hydroxy-2-methyl-6-(((1*S*,3*S*)-3,5,12-trihydroxy-3-(2-hydroxyacetyl)-6,11-dioxo-1,2,3,4,6,11-hexahydrotetracen-1-yl)oxy)tetrahydro-2*H*-pyran-4-yl)carbamate (**31**, FAPi.SA.FAPs.PABC.Dox)

TFA (925 μ L), TIPS (50 μ L) and MilliQ® water (25 μ L) was added to Boc-FAPs.PABC.Dox **30** and stirred at RT for one hour. After removal of the solvents *in vacuo* via co-distillation with MeOH, the residue was taken up in dry DMF (1.5 mL). TEA (300 μ L) and FAPi.SA **24** (29.0 mg, 52.2 μ mol, 1.71 eq) was added and it was stirred for two days at 40 °C. No product could be isolated and no product formation was observed.

4-((S)-1-((6-(4-((tert-butoxycarbonyl)amino)butoxy)quinoline-4-carbonyl)glycyl)-4,4-difluoropyrrolidine-2-carboxamido)benzyl ((S)-1-(((S)-1-(((3R,4S,5R)-1-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-4-yl)(methyl)amino)-3-methyl-1-oxobutan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)(methyl)carbamate (**32**, Boc-FAPs.PABC.MMAE)

Boc-FAPs.PABC-PNP **29** (25.0 mg, 30.5 μ mol, 1.00 eq), MMAE (35.9 mg, 50.0 μ mol, 1.64 eq) and DIPEA (10.4 μ L, 61.0 μ mol, 2.00 eq) were dissolved in dry DMF (1 mL) and stirred at 40 °C for two days. Then, more DIPEA (5.2 μ L, 30.5 μ mol, 1.00 eq) was added and stirred for another 3 days at 50 °C. The solvent was removed under reduced pressure and residual oil was used without further purification in the next step. MS (ESI⁺): m/z (%) = 650.6 (30, [M-Boc+H]²⁺), 672.5 (19, [M-^tBu+H]²⁺), 700.5 (, [M+H]²⁺), 701.4 (12, [M+H]²⁺), 1400.0 (10, [M+H]⁺), calculated for C₇₃H₁₀₄F₂N₁₀O₁₅: 1398.77 [M].

4-((S)-1-((6-(4-((2-((4-((2-((S)-2-Cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)butoxy)quinoline-4-carbonyl)glycyl)-4,4-difluoropyrrolidine-2-carboxamido)benzyl ((S)-1-(((S)-1-(((3R,4S,5R)-1-((S)-2-((1R,2R)-3-(((1S,2R)-1-hydroxy-1-phenylpropan-2-yl)amino)-1-methoxy-2-methyl-3-oxopropyl)pyrrolidin-1-yl)-3-methoxy-5-methyl-1-oxoheptan-4-yl)(methyl)amino)-3-methyl-1-oxobutan-2-yl)amino)-3-methyl-1-oxobutan-2-yl)(methyl)carbamate (**33**, FAPi.SA.FAPs.PABC.MMAE)

Boc-FAPs.PABC.MMAE **32** was dissolved in TFA (925 μ L), TIPS (50 μ L) and MilliQ® water (25 μ L). The solution was stirred at RT for one hour. The solvents were removed under reduced pressure via co-distillation with MeOH and the residual oil was immediately used in the next step. Dry DMF (1.5 mL), TEA (150 μ L) and FAPi.SA **24** (24.0 mg, 43.2 μ mol, 1.42 eq) was added and after stirring at 50 °C for one day TEA (150 μ L) was added before the temperature was increased to 60 °C. Stirring was continued for two days but no product could be isolated neither was any product formation observed.

(S)-4-(1-((6-(4-((tert-Butoxycarbonyl)amino)butoxy)quinoline-4-carbonyl)glycyl)-4,4-difluoropyrrolidine-2-carboxamido)benzyl (4-(8-fluoro-1-oxo-2,3,4,6-tetrahydro-1H-azepino[5,4,3-cd]indol-5-yl)benzyl)(methyl)carbamate (**34**, Boc-FAPs.PABC.Rucaparib)

Boc-FAPs.PABC-PNP **29** (10.0 mg, 12.2 μ mol, 1.00 eq), Rucaparib*H₃PO₄ (7.2 mg, 16.7 μ mol, 1.37 eq) and DIPEA (4.2 μ L, 24.4 μ mol, 2.00 eq) was stirred overnight under argon atmosphere in dry DMF (1 mL) at RT. The solvent was removed under reduced pressure and the yellow oil was used without further purification. MS (ESI⁺): m/z (%) = 475.2 (60, [M-^tBu+H]²⁺), 1005.3 (100, [M+H]⁺), 1006.4 (66, [M+H]⁺), 1007.2 (10, [M+H]⁺), calculated for C₅₃H₅₅F₃N₈O₉: 1004.40 [M].

4-((S)-1-((6-(4-((2-((4-((2-((S)-2-Cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)carbamoyl)quinolin-6-yl)oxy)butyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)butoxy)quinoline-4-carbonyl)glycyl)-4,4-difluoropyrrolidine-2-carboxamido)benzyl (4-(8-fluoro-1-oxo-2,3,4,6-tetrahydro-1H-azepino[5,4,3-cd]indol-5-yl)benzyl)(methyl)carbamate (**35**, FAPi.SA.FAPs.PABC.Rucaparib)

Boc-FAPs.PABC.Rucaparib **34** was suspended in dry MeCN (2 mL) and 4 M HCl in 1,4-dioxane (100 μ L, 0.4 mmol, 32.8 eq) was added. The solvents were removed under reduced pressure after stirring was carried out at RT for 4 hours and the residual solid was taken up in dry DMF (1 mL), dry MeOH (0.5 mL) and TEA (150 μ L). FAPi.SA **24** (11.1 mg, 20.0 μ mol, 1.64 eq) was added and the solution was shaken at 40 °C for four days. No product **35** could be isolated at subsequent RP-HPLC although some product formation was observed via LC/MS. MS (ESI⁺): m/z (%) = 707.9 (100, [M+H]²⁺), 708.9 (10, [M+H]²⁺), 1414.4 (5, [M+H]⁺), calculated for C₇₃H₆₈F₅N₁₃O₁₂: 1413.50 [M].

4.3. In Vitro Studies

4.3.1. Cleavage of FAPs.cytostatic by FAP and PREP (Short Incubation)

The cleavage of the compounds FAPs.Dox **19**, FAPs.MMAE **21** and FAPs.Rucaparib **23** by FAP and/or PREP was evaluated using an in-house developed UPLC-MRM MS assay published by De Decker et al. [33]. Using this method, the concentration of the cleavage product FAPs-COOH **17** was measured at specific time points (0, 2, 4, 6, 8, 10, 15, 20, 25 and 30 min).

To start, a calibration series using FAPs-COOH **17** was prepared (31.25 nM, 62.5 nM, 125 nM, 250 nM, 500 nM, 750 nM, 1000 nM, 2000 nM, 3000 nM and 4000 nM). The calibration series was prepared in FAP assay buffer (50 mM Tris-HCl (pH = 7.5), 140 mM NaCl) and PREP assay buffer (0.1 M Tris-HCl (pH = 7.5), 1 mM EDTA, 3 mM dithiothreitol (DTT)) for FAP and PREP cleavage assays, respectively.

100 mM stock solutions of the compounds **19**, **21** and **23** were prepared in 100% DMSO. Stock solutions of these compounds were subsequently diluted to a final concentration of 10 μ M in protein LoBind Eppendorf tubes using FAP and PREP assay buffer for the respective FAP and PREP cleavage assay experiments. Next, 7.5 μ L of enzyme (final concentration 4.55 nM FAP or 5.2 nM PREP) or buffer (control for compound autohydrolysis) was added to 142.5 μ L of the 10 μ M FAPs.cytostatic solution. Samples were incubated at 37 °C in a Thermomixer. Subsequently, 10 μ L of sample was taken at 0-, 2-, 4-, 6-, 8-, 10-, 15-, 20-, 25- and 30 minutes. 90 μ L of water with 1% formic acid (HFO) was added to deactivate the enzyme and the samples were stored at -80 °C until analysis. Final DMSO concentration during this assay was kept constant and was 0.5%. For all experiments, LC-MS grade -water with 0.01% HFO, -water and - DMSO were used.

UPLC/MS: Chromatographic separation was performed using a Waters XSselect CSH C18 Column XP (2.5 μ m 2.1x75mm). 3 μ L of sample was injected using a flowrate of 0.3 mL/min. Solvent A consisted of water with 0.05% HFO and solvent B consisted of MeOH. Optimized separation conditions were obtained by using a gradient 3–90% B in 7.5 min, then back to 3% over 2.5 min which was kept constant for 2 min. Detection was consecutively performed by using mass spectrometry in multiple reaction monitoring (MRM) mode (ESI⁺).

4.3.2. FAP Inhibition Properties of FAPs.cytostatic

Initial screening of **19**, **21** and **23** were was performed using our screening method for FAP inhibition. Z-Gly-Pro-7-amino-4-methylcoumarine (AMC, Bachem) was used as the substrate at a concentration of 50 μ M at pH = 8 (0.05 M Tris-HCl buffer with 1 mg/mL BSA and 140 mM NaCl). The compounds were tested at two concentrations (80 and 8000 nM) which is the final concentration in the well (final DMSO concentration = 0.5%). The compounds were pre-incubated with the enzyme for 15 minutes or 24 hours at 37 °C, afterwards the Z-Gly-Pro-AMC substrate was added and the velocities of AMC release were measured kinetically at λ_{ex} = 380 nm, λ_{em} = 465 nm for at least 15 minutes at 37 °C. Measurements were performed on the Infinite 200 from Tecan Group Ltd. (Männedorf, Switzerland) and the Magellan software was used to process the data.

4.3.3. Cleavage of FAPs.cytostatic by FAP and PREP (Long Incubation)

The long incubation experiments were carried out similarly to the short incubation experiments (see above). Only the deviations are listed below.

The concentration of the cleavage product FAPs-COOH **17** was measured at specific time points (0, 1 and 24 hours for **19** and **23**; 0, 1, 4, 8 and 24 hours for **21**).

A calibration was done with FAPs-COOH **17** (8 nM, 16 nM, 31.25 nM, 62.5 nM, 125 nM, 250 nM, 500 nM, 750 nM, 1000 nM, 2000 nM, 3000 nM and 4000 nM) in FAP assay buffer (50 mM Tris-HCl (pH = 7.5), 140 mM NaCl) and PREP assay buffer (0.1 M Tris-HCl (pH = 7.5), 1 mM EDTA, 3 mM dithiothreitol (DTT)).

100 mM stock solutions in pure DMSO were subsequently diluted to 10 μ M or 100 nM final concentration in protein LoBind Eppendorf tubes using FAP and PREP assay buffer. After addition of the enzyme (final concentration 4.55 nM FAP or 5.2 nM PREP) or the assay buffer only samples (10 μ L) were taken at 0, 1 and 24 hours **19** and **23** and at 0, 1, 4, 8 and 24 hours for **21**. The enzyme was deactivated as mentioned above (90 μ L water with 1% HFO, storage at -80 °C until analysis).

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Supporting Information

Organic Synthesis - analytical RP-HPLC

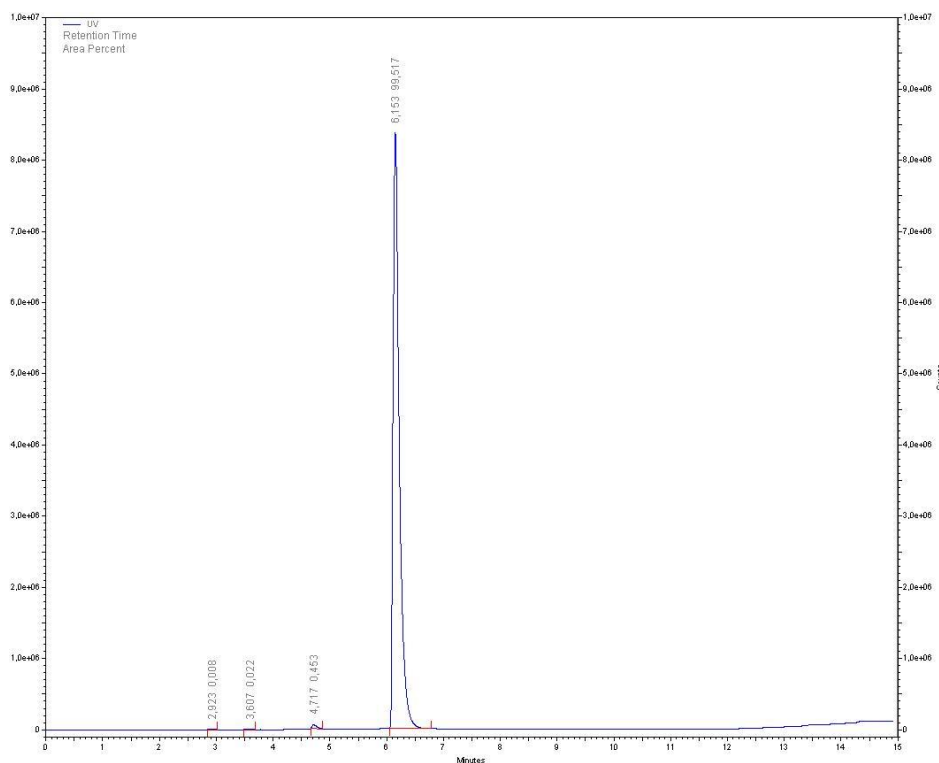


Figure S1: Analytical RP-HPLC of FAPs-COOH **17** (t_R = 6.2 min), 10-50% MeCN + 0.1% TFA (linear gradient) in 10 min, 5 mL/min: purity = 99.5%.

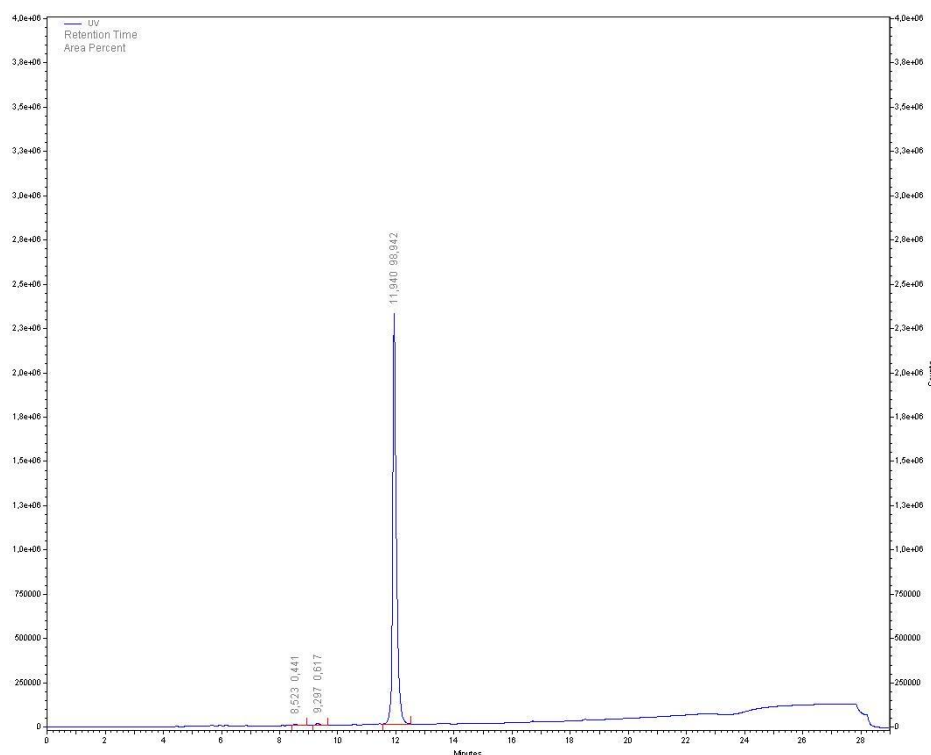


Figure S2: Analytical RP-HPLC of FAPs.Dox **19** (t_R = 11.9 min), 10-90% MeCN + 0.1% TFA (linear gradient) in 20 min, 5 mL/min: purity = 99.9%.

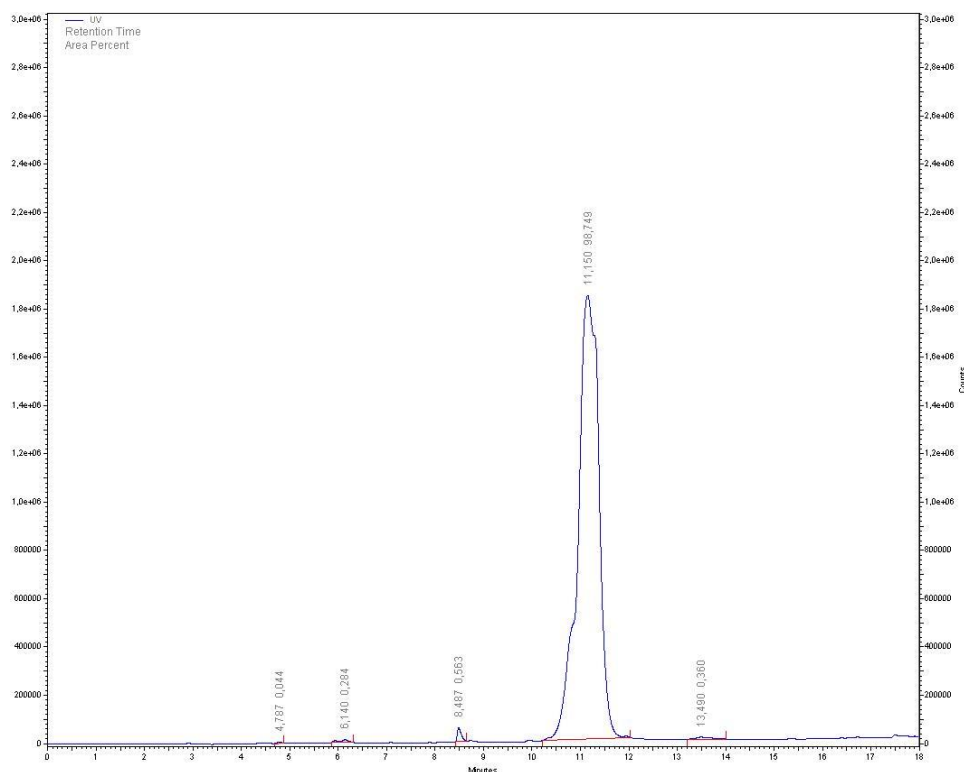


Figure S3: Analytical RP-HPLC of FAPs.MMAE **21** ($t_R = 11.2$ min), 10-90% MeCN + 0.1% TFA (linear gradient) in 18 min, Licrospher® 100 RP18 EC column, 5 mL/min: purity = 98.7%.

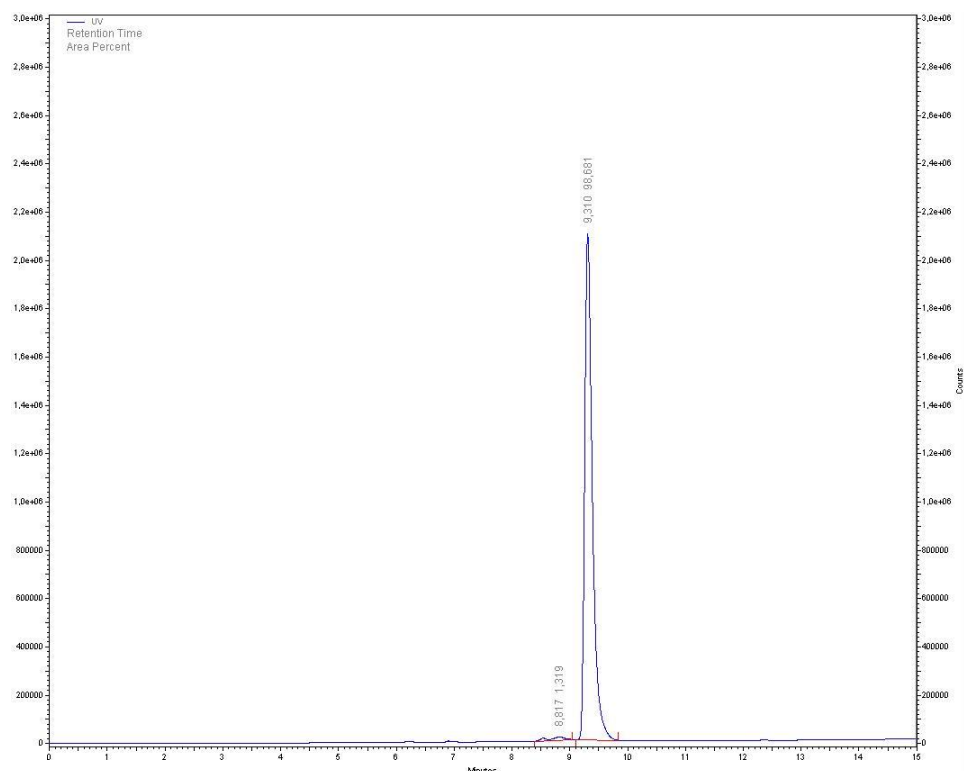


Figure S4: Analytical RP-HPLC of FAPs-COOH **17** ($t_R = 9.3$ min), 10-90% MeCN + 0.1% TFA (linear gradient) in 10 min, 5 mL/min: purity = 98.7%.

6.2. List of abbreviations

%ID/g	Percent injected dose per gram	Bq	Becquerel
4-AN	4-aminonaphthol	B _{Reg}	Regulatory B cells
[¹⁸ F]F-FDG	2-[¹⁸ F]fluoro-2-deoxy-D-glucose	BSA	Bovine serum albumin
[¹⁸ F]FET	<i>O</i> -(2-[¹⁸ F]fluoroethyl)-L-tyrosine	Bz	Benzyl
<i>A</i>	Mass number	(C) _r	Linker group
(A) _p	Linker group	<i>c</i>	Concentration
α-SMA	α-smooth muscle actin	c.a.	Carrier-added
AAZTA ⁵	5-[[6-(<i>N,N'</i> -diacetic acid)amino]-1,4-diacetic acid-1,4-diazepane]-pentanoic acid	CAFs	Cancer-associated fibroblasts
Ac	Acetyl	CAR	chimeric antigen receptor
ADP	Adenine diphosphate	CCL2	CC chemokine ligand 2
Ala	Alanine	cfDNA	Circulating free DNA
AMC	7-amino-4-methylcoumarine	CH	Cyclohexane
AmOAc	Ammonium acetate	Chino	Quinoline
apCAFs	Antigen-presenting CAFs	CL	Central linker
Arg	Arginine	CSC	Cancer stem cells
Asn	Asparagine	CT	Computed tomography
Asp	Aspartate	CXCL12	C-X-C motif chemokine 12 = stromal cell-derived factor 1 (SDF-1)
ATP	Adenine triphosphate	CXR	Cation exchange resin
avg	Average	Cys	Cysteine
(B) _q	Linker group	<i>D</i> _{7.4}	Octanol/water (pH = .74) distribution coefficient
BBN	Bombesine	δ	Chemical shift
BCN	bicyclo[6.1.0]nonyne	Da	Dalton
Boc	<i>tert</i> -butyloxycarbonyl	DAST	Diethylaminosulfur trifluoride
Boc ₂ O	Di- <i>tert</i> -butyldicarbonate	DATA ^{5m}	5-[[6-(<i>N</i> -acetic acid)(<i>N'</i> -methyl)amino]-1,4-diacetic acid-1,4-diazepane]pentanoic acid

DBCO	Dibenzocyclooctin	ECM	Extracellular matrix
DCC	<i>N,N'</i> -Dicyclohexylcarbodiimide	ECs	Endothelial cells
DCM	Dichloromethane	EDC	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
DCU	<i>N,N'</i> -Dicyclohexylurea	EDDA	Ethylenediaminediacetic acid
DFO	Deferoxamine	EDTA	ethylenediaminetetraacetic acid
DIPEA	<i>N,N</i> -diisopropylethylamine	EGF	Endothelial growth factor
DMF	<i>N,N</i> -dimethylformamide	EMA	European Medicines Agency
DMSO	Dimethyl sulfoxide	EMT	Epithelial-mesenchymal transition
DNA	Desoxyribonucleic acid	en	Ethylendiamine
DO3A/ DOTA	1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid	EndMT	Endothelial-mesenchymal transition
DOTAGA	1-glutaric acid-4,7,10-triacetic acid	ERT	Endoradiotherapy
DOTA- GA(^t Bu) ₄	5-(<i>tert</i> -butoxy)-5-oxo-4-(4,7,10-tris(2-(<i>tert</i> -butoxy)-2-oxoethyl)-1,4,7,10-tetra-azacyclododecan-1-yl)pentanoic acid	ESI	Electrospray ionization
DOTA- tri(^t Bu)ester	2-(4,7,10-tris(2-(<i>tert</i> -butoxy)-2-oxoethyl)-1,4,7,10-tetraaza-cyclododecan-1-yl)acetic acid	EtOH	Ethanol
Dox	Doxorubicine	eV	Electron volt
DPP	Dipeptidyl peptidase	EVs	Extracellular vesicles
DTPA	Diethylenetriaminepentaacetic acid	FAP	Fibroblast activation protein alpha
DTT	Dithiothreitol	FAPi	FAP inhibitor
<i>E</i>	Energy	FAPi-NH ₂	6-(4-aminobutoxy)- <i>N</i> -(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide
EA	Ethyl acetate	FAPs	FAP substrate
EB	Evans Blue	FDA	U.S. Food and Drug Administration
EC	Electron capture	FGF	Fibroblast growth factor

Fmoc	Fluorenylmethyloxycarbonyl	IC_{50}	Half-maximal inhibitory concentration
G3	Triglycine	iCAFs	Inflammatory CAFs
Glu	Glumatic acid	IHC	Immunohistochemistry
Gly	Glycine	IL	Interleukin
GRPR	Gastrin-releasing protein receptor	IQR	Interquartile range
GSO	gadolinium oxyorthosilicate	k_{cat}	cleavage rate
Gy	Gray	K_D	Dissociation constant
HATU	1-[bis(dimethylamino)methylene]-1 <i>H</i> -1,2,3-triazolo [4,5- <i>b</i>]pyridinium 3-oxide hexafluorophosphate	K_i	Inhibitory constant
HBED-CC	<i>N,N'</i> -bis[(2-hydroxy-5-propanic acid)benzyl]ethylenediamine- <i>N,N'</i> -diacetic acid	K_m	Michaelis constant
HBTU	2-(1 <i>H</i> -benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate	KuE	Lys-urea-Glu
HEPES	4-(2-hydroxyethyl)-1-piperazineethansulfonic acid	L	Linker
HEGP	high-energy general purpose	LC	Liquid chromatography
HFo	Formic acid	LET	Linear energy transfer
HGF	hematopoietic growth factor	Leu	Leucine
HIF	Hypoxia inducible factor	LoR	Line of response
His	Histidine	LSO	lutetium oxyorthosilicate
HOBt	1-Hydroxybenzotriazole	LU	Labeling unit
HPLC	High-pressure liquid chromatography	Lys	Lysine
HS	Human serum	M	Molecule
HSA	Human serum albumin	<i>m</i>	Mass
hSSTR	human Somatostatin Receptor	MA	Mercapto acetic acid
HYNIC	6-Hydrazinonicotinamide	mAb	Monoclonal antibody
<i>I</i>	Integral	MAG3	mercapto acetyl triglycine

max	Maximum / maximal	OS	Overall survival
MCT	Monocarboxylate transporter	<i>p</i>	Proton
MDSCs	Myeloid-derived suppressor cells	PAB-OH	<i>p</i> -aminobenzyl alcohol
MeCN	Acetonitrile	PABC	<i>p</i> -aminobenzyl carbamate
MEK	Methyl ethyl ketone	PARP	poly (ADP-ribose) polymerase
MeOH	Methanol	PBS	Phosphate-buffered saline
Met	Methionine	PCa	Prostate cancer
MIP	Maximum intensity projection	p.i.	Post-injection
MMAE	Monomethyl auristatin E	PD	Pharmacodynamic
MMP	Matrix metalloproteinase	PD-1	programmed cell death protein 1
MMT	Mesenchymal-mesenchymal transition	PDGF	platelet-derived growth factor
MRI	Magnetic resonance imaging	PD-L1	programmed cell death ligand 1
MS	Mass spectrometry	PEG / PEG _n	Polyethylene glycol
MSC	Mesenchymal stem cells	PEG ₂	Diethylene glycol
myCAFs	Myofibroblast CAFs	PET	Positron emission tomography
<i>N</i> , <i>n</i>	Neutron number	PFS	Progression-free survival
N4	3-((2-aminoethyl)amino)-2-(((2-aminoethyl)amino)methyl)propanoic acid	Ph	Phenyl
ν_e	Electron neutrino	Phe	Phenylalanine
NaOAc	Sodium acetate	PK	Pharmacokinetic
n.c.a.	Non-carrier-added	PKM1/2	pyruvate kinase M1/M2
NET	Neuroendocrine tumors	<i>p</i> NA	<i>p</i> -nitroaniline
NHS	<i>N</i> -hydroxysuccinimide	PNP	(4-nitrophenyl)carbonate
NK	Natural killer cells	ppm	Parts per million
NMR	Nuclear magnetic resonance	PREP	Prolyl endopeptidase
NODAGA	1,4,7-triazacyclononane-1-glutaric acid-4,7-diacetic acid	Pro	Proline
NOTA	1,4,7-Triaazacyclononane-1,4,7-triacetic acid	PSMA	Prostate specific membrane antigen
NPyr	3-Aminopyrrolidine	PTRT	Peptide-Targeted Radionuclide Therapy

Pyr	Pyrrolidine	SPECT	Single photon emission computed tomography
Q	Charge	SSTR	Somatostatin receptor
RCC	Radiochemical conversion	SUL	SUV normalized by lean body mass
RCP	Radiochemical purity	SUV	Standard uptake value
RCY	Radiochemical yield	t	Time
RECIST	Response Evaluation Criteria in Solid Tumors	$t_{1/2}$	Half-life
R_f	Retention factor	TAM	Tumor-associated macrophages
RGD	Arg-Gly-Asp	TAT	Targeted alpha-particle therapy
rhFAP	recombinant human fibroblast activation protein	TATE	D-Phe-cyclo[Cys-Tyr-D-Trp-Lys-Thr-Cys]Thr
RLT	Radioligand therapy	TBR	tumor-to-background ratio
R^n	Side chain	TC	Tumor cell
RNT	Radionuclide therapy	TCO	<i>Trans</i> -cyclooctene
ROS	Reactive oxygen species	TCCA	trichloroisocyanuric acid
RP	Reversed phase	TEA	Triethylamine
RR-DTC	Radioiodine-refractory differentiated thyroid cancer	TEMPO	2,2,6,6-tetramethylpiperidin-1-yl)oxyl
RT	Room temperature	TFA	Trifluoroacetic acid
S	Spacer	TGF	Transforming growth factor
$[S]$	Substrate concentration	T_H	T helper cell
SA	Squaric acid, squaramide	THF	Tetrahydrofuran
SAR	structure-activity relationship	THP	tris(hydroxypyridinone)
Sar	Sarcophagine	TIPS	Triisopropylsilane
SEM	Standard error of the mean	TLC	Thin-layer chromatography
SDDS	Smart Drug Delivery System	TME	Tumor microenvironment
Ser	Serine	TNF- α	Tumor necrosis factor alpha
SI	Selectivity index	TOC	D-Phe-cyclo[Cys-Tyr-D-Trp-Lys-Thr-Cys]Thr(ol)
SNMMI	Society of Nuclear Medicine and Molecular Imaging	ToF	Time-of-flight
SPAAC	strain-promoted alkyne-azide cycloaddition	t_R	Retention time

TRAP	1,4,7-triazacyclononane-1,4,7-tris[methyl(2-carboxyethyl)-phosphinic acid
T _{Reg}	Regulatory B cells
TRIGA	Training, Research, Isotopes, General Atomic
TV	Targeting vector
Tyr	Tyrosine
UAMC-1110	(<i>S</i>)- <i>N</i> -(2-(2-cyano-4,4-difluoropyrrolidin-1-yl)-2-oxoethyl)quinoline-4-carboxamide
UPLC-MRM	ultra performance liquid chromatography in multiple reaction mode
ν	Reaction rate
VAS	Visual analogue scale
VEGF	Vascular endothelial growth factor
VoR	Volume of response
<i>X</i>	Parent (radio)nuclide
<i>Y</i>	Daughter (radio)nuclide
<i>Z</i>	Atomic number or benzyloxycarbonyl
<i>z</i>	Charge

6.3. Danksagung

Ich möchte mich zuallererst bei Prof. Dr. Frank Rösch für die Gelegenheit bedanken die Promotion in Ihrem Arbeitskreis absolviert haben zu dürfen. Herzlichen Dank für die angenehme und stets positive Arbeitsatmosphäre, das super-spannende Thema, an dem ich forschen durfte, sowie die stets hilfreichen fachlichen und organisatorischen Ideen und Anmerkungen in zahlreichen konstruktiven Gesprächen.

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Großer Dank gilt ebenfalls Prof. Pieter Van Der Veken und Prof. Ingrid De Meester sowie Ihren Gruppenmitgliedern, allen voran Yentl Van Rymentant und Emile Verhulst, aber auch Joni De Loose und Sophie Lyssens für die Durchführung der *in vitro* Bindungsaffinitätsstudien.

Auch bedanken möchte ich mich bei Dr. Eleni Gourni für die Arbeit an Zell- und Tierstudien, Prof. Dr. Richard Baum und Lukas sowie dem Team im Curanosticum für die Erfahrungen, die ich dort in der Zusammenarbeit sammeln konnte, sowie Michael Bachmann, Anja Feldmann, Ralf Bergmann und Prof. Dr. Mark Helm für die (finanzielle) Unterstützung während meiner Promotion.

Zuletzt will ich mich noch ganz besonders herzlich bei meiner Familie und meinen Freunden bedanken. Danke dafür, dass Ihr mich jederzeit bedingungslos unterstützt (und wenn nötig gestützt) habt, auch wenn ich keine Zeit hatte oder nicht so viel, wie ich gerne gewollt hätte. Allen voran sind meinen Eltern und Großeltern sowie Alex und Kim zu nennen, da Ihr stets an mich geglaubt habt – und immer noch glaubt. Außerdem vielen Dank Flo, für das offene Ohr und die vielen guten Ratschläge bei unseren abendlichen Unterhaltungen auf dem Balkon. Ohne euch wäre ich bestimmt nicht so weit gekommen. Tausend Dank dafür!

6.4. Eidesstattliche Erklärung

Die vorliegende Arbeit wurde zwischen März 2020 und Mai 2023 unter Anleitung von Prof. Dr. FRANK RÖSCH am Department Chemie – Standort TRIGA (ehemaliges Institut für Kernchemie) angefertigt.

Hiermit versichere ich, MARCEL MARTIN, dass ich diese hier vorliegende Dissertation selbstständig verfasst und alle verwendeten schriftlichen und elektronischen Quellen sowie andere Hilfsmittel angegeben habe. Alle Ausführungen, die anderen Schriften wörtlich oder sinngemäß entnommen wurde, habe ich kenntlich gemacht.

Hiermit versichere ich weiterhin, dass die vorliegende Arbeit nicht für eine andere Prüfung und auch nicht bei einer anderen Fakultät oder Fachbereich eingereicht wurde.

Mainz, den 01. Juni 2023

Marcel Martin

6.5. Curriculum Vitae

Marcel Martin

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Date of Birth: 4 May 1992
Place of Birth: Bingen am Rhein
Nationality: German



Ph.D.

02/2020 – 07/2023 **Scientific associate/Ph.D. student**

Johannes Gutenberg-University Mainz, Department of Chemistry - TRIGA site, research group of Prof. Dr. FRANK ROESCH

“Novel fibroblast activation protein inhibitor (FAPi)-based radiopharmaceuticals for improved theranostic application in various cancer types”

- Organic synthesis of precursors and subsequent radiolabeling optimization with ^{68}Ga , ^{177}Lu , ^{225}Ac , ^{90}Y , ^{44}Sc
- Close translational cooperation with partners in nuclear medicine (University Medical Center Rostock, AIIMS Delhi: optimization of automated radiosynthesis, human *in vivo* studies)
- >50 radiosyntheses for clinical application ($^{177}\text{Lu}/^{68}\text{Ga}$ -PSMA und -DOTATOC)
- Drafted several patents
- Participation in international conferences (poster at 6th TWC 2022, presentation at 54th SNMICON 2022)
- Supervision of 2 master, 4 bachelor theses and 4 research internships
- Supervision of practical course “Organic Chemistry for Pharmacists” for four semesters

Anticipated Disputation: 06 – 07/ 2023

Education and School

10/2017 – 12/2019 **Master of Science Chemistry**

Johannes Gutenberg-University Mainz, Germany (grade: 1.3)

Master thesis: “Squaric acid-based PSMA tracer with EuEK targeting vector and DNP group for use in prostate cancer” (grade: 1.0)

10/2012 – 09/2017 **Bachelor of Science Chemistry**

Johannes Gutenberg-University Mainz, Germany (grade: 1.9)

Bachelor thesis: “Synthesis of aminofallypride and its coupling to DTPA and DFO derivatives” (grade: 1.0)

08/2002 – 03/2011 **General qualification for university entrance (Abitur)**
Stefan-George-Gymnasium, Bingen am Rhein, Germany (grade 2.1)

Internships

08/2018 – 10/2018 **Student Trainee** (Biological Lab Technician) in the group of Prof. Dr. JÖRG VON HAGEN (Department Cosmetics R&D (Active Ingredients) of Merck KGaA, Darmstadt, Germany)

- Cell culture (HaCaT cells)
- Establishing of a Mitochondrial Membrane Potential (MMP) assay
- Screening of >800 plant extracts incl. data analysis

07/2011 Internship as Chemical Lab Technician (Chemical Production/ Production Support and Analytics, Boehringer Ingelheim Pharma GmbH & Co. KG, Ingelheim, Germany)

Further Education

06/2020 GMP Basic Training B1 (Concept Heidelberg GmbH, Germany)

06/2020 GMP Advanced Course B2 (Concept Heidelberg GmbH, Germany)

Skills

Languages	German	Native speaker
	English	Fluent (writing and speaking)
	French	Basic
IT	MS Office	Very good
	Photoshop	Good
	MatLab, LaTeX	Basic
Personal strenghts	Reliability	Radiosyntheses for clinical application
	Attention to detail	Drafting of patents and publications
	Leadership skills	Supervision of numerous theses and students

Personal Interests

Playing guitar and drums
Band (incl. songwriting, organization, gigs)
Cycling

Volunteer Experience

08/2011 – 07/2012 **Voluntary Social Year** at the Elementary School Gensingen, Germany

- Supporting and supervising during lessons
- Private tutoring and homework assistance
- Sports club and support of other clubs