The potential of the human kidney cell line, Caki-1, as an in vitro screening system for drug interactions with organic cation transporters

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“research is discovering God’s secrets”

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Abbreviations

ΔC_T	Change in threshold cycle
Ω cm^2	ohms cm^2
ABC	ATP-Binding Cassette
AJ	Adherent junction
AP	Alkaline phosphatase
APM	Aminopeptidase M
ATCC	American type culture collection
ATP	Adenosine triphosphate
AUC	Area under the curve
$B^{o,+}$	amino acid transporter
BBM	Brush-border membrane
BCA	bicinchoninic acid
BCIP/NBT	5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium
BLM	Basolateral membrane
BLQ	Below the level of quantification
BS	Biosafety
Caco-2	Human **co**lon **ca**rcinoma cell line
Caki-1	Human **ki**dney **ca**rcinoma cell line
CHO-K1	Chinese hamster ovary cell line
cDNA	Complementary DNA
$CL_{filtration}$	Filtration clearance
$CL_{reabsorption}$	Reabsorption clearance
CL_{RENAL}	Renal clearance
$CL_{secretion}$	Secretion clearance
C_{max}	Maximum plasma concentration
C_T	Threshold cycle
CYP450	Cytochrome P450
d	Day
DAPI	4', 6-diamidino-2-phenylindole
DDI	Drug-drug interaction
DME	Drug metabolizing enzyme
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPM	Disintegration per minute
DPP IV	Dipeptidyl peptidase IV
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH
EDTA	Ethylenediaminetetraacetic acid
EMT	Extraneuronal monoamine transporter
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FITC	fluorescein isothiocyanate
f_u	Fraction unbound (free) drug

GF	Glomerular filtration
GFR	Glomerular filtration rate
GGT	Gamma glutamyl transferase
GMNA	L-glutamic acid γ-(4-methoxy-ß-napthylamide)
h	Hour
HBSS	Hank's Balanced Salt Solution
HEK-293	Human embryonic kidney cell line
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HK-2	Human kidney cell line
HPT	Primary human proximal tubule cells
HRPE	Human retinal pigment epithelial cell line
HUGO	The human genome organization
IC_{50}	50% inhibition constant
IgG	Immunoglobulin subtype G
IPK	Isolated perfused kidney
K_i	Inhibition constant
K_m	Michaelis-Menten constant
LLC-PK1	Porcine proximal tubule cell line
MATE	Multi-drug and toxin extrusion (transporter family)
MDCK	Madin Darby canine kidney cell line
MES	2-(*N*-morpholino) ethanesulfonic acid
MFS	Major Facilitator Superfamily
Min	Minute
mM	millimolar
MPP^+	1-methyl-4-phenylpyridinium
mRNA	Messenger RNA
MRPs	Multi-drug resistance-associated protein
N	molar
NaOH	Sodium hydroxide
Na^+/K^+-ATPase	Sodium potassium pump
NHE3	Sodium-proton exchanger subtype 3
NRK-52E	Rat kidney epithelial-like cell line
OA	Organic anion
OAT	Organic anion transporter
OCT	Organic cation transporter
OK	Opossum kidney cell line
OCTN	novel organic cation transporter (subfamily)
PBS	Phosphate buffered saline solution
PC	Polycarbonate
PE	Polyethylene
P-gp (MDR1)	P-glycoprotein
PFA	Paraformaldehyde
PT	proximal tubule
RCC	Renal clear cell carcinoma
RNA	Ribonucleic acid
RPLP0	large ribosomal protein P0

RPM	Revolution per minute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
s	Second
SCD	Systemic carnitine deficiency
SEM	Standard error of the mean
SLC	Solute Carrier family (transporter family)
SLC22	Solute Carrier family 22 (transporter subfamily)
SLCO/OATP	Organic anion transporter polypeptide (transporter family)
TAE buffer	Tris-acetate EDTA buffer
TEA	Tetraethylammonium
TEM	Transmission electron microscopy
TER	Transepithelial electrical resistance
TMDs	Transmembrane domains
µM	micromolar
XTT	(2, 3-bis [2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt)

Note: Gene and protein names of specific transport proteins can be found in Tables 1.1 and 1.2.

1. Introduction

1.1 The pharmacological role of the kidney

1.1.1 General structural and functional organization of the kidney

The kidneys constitute a major portion of the urinary system and are major elimination organs. They comprise less than 1% body mass but receive 20% of the resting cardiac output. The kidney is a retroperitoneal organ and upon bisection, two distinct regions are observable: the outer cortex and inner medulla; considered to be the working tissue of the kidney (Figure 1.1).

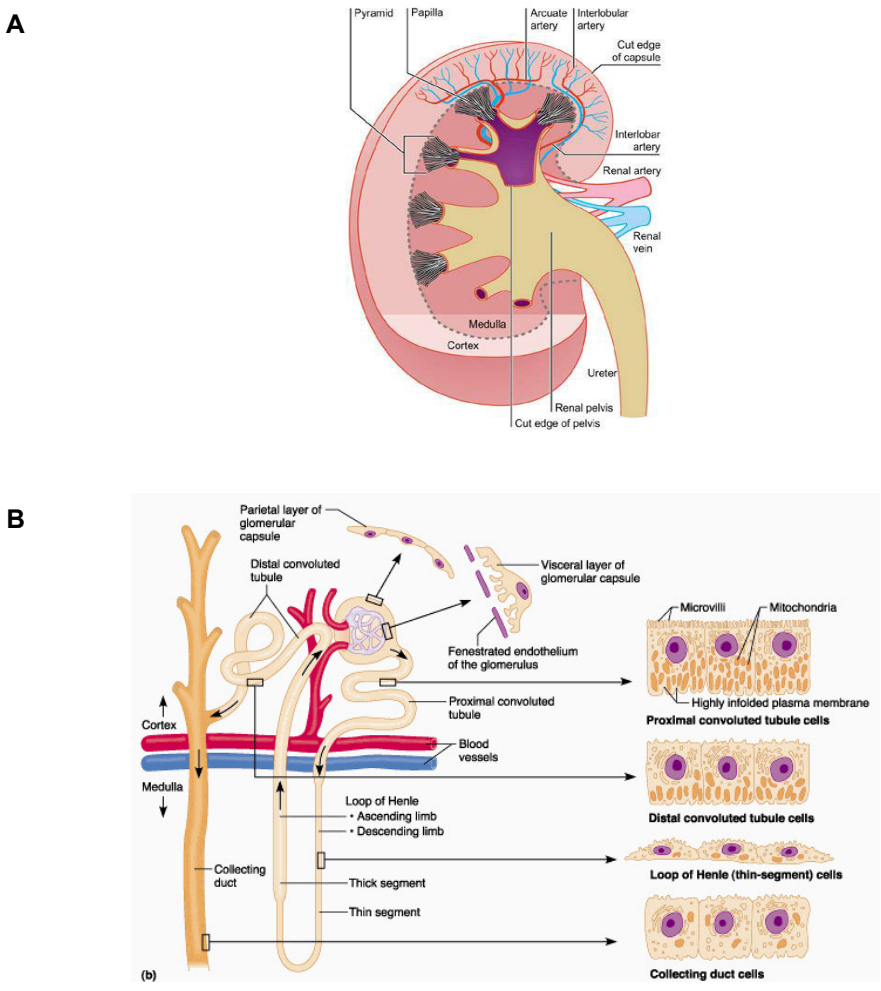

Figure 1.1. Structure of the kidney and nephron.
A: Cross-section of a human kidney
B: Illustration of one functional unit of the kidney, the nephron, depicting the individual functional segments and respective cell types (www.fleshandbones.com)

The functional unit of the kidney is the nephron, with approximately 1 million units present per kidney. Each nephron is composed of a renal corpuscle, proximal tubule (PT), thin limbs, distal tubule and connecting segment (see Figure 1.1). The tubules and blood vessels are arranged in parallel arrays allowing interactions and transfer of material. The renal corpuscle, consisting of the glomerulus, is surrounded by a Bowman's capsule and is involved in the first step of urine formation via the separation of protein-free filtrate from plasma. In brief, blood enters the kidney via the renal artery which branches into smaller arteries until reaching the afferent arteriole, leading to the glomerulus. Blood is filtered by the glomerulus, resulting in an ultrafiltrate of plasma entering the Bowman's capsule. The ultrafiltrate consists of plasma, water and solutes that are capable of passing through the glomerular sieve. Upon leaving Bowman's space, the ultrafiltrate enters the PT. The PT can be subdivided into three distinct sections, namely, the S1, S2, and S3 segments. Approximately 80% of filtered water is reabsorbed at the PT, the remainder being absorbed in the latter portions of the nephron. Continuing from the PT is the thin descending limb of the loop of Henle, which eventually loops upwards to become the thin ascending limb. The following segment of the nephron is represented by the distal tubule and begins at the thick ascending limb of Henle, continuing through the macula densa. The most distal portion of the nephron is the collecting duct; which is subdivided into distinct sections based upon its location in the kidney. These sections include the cortical collecting segment and outer and inner medullary segments. Only a small volume of ultrafiltrate leaves the kidney in the form of urine. Blood which is not filtered leaves the glomerulus via the efferent arterioles and enters the peritubular capillaries. Venous blood collected from the peritubular capillaries is drained into a network of venous vessels which culminate at various junctions within the kidney and ultimately at the renal vein.

1.1.2 General functions of the kidney

The kidney is a dynamic organ and represents the major control system maintaining homeostasis. The kidneys perform a wide array of functions including the regulation of water and electrolyte balance, excretion of metabolic waste, regulation of arterial blood pressure, regulation of red blood cell production and the clearance of bioactive substances including hormones and many foreign substances such as several commonly prescribed therapeutic agents and their respective metabolites. The latter being the focus of the current work.

1.1.3 Renal drug-handling processes (renal clearance)

Three physiological events in the kidney, namely glomerular filtration, tubular secretion and tubular reabsorption, contribute to the overall drug excretion into the urine. Accordingly, renal clearance (CL_{renal}) of a given compound is the sum of its clearance by filtration and secretion, minus reabsorption (Equation 1):

$$CL_{renal} = CL_{filtration} + CL_{secretion} - CL_{reabsorption}$$

1.1.4 Glomerular filtration (GF)

GF is a passive process, which may be affected by haemodynamic changes and renal disease state. Only unbound drug is filtered. For compounds (e.g., inulin) that are not protein-bound, neither secreted nor reabsorbed, the renal clearance approximates the glomerular filtration rate (GFR). However, the renal filtration clearance ($CL_{filtration}$) of a protein bound drug depends on the free fraction of drug (f_u) in plasma (i.e., $CL_{filtration} = f_u$ x GFR), and protein binding displacement may result in a change in the GF clearance [1].

1.1.5 Tubular secretion

Tubular secretion involves the transeptihelial flux of drug molecules from blood into the tubular lumen. Secretion is evident when the CL_{renal} of free drug >> GFR. However, secretion can also occur with drugs that have a $CL_{renal} \approx$ or < GFR, if tubular reabsorption is extensive [1]. Tubular secretion occurs mainly in the PT of the nephron. The vectorial transport/secretion of organic anions (OAs) and organic cations (OCs) is carried out by the concerted effort of membrane transporters at the basolateral membrane (BLM) and brush-border membrane (BBM) of the PT. Therefore, tubular secretion exhibits characteristics typically associated with transport proteins: saturability, substrate/inhibitor specificity, and temperature dependence [1].

1.1.6 Tubular reabsorption

Tubular reabsorption may be either passive or active and represents the back-flux of drug molecules from the lumen to blood. Passive back-diffusion is driven by the large concentration gradient of a filtered molecule, developed as the majority of water in the filtrate is reabsorbed along the nephron. Since this movement is passive, only unionized and nonpolar drugs with sufficient lipophilicity undergo passive reabsorption. As a change in urinary pH alters the fraction of ionization of weak bases and acids, passive reabsorption is affected by urinary pH changes. In addition to passive reabsorption, certain endogenous substances and drugs undergo active or carrier-mediated reabsorption, assisted by transporters in the PTs. Active reabsorption exhibits characteristics of carrier proteins, as those for tubular secretion.

1.2 Proximal tubule (PT) epithelium

1.2.1 Morphological, biochemical and electrophysiological characteristics of the PT epithelium

In vivo the human kidney cortex contains at least 18 different resident cell types, each with individual characteristics [2]. The human PT ranges in length from approximately 17.8 to 21.5 mm, dependent on the location of the nephron in the kidney [3]. The length of a single PT can be divided into three segments, S1, S2 and S3, with cell populations of distinct

size, shape, microvilli density, histochemical staining and the quantity and type of intracellular organelles [4].

The PT epithelium is composed of polarized epithelial cells which are columnar and possess a prominent brush border (microvilli) (Figure 1.2). The PT is an example of a leaky epithelium. This conclusion was based on several distinct pieces of experimental evidence including electrophysiological studies, studies employing extracellular markers and electron microscopic investigations [4]. The in vivo PT epithelium maintains a low transepithelial electrical resistance (TER) of approximately 5-12 Ω cm^2. The formation of the TER is closely associated with the tight junctions located to the apical end of the basolateral membrane. These junctions represent the combined effort of several membrane proteins including the cadherins, occludin and claudins and are involved in the regulation of epithelial cell polarity [5]. The PT can be further distinguished from other nephron segments based on antigen expression, hormonal response patterns, transport processes and the expression and/or activity of many marker proteins or enzymes e.g., alkaline phosphatase (AP), aminopeptidase M (APM), dipeptidyl peptidase IV (DPP IV), gamma glutamyl transferase (GGT) and the Na^+/H^+-exchanger 3 (NHE3) [2].

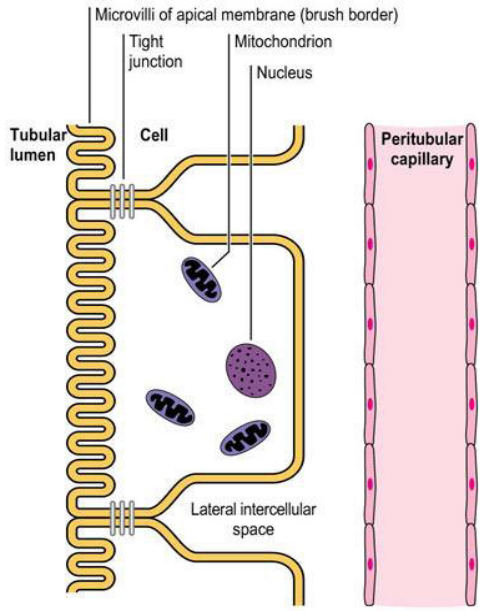

Figure 1.2. Illustration of a typical proximal tubule cell with apically situated microvilli, tight junctions, multiple mitochondria and alignment with the blood capillary system. (www.fleshanbones.com)

A proton concentration gradient exists between the urine, PT cell and extracellular fluid (blood) and is represented by the approximate pH values of 6.7, 7.2 and 7.4, respectively. Thus, the concentration of protons is highest in the urine and lowest in the blood. Additionally, an electrical gradient is maintained between the renal tubular lumen (0 mV), renal tubular cell (-70 mV) and blood (-3 mV).

1.2.2 Carrier-mediated drug transport in the PT epithelium

Drugs cross the membranes of PT cells via the typical paracellular or transcellular routes. The paracellular pathway refers to the passage of drugs in between the adjacent cells, while the transcellular pathway involves the transepithelial passage of the drug across the cells (Figure 1.3). The major pathway of transport of a drug depends on its physicochemical characteristics as well as membrane features, such as the presence of membrane transport proteins (discussed in detail in section 1.3).

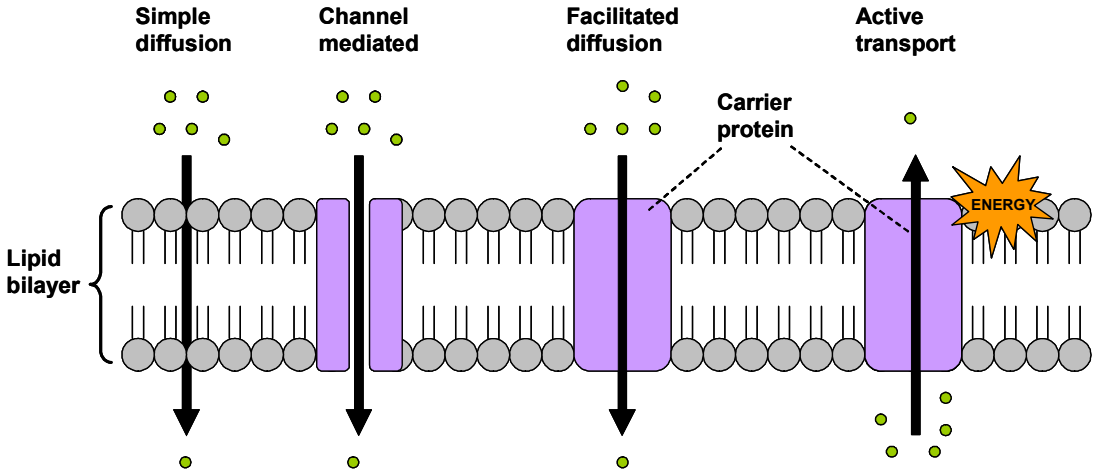

Figure 1.3. Paracellular and transcellular routes of transport of molecules across cellular membranes

In general lipophilic drugs cross the biological membrane transcellularly and hydrophilic drugs cross the membrane paracellularly.

The PT is the primary site of drug secretion in the kidney, as determined by stop-flow micropuncture and microperfusion studies [6]. The PT is also responsible for the more limited reabsorption of certain drugs. Although a number of endogenous compounds are accepted as substrates of these transport pathways, it is generally accepted that the principal function of this process is clearing the body of xenobiotic agents [6]; among these substances are several therapeutic drugs of cationic structure. The secretory process of the PT is also a site of clinically significant interactions between OCs in humans (see section 1.4). The PT cells are subject to the highest degree of toxicity due to the expression of relevant drug transport systems and metabolizing enzymes.

1.2.3 PT Drug Metabolism

Although drug metabolism takes place predominantly in the liver, the kidney plays a significant role in drug metabolism during renal excretion. Renal biotransformation pathways involve phase I oxidative, reductive and hydrolytic reactions and conjugation into glucoronides, sulphate esters or glutathiones in phase II reactions [7, 8]. Microsomal oxidation takes place primarily in the PTs of the kidney where the largest concentration of metabolizing enzymes has been detected [8]. Metabolic reactions occur in the endoplasmic reticulum (ER) and are related to membrane bound cytochrome P450s (CYP450) and comparable enzymes and subsequently by Phase II reaction enzymes. In the kidney the most extensively characterized pathways for metabolism are those occurring via isozymes of the CYP450 family. Phase II conjugating enzymes within and outside of the ER generate drug metabolites which are suitable for rapid excretion. Glucuronides are formed through enzymatic activity from UDP-glucuronosyl-transferases of which various enzymes have been identified in the kidney. Sulfate conjugation in the kidney is catalyzed by sulfotransferases but is quantitatively less than that of glucuronidation [8]. Glutathione conjugation is catalyzed by the enzyme GSH-transferase and the conjugates can be subsequently transformed into cysteine conjugates and mercapturates [9]. The phase II reaction enzymes are not evenly distributed in the kidney but expression and activity has been shown to be concentrated to the PT epithelium.

1.3 Drug Transporters

1.3.1 General characteristics of drug transporters

Until, recently, the general role of transporters in the reabsorption and excretion of drugs had received only minor attention. However, membrane transport proteins are crucial determinants in the absorption, distribution and excretion of drugs and metabolites across cellular membranes. These proteins therefore govern the itinerary of many drugs in the body and their respective pharmacological activity and/or toxicities. Transport proteins for carrier-mediated processes are more commonly referred to as transporters. These proteins function in the translocation of substrates at both sides of the cellular membrane (BLM and BBM), binding their substrate at one side of the plasma membrane and undergoing a conformational change to release them at the opposite membrane. The polarized expression of uptake and efflux transporters on the BLM and BBM of PTs allows for efficient vectorial or directional movement of substrates from the blood capillaries into the urine or vice versa. This is unlike ion channels, which provide hydrophilic pathways through the lipid bilayers. Transporters are characterized by their saturation and competition kinetics and in some cases, energy-dependent uphill transport (active transport dependent on ATP). Greater than 80% of the transporters located in plasma membranes, transport specific metabolic and nutritional compounds and are referred to as oligospecific (e.g. for amino acids, glucose, nucleosides) [10]. In contrast, drugs and toxins are most often translocated by polyspecific transporters, which accept a wide range of compounds of various size and molecular structure. Nevertheless, these transporters can quantitatively distinguish between solute substrates. Such distinctions are evident in the large variation in the apparent Michaelis-Menten kinetics (K_m and IC_{50}) of various compounds. Clinically

significant interactions due to the co-medication with drugs which are substrates and others which inhibit the same transport system can lead to potentially adverse side-effects. The appreciation for the role of drug transporters in detoxification and as sites for drug-targeting is constantly growing. These transporters additionally have specific physiological roles and implications in certain disease states.

Drug transporters can be generally divided into two groups: the solute carriers (SLC) and ATP-binding cassette (ABC) superfamilies. SLC variants generally use electrochemical gradients of inorganic solutes as a driving force (facilitated diffusion), whereas, ABCs are believed to be dependent on ATP hydrolytic energy for their driving force (active transport). Overall, 49 ABC genes (SLC1-SLC43) and 319 SLC genes (ABCA-ABCG) have been identified. However, from the aforementioned transporters there are few which recognize therapeutic agents as substrates.

1.3.2 Transporter expression in the PT

The kidney is a principal excretory organ for drugs and their metabolites and hence has developed high capacity systems to rapidly eliminate large quantities of foreign compounds. GF remains the main route of drug excretion but renal tubular epithelial cells express a variety of transport systems with diverse substrate specificities for the carrier-mediated renal secretion and in some cases reabsorption of many xenobiotics. Such transporters are predominantly localized to the PTs but additionally found in other cell types of the renal cortical tissue. Numerous drugs and endogenous compounds are efficiently transported within the PT epithelium, primarily via two groups of carrier-mediated pathways: organic anion and cation transport systems (OAT and OCT respectively), the latter of focus of the current work. It has also been suggested that ABC transporters such as P-glycoprotein (P-gp) and multi-drug resistance proteins (MRPs) function as efflux pumps in renal tubular cells, for more hydrophobic molecules and anionic conjugates. Further molecular determinations have led to the detection of other transport systems expressed in the PT epithelium including those for peptides (PEPT1 and 2), nucleosides (CNT and ENT) and bile salts (OATPs). A significant portion of renal disposition is achieved through an interaction of these diverse secretory and absorptive transporters in renal tubules.

The current work focused on specific members of the OCTs, belonging to the SLC22 family, which is a subgroup of the Major Facilitator Superfamily (MFS). However, the mRNA expression of several additional transport proteins belonging to the ABC, SLC and organic acid transporting polypeptide (OATP or SLCO) superfamilies were as well investigated. Therefore, details regarding the tissue distribution, function and substrate specificity of all transporter proteins investigated in the current work and relevant to renal secretion and reabsorption are summarized in Tables 1.1 and 1.2 for the ABC and SLC/SLCO transporters, respectively. A subsequent in depth discussion into the OCTs is given in section 1.3.5. The focus will be on the tissue distribution, physiological roles, functional properties and substrate or inhibitor specificities. Several other transporters which interact with OCs but are beyond the scope of the current work include the monoamine neurotransmitter, choline, acetylcholine and thiamine transporters [11].

1.3.3 ABC transporters

The ABC transporter superfamily is among the largest and most broadly expressed protein superfamilies known. The vast majority of its members are responsible for the active transport of a wide variety of compounds across biological membranes, including ions, peptides, bile acids, drugs and other xenobiotics [12]. In humans, 49 genes which are organized into seven subfamilies (ABCA-ABCG) have been described. For the majority of ABC transporters the binding and subsequent hydrolysis of ATP at their nucleotide binding domains is required to provide energy for the movement of substrates across membranes. A major function of several ABC variants is their ability to confer multi-drug resistance. Several ABC members are now known to be involved and include: P-gp (ABCB1), and MRP1-5 (ABCC1-5). A more distantly related transport protein, namely BCRP (ABCG2) is also known to be involved in drug resistance in human tissue. A summary of the ABC transporters relevant to the current work is given in Table 1.1.

1.3.4 SLC and SLCO transporters

The SLC superfamily comprises 43 subfamilies and 300 human genes are assumed to encode SLC carriers [13]. Many of these carriers transport physiological substrates alone; several variants transport a few foreign compounds in addition to endogenous compounds and certain members transport predominantly xenobiotics. The SLC members have been assigned sequential numbers from the Human Genome Organization (HUGO) nomenclature committee. OATPs also referred to as SLCO, comprise a total of at least 14 members and are responsible for the transport of bile acids and a plethora of drugs. Table 1.2 lists the details of the SLC and SLCO transport proteins relevant to the current work.

1.3.5 Organic Cation Transporters (OCTs): structure, function, physiological roles and biopharmaceutical implications

This section is intended to summarize the current knowledge in regards to the structure, distribution (PT cells in specific), specificity, physiological (in regards to the kidney) and biopharmaceutical roles of the OCTs. OCTs belong to the SLC22 family. The SLC22 family contains the three facilitated diffusion transporters OCT1 (SLC22A1), OCT2 (SLC22A2) and OCT3 (SLC22A3), the cation and carnitine transporter OCTN1 (SLC22A4), which may function as a proton cation exchanger, Na^+-carnitine cotransporter OCTN2 (SLC22A5), which can additionally operate as a Na^+-independent transporter of OCs and the carnitine and cation transporter OCT6 (SLC22A16). The current discussion, will detail only those subtypes expressed in humans, however, much information is known regarding the species differences of the various subtypes; this is nevertheless beyond the scope of the current work. Furthermore, the regulation and structure-function relationships of these transporters will not be touched upon.

Table 1.1. ABC transporters. Summary of the details of the ABC transporters investigated at the gene level in the current work.

Gene name	Protein name	Example tissue distribution and/ or subcellular localization	General Function	Sample substrates
ABCB1	P-gp/MDR1	intestine, liver (canicular membrane hepatocytes), kidney (BBM of proximal tubule cells), brain	multi-drug resistance	steroid hormones, digoxin, talinolol, cyclosporine
ABCB4	MDR3	liver	phosphatidylcholine transport	phosphatidylcholine
ABCB11	BSEP	liver (canicular membrane of hepatocytes)	bile acid transport	bile acids
ABCC1	MRP1	ubiquitous, (BLM of polarized cells)	multi-drug resistance	glutathione, glucuronate and sulfate conjugates
ABCC2	MRP2/cMOAT	liver, kidney (BBM of proximal tubule cells)	multi-drug resistance	HIV protease inhibitor, vinblastine, phase-II conjugates
ABCC3	MRP3	lung, intestine, liver, prostate, kidney (BLM of proximal tubule cells)	multi-drug resistance	glucoronide and glutathione conjugates, methotrexate
ABCC4	MRP4	kidney (BBM of proximal tubule cells), ubiquitous	multi-drug resistance	estradiol-17ß-glucuronide, methotrexate, urate, nucleotides
ABCC5	MRP5	kidney (BLM of proximal tubule cells), liver	multi-drug resistance	glutathione conjugates, nucleotides
ABCC6	MRP6	kidney (BLM of proximal tubule cells), liver	multi-drug resistance	BQ123 (endothelin antagonist), organic anions
ABCG2	BCRP	brain, liver, kidney, small intestine, placenta, prostate	multi-drug resistance	anticancer drugs, phase-II conjugates

Table 1.2. SLC transporters. Summary of the details of the SLC transporters investigated at the gene level in the current work

Gene name	Protein name	Example tissue distribution and/or subcellular localization	Sample/predominant substrates
SLC10A1	NTCP	liver, pancreas	bile acids
SLC10A2	ASAT	biliary tract, ileum, kidney	bile acids
SLC15A1	PEPT1	intestine, kidney	di- and tripeptides
SLC15A2	PEPT2	kidney, lung, brain, mammary gland, bronchial epithelium	di- and tripeptides
SLC16A1	MCT1	ubiquitous	lactate, pyruvate, ketone bodies
SLC16A4	MCT4	brain, muscle, liver, kidney, lung, ovary, heart	lactate, monocarboxylates
SLC22A1	OCT1	liver, sinusoidal membrane of hepatocytes, kidney	organic cations
SLC22A2	OCT2	kidney (BLM of proximal tubule cells), brain	organic cations
SLC22A3	OCT3	liver, skeletal muscle, placenta, kidney (BLM of proximal tubule cells), heart, lung, brain	organic cations
SLC22A4	OCTN1	kidney, skeletal muscle, placenta, prostate, heart	organic cations
SLC22A5	OCTN2	skeletal muscle, kidney (BBM of proximal tubule cells), prostate, lung, pancreas, heart, small intestine, adrenal gland, thyroid etc.	l-carnitine, organic cations
SLC22A6	OAT1	kidney (BLM of proximal tubule cells), placenta, brain	organic anions
SLC22A7	OAT2	liver, kidney (BLM of proximal tubule cells)	organic anions
SLC22A8	OAT3	kidney (BLM of proximal tubule cells), brain, skeletal muscle	organic anions
SLC22A9	UST3	liver	not determined
SLC22A11	OAT4	kidney (BBM of proximal tubule cells), placenta	organic anions
SLC28A3	CNT1	liver, kidney, small intestine	pyrimidine, nucleosides
SLC29A1	ENT1	ubiquitous	purine and pyrimidine nucleosides
SLCO1A2	OATPA	brain, liver, lung, biliary epithelial cells, kidney and testes	bile acids, steroid hormone conjugates, thyroid hormones, oligopeptides
SLCO1B1	OATPC	liver	taurocholate, bilirubin, BSP, steroid hormone conjugates, thyroid hormones and drugs
SLCO1B3	OATP8	liver, cancer tissues	BSP, steroid hormone conjugates and bile salts
SLCO1C1	OATPF	testes, brain	thyroxine
SLCO2B1	OATPB	liver, spleen, placenta, lung, kidney, heart, ovary, small intestine, brain	BSP, estrone-3-sulfate
SLCO3A1	OATPD	ubiquitous	estrone-3-sulfate, prostaglandin E_2, benzylpenicillin
SLCO4A1	OATPE	brain, several other tissues	thyroxine
SLCO4C1	OATPH	kidney	not determined

1.3.5.1 Cloning of the polyspecific organic cation transporters: OCTs, OCTNs and MATEs

OCT and OCTN transporters

The SLC22 family is a member of the major facilitator superfamily (MFS) which comprises transporters from bacteria, plants, animals and humans [14]. The first transporter of the SLC22 family in mammals, the rat organic cation transporter OCT1, was cloned in 1994. Since then, 16 additional human family members and several orthologs from different species have been identified [11]. In addition to four organic anion transporters OAT1-4 (SLC22A6-8, 11) and one urate transporter URAT1 (SLC22A12), the SLC22 family includes three organic cation transporters (OCT1-3 or SLC22A1-3) and three transporters for carnitine and/or OCs (OCTN1 or SLC22A4, OCTN2 or SLC22A5, hCT2 or OCT6 or SLC22A16). Similar to most members of the SLC22 family the organic cation and carnitine transporters have the predicted membrane topology that comprises 12 α-helical transmembrane domains (TMDs), an intracellular N-terminus, a large glycosylated extracellular loop between TMDs 1 and 2, a large intracellular loop with phosphorylation sites between TMDs 6 and 7 and an intracellular C-terminus (Figure 1.4) . OCT1 has been cloned from human, rat, mouse and rabbit [15-18]; OCT2 has been cloned from human, rat, mouse rabbit and pig and OCT3 from human, rat and mouse [16, 17, 19-23]. Functional isoforms of OCT1 and OCT2 have been identified [24, 25]. OCTN1 and OCTN2 have been cloned from human, rat and mouse [26-31]. hCT2 also referred to as OCT6 has been cloned from humans [32, 33].

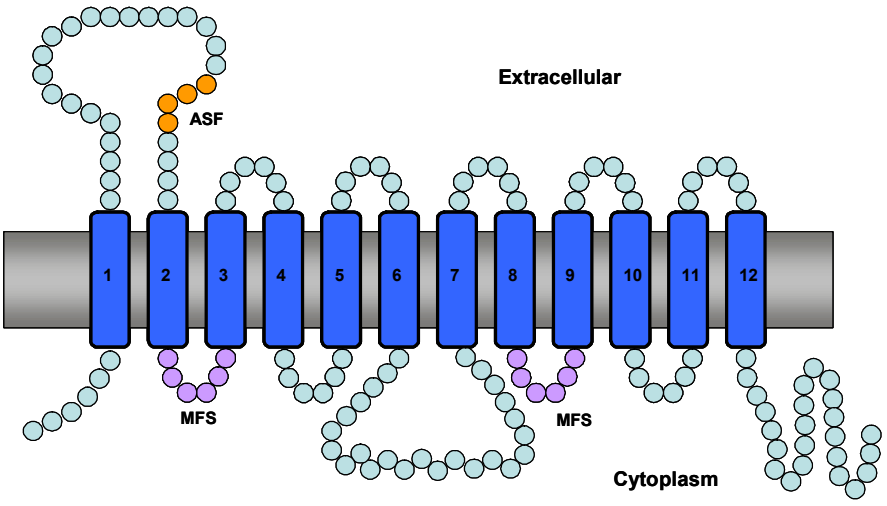

Figure 1.5. Schematic illustration of the postulated secondary structure of the human ortholog OCT2. Features common to other members of the OCT transporter family include 12 transmembrane spanning domains (TMDs), a large extracellular loop between TMDs 1 and 2, and a large cytoplasmic loop between TMDs 6 and 7. Indicated in purple and orange are conserved MFS and ASF motifs, respectively.

1.3.5.2 MATE transporters

Recently three mammalian proton cation transporters known as MATE1, MATE2-K and MATE2-B have been cloned. They belong to the multi-drug and toxic compound extrusion (MATE) protein family. MATE1 has been cloned from human, rat and mouse [34-36]. All three MATE variants are expressed in humans, however, only for the MATE2-K variant has functional activity been demonstrated. A typical membrane topology of 12 α-helical TMDs has been predicted for the MATE transporters.

1.3.5.2 Tissue distribution and membrane localization (see Figure 1.5 for an overview)

OCT1. OCT1 exhibits a broad tissue distribution. OCT1 is expressed in epithelial cells and in some neurons [11, 15]. In humans it is most strongly expressed in the liver whereas in rodents, OCT1 is strongly expressed in the liver, kidney and small intestine. OCT1 has been detected in many other organs of humans and tumor cells [19, 37, 38]. In human and rat liver, OCT1 was located to the sinusoidal membrane of hepatocytes [39]. OCT1 was further localized to the basolateral membrane of the S1 and S2 segments of the PTs in rat kidney [40].

OCT2. OCT2 has a more restricted expression pattern than OCT1 or OCT3. Similar to OCT1, OCT2 is expressed in epithelial cells and neurons. OCT2 is most strongly expressed in the kidney [16] but also in a variety of other organs including the small intestine, lung, skin and brain [14, 16, 33, 37, 38]. In the human kidney OCT2 is expressed in all three segments of the PTs.

OCT3. OCT3 exhibits a wide tissue distribution and in contrast to OCT1 and OCT2, OCT3 is additionally expressed in muscle and glial cells [41-44]. In humans OCT3 is strongest expressed in the skeletal muscle, liver, placenta and heart, however, additionally in many other organs including the kidney and brain. OCT3 has been localized to the basolateral membrane of the trophoblast in placenta, to the sinusoidal membrane or hepatocytes and a probable localization to the basolateral membrane of human kidney PT cells has been determined in the current work (submitted data from Glube et al., Nephron Experimental Neph 2007) [45]. OCT3 has additionally been localized to the apical membranes of the small intestine and bronchial epithelium [37, 46].

OCTN1. OCTN1 is expressed in the epithelial and muscle cells of various tissues. In humans the strongest expression has been observed in kidney, skeletal muscle, bone marrow and trachea tissue [27]. Weaker expression has been observed in several other organs. It has additionally been reported that OCTN1 is also located to the mitochondria in humans [47].

OCTN2. OCTN2 has a relatively ubiquitous distribution and is expressed in epithelial cells, muscle cells, glial cells, macrophages, lymphocytes and sperm [14, 29]. OCTN2 has been localized to the apical membrane of PT cells [48, 49].

OCT6 or CT2. OCT6 is mainly expressed in the testis but additionally in the embryonic liver and hematopoietic cells [32, 33]. In the testis OCT6 is located to the plasma membrane of Sertoli cells [32].

MATE1. In humans MATE1 is expressed in the liver, kidney and skeletal muscle and has also been detected in the heart [34]. MATE1 has been detected at the BBM of mouse PTs and is assumed to be located to BBM of human PTs [34].

MATE2. MATE2-K is predominantly expressed in the human kidney, where it has been located to the BBM of human PTs. MATE2-B has been located in many organs but not the kidney [50].

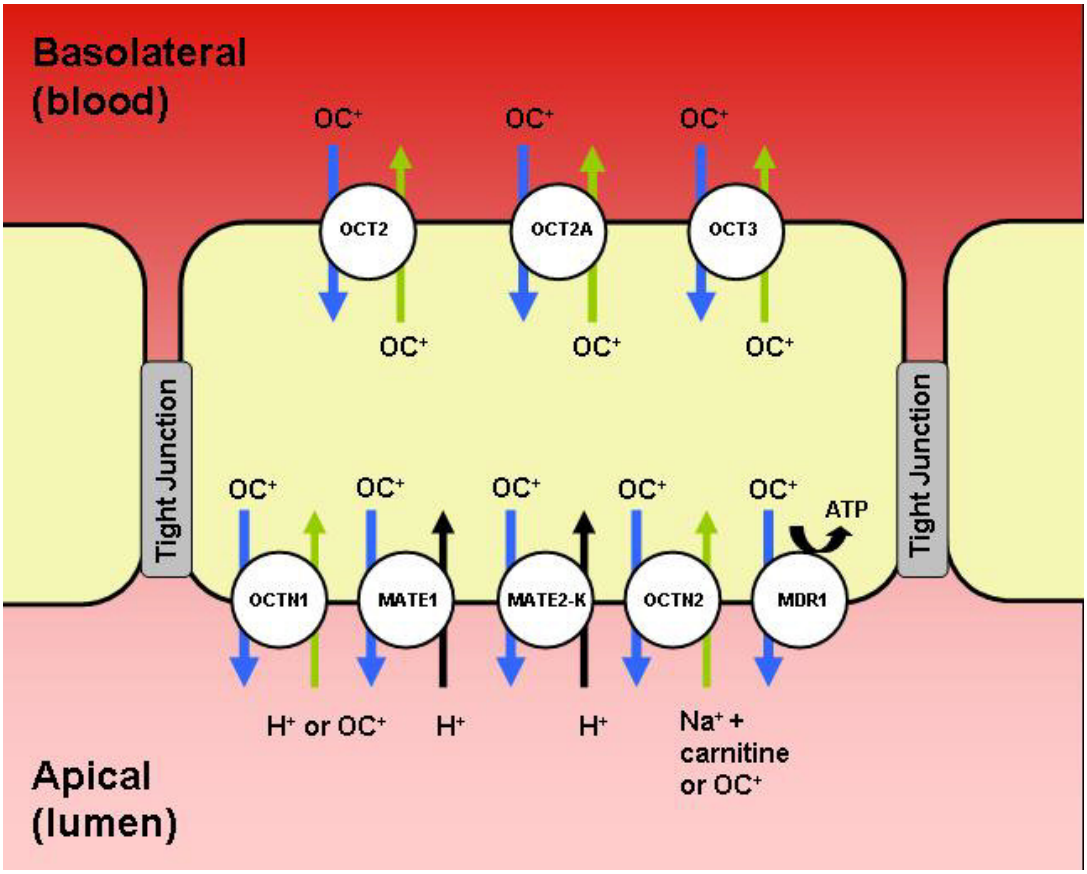

Figure 1.5. Organic cation transporters located in the plasma membranes of human renal proximal tubule cells. Blue arrows indicate transport activities that are involved in cation secretion, whereas green arrows indicate transport activities involved in cation reabsorption (modified from Koepsell et al., 2007).

1.3.5.3 Functional properties and substrate specificities

Common functional properties of OCT1-3

The basic transport characteristics of OCT1-3 in various species is similar [14]. OCT1-3 translocate a variety of OCs with diverse molecular structures and are inhibited by a large number of additional compounds that are not transported. The approximate molecular mass of most compounds that are transported by OCT1-3 is below 500. OCTs translocate OCs in an electrogenic fashion [14]. OCT1-3 operate independently of Na^+ and have the ability to function bidirectionally. The majority of substrates translocated by OCT1-3 are OCs and weak bases that are positively charged at physiological pH but non-charged compounds at physiological pH (e.g. cimetidine) may also be transported. Transported substrates of the OCTs include endogenous compounds, drugs, xenobiotics and model compounds such as 1-methyl-4-phenylpyridinium (MPP^+); MPP^+ is transported by all three OCT variants with similar Michaelis-Menten K_m values. Several compounds inhibit OCT transport (e.g. decynium22) but are not transported themselves. The substrate and inhibitor specificities of OCT1-3 broadly overlap but there are significant differences in the specificity of the individual subtypes and between identical subtypes of different species, which allow for their in vitro investigation [11].

1.3.5.4 Substrate and inhibitor specificities of the individual OCTs

OCT1. Compounds which are transported by OCT1 include but are not limited to the model cations MPP^+, tetraethylammonium (TEA), the endogenous compounds choline, acetylcholine and agmatine and the drugs quinidine, quinine and metformin [14]. The inhibitors of highest affinity for OCT1 are atropine (IC_{50} 1.2 µM) and prazosin (IC_{50} 1.8 µM).

OCT2. OCT2 transports several cations in common with OCT1 (MPP^+, TEA, quinine and metformin) with similar K_m values. However, difference in affinities between OCT1 and OCT2 do exist and other endogenous and exogenous compounds including histamine, cimetidine, famotidine and ranitidine were found to be transported by OCT2.

OCT3. A similar affinity for the transport of MPP^+ in comparison to OCT1 and OCT2 was determined for OCT3, however significant differences in the transport of TEA via OCT3 was determined (much larger K_m for OCT3) [14]. The substrates MPP^+ and histamine exhibited the highest affinities for transport via OCT3 with K_m values of 47 µM and 180-200 µM respectively [14]. Potent inhibitors of the OCT3 subtype include corticosterone and cyanide dyes such as decynium22.

1.3.5.5 Function and substrate specificities of OCTN1 and OCTN2

OCTN1. OCTN1 transports the zwitterions ergothioneine, and l-carnitine and the OCs TEA, quinidine, pyrilamine and verapamil. OCTN1 can be further inhibited by several different compounds for example cephaloridine, cimetidine, procainamide etc. OCTN1 apparently employs different translocation methods for different substrates and has the ability to operate in both directions [14].

OCTN2. The Na^+-dependent carnitine transporter is a high affinity transporter of l-carnitine, acetyl-l-carnitine and the zwitterionic ß-lactam antibiotic cephaloridine but can function alternatively as a Na^+-independent transporter of cations [48, 51, 52]. Cationic substrates of OCTN2 include TEA and verapamil. OCTN2 uptake can be inhibited by compounds such as cimetidine, clonidine, quinine and MPP^+. The Na^+-dependent transport of l-carnitine is electrogenic and stereospecific [52, 53]. Uptake measurements provided evidence that OCTN2 is a Na^+-l-carnitine cotransporter and is induced by an inwardly directed Na^+-gradient [48]. For OCTN2 a K_m for l-carnitine of approximately 4-5 µM has been determined experimentally [51]. Experimental data suggests that OCTN2 is responsible for l-carnitine uptake into several cell types including renal epithelial cells.

OCT6 or CT2. OCT6 is a high affinity transporter of carnitine but can additionally translocate several cations such as TEA and doxorubicin [32, 54]. OC uptake could be inhibited by several other OCs and carnitine transport was partially dependent on sodium.

1.3.5.6 Function and specificity of MATE transporters

MATE1. MATE1 mediates the uptake of TEA (K_m 220 µM) [14]. TEA uptake was independent of the membrane potential and the extracellular concentration of sodium. MATE1 is assumed to be a proton-cation exchanger which functions in both directions [34]. TEA uptake via MATE1 can be inhibited by several cationic compounds such as MPP^+, cimetidine, verapamil and quinidine suggesting a polyspecificity similar to that of the OCTs and OCTNs.

MATE2. MATE2-K is the single MATE2 protein variant for which transport has been detected in humans [50]. MATE2-K is located to the BBM of PTs and represents a polyspecific proton-cation exchanger which can translocate cations such as TEA, cimetidine, MPP^+, procainamide and metformin.

1.3.5.7 Function of polyspecific OCTs in the kidney

OCs which are not bound to plasma proteins may be readily ultrafiltrated in the glomeruli of the kidney and subsequently secreted and/or reabsorbed in the PTs. Reabsorption is dependent upon the concentrations of the respective cation in the blood and the primary filtrate. Several endogenous cations and cationic drugs which are bound to plasma proteins are efficiently filtrated and many of these are actively secreted. Secretion and reabsorption may occur in the PTs, distal tubules and collecting but it is assumed that the PTs are the major site of these processes and the most research has been concerned with the PTs [55]. In the first step of OC secretion across the PT, OCs are translocated across the BLM. In humans OCT2 and OCT3 are assumed important variants in this process [14]. In the second step of secretion, OCs are released at the BBM by the proton-cation exchangers MATE1, MATE2-K and/or OCTN1 or OCTN2 [14, 27, 34, 49]. The proton-cation antiport mechanism used by these transporters helps to overcome the membrane potential during cation efflux; this step is energized by an extracellular>intracellular proton concentration difference, which is generated partially by the sodium-proton exchanger (NHE3) [6, 14].

1.3.5.8 Biomedical implications of polyspecific OCTs in the kidney

Renal excretion is one of the major determinants for the pharmacokinetics of several hydrophilic cationic drugs. The localization of OCT2 and OCT3 to the PT cells of the human kidney implies critical sites for clinical drug-drug interactions. OCT2 has been implicated in the renal secretion of many neurotransmitters including dopamine, serotonin and epinephrine and additionally appears to be critically involved in the renal excretion of agonists and antagonists of various receptors, of various blockers of ion channels and transporters and of various other drugs [14]. OCT3 is assumed to be involved in the excretion of epinephrine, histamine and norepinephrine and OCT3 is inhibited by several drugs of various therapeutic classes which could be potential substrates but remains to be determined (submitted data Glube et al.).

1.4 Drug-drug interactions (DDIs) in the PT via transport proteins

Renal excretion makes up a primary elimination pathway for drugs and drug metabolites; approximately 25 % of the top 50 prescribed drugs on the current market are eliminated via renal excretion [1]. Given the complexity of renal drug-handling processes, DDIs in the kidney may occur via one or more of the following pathways: (1) displacement of protein bound drug, resulting in a change in $CL_{filtration}$, (2) competition at a tubular secretion site resulting in a decrease in drug excretion, (3) competition at a site of tubular reabsorption resulting in an increase in drug excretion and (4) a change in urinary pH and/or urine flow rate that alters the passive reabsorption, depending on the pKa of a drug. Among the aforementioned mechanisms, the most well known type of interactions results from the competitive inhibition of drug transporters via the secretory pathways, resulting in a reduced secretion clearance and plausible increases in plasma concentrations [1].

For competitive inhibition of renal secretion, certain conditions must be satisfied for clinically relevant interactions to occur: (1) the affected drug must be actively secreted by the kidney and transporter mediated clearance must account for the majority of the total clearance of the affected drug and (2) clinical unbound concentration of the interacting drug must be high enough in order to produce a pronounced effect (when plasma concentrations are less than the Michaelis-Menten inhibitory constant (K_i)), the potential for a significant drug interaction is minimal, unless the drug has a narrow therapeutic window. The following section offers a few examples of renal DDIs where in vitro data are available to support the involvement of individual polyspecific organic cation transporters.

1.4.1 Interactions involving OCTs

Many pharmacokinetic renal interactions between cationic drugs have been reported and were attributed to competition for an OC secretion system in the PT [1]. A number of these interactions involve the H_2-receptor antagonist cimetidine. Cimetidine is a prototypical OC transport inhibitor and is extensively renally eliminated with a CL_{renal} averaged at 400 ml/min [56]. Via in vitro expression systems, OCT2 has been identified as the major BLM located transporter involved in the uptake of OCs in the PT and has been shown to

transport cimetidine. Therefore, it is assumed that OCT2 plays a major role in the active secretion of cimetidine and the resulting DDIs between cimetidine and other OCs.

Metformin is a commonly used oral antihyperglycaemic agent and undergoes extensive renal secretion. It has been clinically proven that cimetidine significantly increased the area under the plasma metformin concentration-time curve (AUC) by an average of 50% and reduced its renal clearance by 28% from ~ 537 to 378 ml/min, whereas, cimetidine kinetics were unaltered by metformin [57]. In vitro it has been shown that metformin and cimetidine are transported by OCT2 and both drugs have the ability to mutually inhibit each others uptake by OCT2 [58, 59]. The apparent IC_{50} value of cimetidine for OCT2-mediated metformin uptake is 72 µM whereas a much higher IC_{50} value (1700 µM) was observed for metformin inhibition on cimetidine uptake; suggesting OCT2 as the molecular site for cimetidine-metformin interactions.

Another clinically significant interaction occurs between cimetidine and amiloride. Amiloride a potassium-sparing diuretic is eliminated predominantly by urinary excretion as intact dug. Co-administration with cimetidine reduced the amiloride CL_{renal} from 358 to 299 ml/min [60]. Using the in vitro test system, HEK-293 cells, expressing OCT2, carrier-mediated uptake of amiloride via OCT2 was shown (K_m 95 µM) [61]. Cimetidine inhibited OCT-mediated amiloride uptake with an IC_{50} of 14 µM which is close to the therapeutic concentration range of cimetidine (4-12 µM), suggesting that OCT2 is the potential site for the cimetidine-amiloride in vivo renal interaction [56, 61].

Aside from the aforementioned renal DDI examples, a number of cationic drug interactions have been reported including cimetidine-procainamide, pindolol-cimetidine and trimethoprim-procainamide, presumably via competition of a OC transport pathway [62-66]. Whether OCT2, or other OCT subtypes, such as OCT3, may be involved in these interactions remains to be investigated and was partially completed in the current work.

1.5 In vitro model systems for studying the PT epithelium and more specifically drug transport in the PT

It was well appreciated a decade ago that expression of drug metabolizing enzymes in vitro could provide valuable analytic tools for toxicology [67]. However, the introduction of comparable tools for the assessment of the role transport proteins play in toxicity is a more recent development. This is due to the fact that the general importance of membrane transport proteins in respect to toxicity was not broadly recognized and secondly membrane transport proteins are in low abundance, therefore hampering the ability to purify sufficient quantities of the proteins for molecular investigations [67].

To study transporter activity one requires a system endogenously/natively expressing the transport protein(s) of interest or the purified form of the transport protein which can then be transfected into an expression system of interest. Often it is valuable to have systems singly and co-expressing transport proteins. Several expression systems exist, varying from cell-free systems to heterologous expression in bacteria, yeast, insect and mammalian cell lines. Reviewing the literature one will realize that there is a lack of

success in the stable transfection of membrane transport proteins, however, adequate expression has been achieved, in Xenopus oocytes and insect and mammalian cell lines.

1.5.1 Experimental considerations

Basic properties of transporters can be observed experimentally including specificity, energetics (mechanism of transport), kinetics, structure/function relationships etc. The ideal experimental system would provide the following characteristics: (1) stability of the model system, (2) ease of initial transfection (where applicable), (3) ability to assay expression and function and (4) retention of all other properties common to the tissue in which the transporter is natively expressed. However, all of these constraints are rarely met in a single system [67]. The downside to several systems is the placement of a transport protein in a foreign micro-environment such as an insect cell, amphibian oocyte or cell line; perhaps leading to altered function and regulation. Such systems can be further engineered to express additional co-factors, receptors and other regulatory factors perhaps lost during de-differentiation [67]. A system in which all key factors of a given pathway are expressed and additionally trafficked to the correct subcellular location, provides a powerful tool for hypothesis testing. Overall, validation of an effect observed in a heterologous system is best confirmed in a cell or tissue system natively expressing the specific transport protein

One would like to assume that for a given transporter, relative affinities of substrates and inhibitors would be independent of the expression system. However, this is not the case as can be seen in the variances in the kinetics determined for OCT1 and OCT2 when expressed in either Xenopus oocytes or MDCK cells [68, 69]. These results argue that differences in the plasma membrane environment, for example lipid and protein composition, may underlie disparities in transporter specificity measured in different expression systems.

Furthering complicating the vitro study of transport processes are factors such as: non-specific binding of substrates to cellular membranes, transport via more than one route, and metabolic events.

1.5.2 Higher-order experimental systems

Isolated perfused kidney (IPK). The IPK, most commonly obtained from rat or rabbit, either perfused cell free or in the presence of erythrocytes represents the most appropriate system for studying potentially nephrotoxic xenobiotics, when intact tubulovascular integrity is required. This system is not influenced by higher-order systems such as nervous, hormonal or blood-borne factors. This system allows the precise control of the drug being administered, however, with the disadvantages that renal function is maintained for a limited period of time (approximately 2h), tissue availability, reproducibility etc.

Isolated perfused nephrons. Isolated perfused nephrons or more precisely nephron segments, have allowed valuable insights into the functional nature of specific nephron segments (transport and electrical properties) and has allowed the localization and distribution of specific transport and enzyme systems. This is however not a convenient method for routine analysis.

Renal tissue slices. The use of renal tissues slices represents one of the earliest in vitro techniques for studying transport and toxicity in the kidney. It is still commonly employed but this model suffers from certain disadvantages including: heterogeneous cell populations making the assessment of functional aspects of a single cell type complicated and cell surfaces are often damaged upon preparation/cutting of the slices.

1.5.3 Cell culture models

Isolated proximal tubular cells are advantageous in studying the overall cellular uptake of and accumulation of drugs (kinetics). Isolated cells can be used in various formats including cell suspensions and monolayers. Monolayers can be plated on solid surfaces or semi-permeable filters. The latter of which allows organized trafficking of molecules but suffers the downside that transport proteins, such as OATs, are often 'lost' during such cultivation techniques [70]. Moreover, freshly isolated (primary) proximal tubular cells generally more effectively maintain their morphologically polarized nature in comparison to immortalized cell lines. Important to note here cancerous cell lines of various species have been employed as model systems for studying renal drug handling and have been shown to grow in an organized polarized fashion, similar to that of primary PT cell cultures. However, to date, data has often been extrapolated from other species into humans, due to the lack of a well-characterized PT cell culture system based on a human cell line; hence the major goal of the current doctoral work was to develop/characterize a human cell line system for studying drug transport in the PT.

Cell cultures have become the model system of choice for studying toxicity in vitro; this is largely due to the improved methodologies for growing homogenous cultures of specific renal cells. Two major strategies have been pursued: the use of primary cultures of renal epithelial cells from various sites along the nephron and the use of immortal renal epithelial cell lines. Renal cell culture models have the advantage of providing an experimental model uninfluenced form higher-order regulatory systems. For successful applications, the following requirements should be met in an ideal cell culture model system intended for simulating drug transport processes occurring in the human PT epithelium:

- Cells should retain a polar organization and tight junction assembly representative of the PT epithelia (e.g. transeptihelial electrophysiological characteristics)
- Cells should express the proper polar distribution of transport systems, resulting in the vectorial transport of solutes (the cellular uptake of xenobiotics should occur from either the BLM or BBM via specific transport processes as expected in vivo)
- Cultured cells should retain PT specific characteristics including distinct metabolic and transport properties and hormone responsiveness

1.5.3.1 Primary PT cell cultures

Generally, the aforementioned requirements (section 1.5.3) are best met with primary cultures of renal epithelial cells. It is generally assumed that the characteristics of primary cultures more closely resemble the in vivo situation as compared to cell lines. Primary cultures however are known to dedifferentiate within hours of culturing [71]. The downsides of primary cultures include: labor intensive preparations, difficulties in passaging (de-differentiation), costs, reproducibility of data etc. However, despite these limitations primary cultures represent a suitable method to study basic renal cellular processes and their modulation via interaction with xenobiotics.

1.5.3.2 PT cell lines

Renal cell lines were initially extensively used for investigating factors involved in the growth and function of the kidney but have been more recently employed for studying renal drug transport processes. They represent powerful tools with extensive literature resources in regards to characterization, methodology etc. Furthermore, cell lines often represent homogenous cultures, which is an important feature, considering the heterogeneity of the kidney. Cell lines can represent well-defined segments of the human nephron, as is the case with PT nature of the Caki-1 cells discussed in the current work.

Overall, cell lines are currently the system of choice when studying PT toxicity and interactions with xenobiotics. The advantages of these systems include their unlimited life-spans, controlled growth and experimental conditions and lack of time-consuming isolation procedures. Naturally, there are drawbacks to these systems. Cells lines generally retain certain de-differentiated functions from their in vivo ancestor cells but have the ability in many respects to remain differentiated in vitro. Therefore, it is likely that several of the currently employed systems claimed to be of PT origin exhibit a combination of properties, characteristic of different segments of the nephron. The most commonly used PT systems (cell lines) to date are that of the HK-2, LLC-PK1 and OK of human, pig and north opossum origin, respectively [71]. The disadvantage that one or more functions may not be expressed in vitro in a cell line should however be taken into consideration when establishing a model system and the use of primary cultures should be implemented when the missing function is of foremost importance. Methods can be employed to re-express the lost functions, to tailor a cell line to more closely resemble the in vivo situation.

1.5.3.3 Membrane vesicles

An alternative system is the use of purified membrane vesicles, isolated from cells either endogenously or over-expressing a transporter of interest. This type of preparation allows one to study uptake or efflux of substrates where the intra- and extracellular conditions can be stringently controlled. The downside to such preparations are that vesicles isolated from polarized cells contain a reduced number of BBM vesicles, which are inside out, therefore, they are less useful in the investigation of ATP-driven transporters (efflux).

1.5.3.4 In vitro systems for OC transport

Great efforts have and are being made in understanding the molecular and cellular physiology of renal OC transport, through studies employing cloned transport proteins in heterologous expression systems. However, the observations obtained from such systems must be interpreted with the caveat that they indicate how a particular process works outside of its native physiological milieu. A number of processes are involved in the net secretion of OCs and the characterization of this concerted activity is essential and best understood within the context of physiologically intact systems.

OC transport has been studied with intact tubules and isolated membrane vesicles from a wide variety of species including mammalian, avian, reptilian and teleost species. The physiological data suggests that the general cellular mechanism of renal OC secretion is conserved over several phyla. OC transport has been further studied in various heterologous expression systems including Xenopus oocytes, HEK-293 cells, HeLa cells, MDCK cells, CHO-K1 cells, HRPE cells and as well as systems endogenously expressing transport proteins (e.g. primary cultures and cell lines).

Functional activity is commonly determined via various methods including the uptake of radiolabeled compounds, changes in electrical currents across membranes or measuring the cytoplasmic fluorescence elicited by the uptake of fluorescing substrates.

2. Aims of the thesis

The human kidney plays a major role in the elimination (transport and metabolism) of a broad spectrum of endogenous compounds and xenobiotics, many of the latter being commonly prescribed therapeutic agents. The elimination of these substances, partially dependent on their physiochemical parameters, occurs either via the paracellular or transcellular route (tubular secretion processes). The transcellular route involves the uptake of a substrate across the basolateral membrane (blood) and efflux at the apical membrane (tubular lumen), via one or more of several xenobiotic transport proteins. A broad range of xenobiotic transport proteins for drug elimination are concentrated in the PTs of the kidneys and can be distinguished from one another based on their substrate profiles. Xenobiotic transporters are generally divided into two major groups: the ATP-binding cassette (ABC) and solute carrier (SLC) proteins. The organic cation and novel organic cation transporters (OCTs and OCTNs) are categorized into the SLC22 subfamily of the SLC superfamily and function primarily for the transport of organic cations and l-carnitine, respectively. The OCT subtypes, OCT3 and OCTN2, were of key focus to the current work.

Due to their expression in the major absorption and elimination organs, drug transporters define the pharmacological and/or toxicological aspects of several drugs. Competitive inhibition, a general characteristic of carrier-mediated transport, can lead to interactions between two therapeutic agents for transport via the same transport protein, potentially resulting in drastic drug-drug interactions (via the tubular route).

In vitro drug transport kinetics is often studied at the cellular level using cell culture models. To date the most characterized and established system is that of the Caco-2 cells, implemented in many fields of research but particularly as an absorption/permeability model of the human small intestine. To date, an in depth characterization of a human cell culture model of the human PT epithelium has been lacking in the field of drug transport. Hence, the **goal** of the current doctoral thesis was to select and characterize a human kidney cell line for its appropriateness as a model system for investigations on carrier-mediated drug transport via OCT3 and OCTN2.

The human kidney cell line, **Caki-1**, was selected as a possible candidate. The goal was to stepwise determine how close the Caki-1 cells simulate the in vivo PT epithelium based on the following considerations:

STEP 1: Morphology, biochemistry, electrophysiology

STEP 2: Expression of drug transport proteins (ABC and SLC) and metabolizing enzymes (CYP 450) at the gene level

STEP 3: Expression of the OCTs (OCT3 and OCTN2) at the protein level

STEP 4: Functional activity of OCT3 and OCTN2

The PT epithelium can be distinguished from other segments of the nephron based on specific morphological (e.g., polarized monolayer, apical microvilli, tight junctions etc.), biochemical (enzyme expression, transport processes, hormone response etc.) and electrophysiological (transepithelial electrical resistance, barrier proteins etc.) characteristics and this served as the basis for the initial characterization of the Caki-1 cell line. In respects to drug transport and metabolism, the most relevant transport proteins of the ABC and SLC superfamilies and enzymes of the CYP 450 family, either involved in drug transport, metabolism or known to be expressed in the kidney were selected for detection in Caki-1 cells. Primary cultures of human PT cells (HPT) were selected as the control for this step. As the highlight of the current work was to introduce an in vitro model system for investigating carrier-mediated organic cation transport in the PT, the OCTs determined in step 2 were characterized for their expression at the protein level and functional activity in Caki-1 cells. Where possible, HPT cell cultures were used to determine the relevance of the data obtained for the Caki-1 cells.

Note: Over the last decade the molecular and functional aspects of several OCTs have been clarified and sufficient information exists in regards to substrate and inhibitor profiles. Therefore, the selection of substrates, inhibitors and experimental design was based on the existing information obtained from the literature.

3. Caki-1 cells represent an in vitro model system for studying the human proximal tubule epithelium

(Running chapter head: Caki-1 cells: a proximal tubule cell line)

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3.1 Abstract

Background/Aims: The human proximal tubule (PT) epithelium is distinguished from other nephron segments via several unique characteristics. Studies assessing PT epithelium increasingly employ cell lines, bypassing the complexity of primary cell cultures. However, few human model systems exist for studying PT cells in vitro. The current work involves an intensive characterization of Caki-1 cells, a commercially available human renal cell line.

Methods: Caki-1 cells were validated as a representative model system for PT cell research via morphological, physiological and biochemical investigations including light and transmission electron microscopy, transepithelial electrical resistance (TER) measurements and the detection of PT markers.

Results: Morphologically, these cells form a polarized monolayer with apical located microvilli and multiple mitochondria per cell. Low TER ranging from 2-28 Ω cm^2 was determined for Caki-1 cells, characteristic of the 'leaky' PT epithelium in vivo. The expression of the PT markers: NHE3, GGT, DPP IV, APM and AP were present in Caki-1 cells. Two epithelial markers, E-cadherin and the Na^+/K^+-ATPase were additionally observed.

Conclusion: The current work is a concise summary which confirms that Caki-1 cells represent well-differentiated polarized PT cells in vitro, regardless to its cancerous origin and multiple passaging. They prove to be a significant contribution to the field of PT research.

3.2 Introduction

The mammalian kidney in regards to morphology, physiology and metabolism is a dynamic organ composed of various cell types (15-20 cells types) [2, 71]. The tubular segment of the nephron consists of the proximal, distal and collecting ducts, each having its own distinct functional capacities [72]. The human proximal tubule (PT) epithelium in specific is a complex metabolically active epithelial system [73]. The availability of in vitro cell culture model systems of human PT epithelia, whether from primary or immortalized origins is very limited. Primary PT cell cultures can be routinely obtained from pig, rabbit, mouse, rat and human; human cultures most closely simulating the clinical situation [72]. Differentiated immortalized human PT cell lines which indicate extended in vitro growth potential, provide a convenient alternative to labour intensive whole tissue and primary cell cultures [74]. However, most models with a few exceptions such as the HK-2 cell line, stem from non-human origins (e.g. LLC-PK_1 (porcine), OK (opossum) and NRK-52E (rat)) [75-79]. Additionally, immortalization of the HK-2 cell line was accomplished via transfection with a recombinant retrovirus, placing it in the second biosafety (BS) level of the BS classification system, according to the ATCC (www.lgcpromochem-atcc.com). The BS level is often the sole limitation of many researchers, lacking the capacity to work at a higher BS level than BS1. Therefore, the introduction of a standardized cell line belonging to the lowest BS level would be a significant contribution to PT epithelium research. The cells investigated in the current work, Caki-1, are commercially available and therefore avoid the extensive work of

isolation and culturing from fresh tissue. Caki-1 cells were originally obtained from a 49-year old male Caucasian patient, with a clear cell renal carcinoma. They have been employed thus far in various fields of research, where they have been shown to functionally express molecules relevant to the kidney and/or proximal tubule in vivo [49, 80-82].

The isolation of pure cultures of PT cells and the characterization of new PT systems is commonly accomplished via the detection of various morphological, physiological and biochemical unique characteristics [83]. Morphologically characteristic of PT cells is the presence of multiple mitochondria per cell and microvilli isolated to the apical portion of the cell membrane, the latter of which is associated with the polarized nature of the PT epithelium [84, 85]. Physiologically, the PT epithelium forms a 'leaky' low resistance barrier in vivo, which should be reflected in the cell culture model system [75, 86]. Biochemical analysis includes the detection of epithelial and/or PT specific markers such as the Na^+/H^+-exchanger isoform NHE3, alkaline phosphatase (AP), γ-glutamyl transferase (GGT), dipeptidyl peptidase IV (DPP IV), aminopeptidase M (APM) and Na^+/K^+-ATPase [87, 88]. Additionally, members of the epithelial barrier proteins of the tight and adherent junctions (AJ) including the cadherins, catenins, occludins, claudins and zonula occludens are often investigated [86, 89].

The focus of this study was to determine the relevance of the human renal carcinoma cell line, Caki-1, as a representative PT in vitro cell system, on the basis of a palette of morphological, physiological and biochemical characterizations.

3.3 Methods

Growth of Cells in Culture

Caki-1 and Caco-2 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig, Germany). Caki-1 and Caco-2 cells were grown in McCoy's 5A and Dulbecco's modified Eagle's medium respectively (Biochrom AG; Berlin, Germany) and supplemented with 10% FBS, (Biochrom AG), 1.14% penicillin/streptomycin (Biochrom AG) and 1.14% non-essential amino acid solution (Biochrom AG). Caki-1 McCoy's 5A medium was additionally supplemented with 0.7% L-glutamine (Biochrom AG). Primary human proximal tubule cell lots (HPT) were obtained from In vitro Technologies (In vitro Technologies; Baltimore, MD, USA) and were used at passage 0 without further subcultivation. HPT cells were grown in *Invitro*GRO PT complete medium (In vitro Technologies). All cell cultures were routinely grown in 75 cm^2 cell culture flasks and were maintained at 37°C, 5% CO_2 and 95% relative humidity.

Transmission Electron Microscopy (TEM)

Caki-1 cells grown on polycarbonate (PC) filters were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 for 1 h. The tissue was then post fixed with 2% OsO_4 in cacodylate buffer for 1 h at room temperature (RT), dehydrated in a graded series of ethanol (30-100%), infiltrated 2 times with propylene oxide and with a 1:1 mixture of propylene oxide and araldite resin overnight. For embedding, samples were transferred to

pure araldite resin (Plano; Wetzlar, Germany) and polymerized for 48 h at 60°C. Ultra thin sections were cut with a diamond knife on a Leica Ultracut S. Counterstained ultra thin sections were analyzed with a FEI Technai 12 BioTwin transmission electron microscope and imaged with a SIS MegaView III SCCD camera.

Transepithelial Electrical Resistance (TER)

For the routine determination of tightness and integrity of the monolayers, Caki-1 and/or HPT cells were seeded at a density of 100 000 cells per 1.13 cm^2 PC filter, with a filter pore size of 0.4 µm (Transwell™, Corning Costar Corporation; Cambridge, MA, USA). For standard TER measurements, cells were fed and TER was measured every second or third day for a time span of 7 d in a similar manner as has been previously reported [75]. Briefly, TER measurements were conducted at a constant RT (25°C ± 2°C) using a 'chopstick' electrode and Millicell-ERS device (Millipore; Schwalbach, Germany). The electrode was pre-calibrated for a minimum of 2 h in incubation medium prior to all measurements. The results were calculated as Ω cm^2 after subtracting the TER value of the membrane filter supports alone. The development of TER in Caki-1 cells was assessed over a time span of 10 d, when grown under routine conditions (see above). The influence of cell passage number and substratum type on the tightness of the Caki-1 monolayers was determined by observing the stability of the TER values between passages 8 and 71 and the employment of various growth substrata. All growth substrata were purchased from Corning Costar.

RT-PCR of NHE3

The RNA was isolated from the cells using the RNA STAT-60™ (Tel-Test Inc.; Friendswood, TX, USA), according to the company's protocol for RNA isolation. The RNA was purified using a DNA-free™ purification kit (Ambion Ltd.; Cambridgeshire, UK). Quantification of isolated RNA was based on spectrophotometric analysis (GeneQuant Pharmacia Biotech; Freiburg, Germany) at a wavelength of 260 nm (RNase-free water served as a blank). The integrity of the isolated RNA was checked by standard gel electrophoresis with 1% agarose in 1xTAE. The total RNA was reverse transcribed into cDNA using a SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen™ Ltd.; Paisley, UK), according to the manufacturer's guidance. cDNA was then amplified by PCR. The sense and antisense primer sequences used for the NHE3 detection were 5'-GGAAATCTTCCACAGGACCAT and 3'-CACTCATCTCCTCATCATAGTTGG respectively (Operon Biotechnologies GmbH; Cologne, Germany). The expected product size was 245 base pair (bp). The PCR was carried out using the Expand™ High Fidelity PCR System kit (Roche Diagnostics; Mannheim, Germany). Samples were initially heated for 4 min and 94°C and PCR amplification cycling conditions were the following: 1 min denaturation at 94°C, 2 min annealing at 60°C and 3 min extension at 72°C, for a total of 35 cycles. Subsequently, the amplified PCR products were analyzed by 2% agarose gel electrophoresis with ethidium bromide staining along with a 100 bp DNA ladder (GeneRuler™, Fermentas GmbH; Leon-Rot, Germany).

Immunofluorescent Visualization of Molecules

E-cadherin and Na^+/K^+-ATPase were visualized by indirect immunofluorescence labelling procedures using confluent cells grown on glass cover slips. The samples were fixed and permeabilized at -20°C in methanol for 10 min and subsequently dried at RT for 30 min. The cells were washed twice with PBS and incubated with Tween 20 (0.04% in PBS) for 20 min. After washing with PBS and blocking (0.5% cold-water fish gelatine (Sigma-Aldrich Chemie GmbH; Schnelldorf, Germany) plus 0.1% ovalbumin (Sigma-Aldrich) in PBS) for 30 min, the cells were incubated with primary antibody, anti- E-cadherin (BD Biosciences; Heidelberg, Germany) or Na^+/K^+-ATPase (Upstate Biotechnology; Lake Placid, NY, USA) diluted 1:25 and 1:200, respectively, in blocking solution, at 4°C overnight. The cells were washed with PBS and subsequently incubated with the secondary antibody conjugated to Alexa® 488 (Invitrogen GmbH; Karlsruhe, Germany) (1:400) and DAPI (1:8000) in blocking solution for 2 h in the dark. Washed cells were mounted in Mowiol 4.88 (Hoechst; Frankfurt, Germany). Mounted sections were examined by fluorescence microscopy with a Leica DMRP fluorescence microscope. Images were obtained with a Hamamatsu Orca ER CCD camera and processed with Adobe Photoshop (Adobe Systems; San Jose, CA, USA). To assess background labelling, all experiments included a series of samples in which primary antibodies were omitted. No significant labelling was observed for the controls. All experiments were repeated inter- and intra-day at a minimum of three replications with good reproducibility.

Assay of Cellular DPP IV and APM Enzyme Activity

DPPIV and APM enzyme activity was measured spectrophotometrically using Gly-Pro-pNA and L-alanine-nitroanilide hydrochloride as DPP IV and APM substrates respectively. The confluent cell monolayers in 96-well flat-bottomed microtiter culture plates were washed twice with PBS; 100µl of either 1 mM Gly-Pro-pNA or 1.66 mM L-alanine-nitroanilide hydrochloride in 0.1 M Tris buffer (pH 8.0) containing Trizma Base were applied and incubated at 25°C for 30 min. Real-time measurements were taken at 2 min intervals at a wavelength of 405 nm in a microplate reader. The enzyme activity was calculated after subtraction of the absorbance values for cell-free controls. Values are displayed as end concentrations of the reaction product p-nitroaniline, after 30 min. A standard curve was constructed using p-nitroaniline. All chemicals were purchased from Sigma-Aldrich.

GGT Staining

Caki-1 cells were grown in 24 multi-well plates with McCoy's 5A complete medium at a seeding density of 12000 cells/well until confluence. The cells were washed three times with 0.85% saline solution; 0.5 ml of completed reaction mixture (14 ml 0.85% saline, 5 ml 0.1 M Tris buffer pH 7.5, 10 mg Gly-Gly, 10 mg Fast Blue and 1 ml L-glutamic acid γ-(4-methoxy-ß-napthylamide) (GMNA) stock solution (15.2 ml distilled water, 0.4 ml DMSO, 0.1 ml NaOH and 10 mg GMNA)) was added and incubated at RT for 3 h in the dark. The cells were washed with 0.85% saline solution. Subsequently, each well was incubated with 1 ml of a 2.5% $CuSO_4$ solution for 2 min and washed with 0.85% saline solution. The cells were

fixed in a 50:50 glycerol : water solution. Controls were carried out with a reaction mixture containing serine borate. All chemicals were purchased from Sigma-Aldrich.

AP Staining

Caki-1 cells were grown in 24 multi-well plates in McCoy's 5A complete medium at a seeding density of 12000 cells/well. The cells were washed with PBS and fixed in a 4% formaldehyde mixture at RT. The cells were stained using a SIGMA FAST BCIP/NBT substrate solution (Sigma-Aldrich), dissolved in deionised water. Cells were incubated at 37°C for 30 min with the substrate solution. Positive bluish-purple staining was observed via light microscopy for the treated cells.

3.4 Results

Cell Morphology. With regards to general morphology, Caki-1 cells grow to form a complete monolayer after approximately 7-8 d in culture. Directly after seeding, up until 4-5 days post-plating, the cells take on the typical elongated shape of healthy (primary) PT cells and after growth into a dense monolayer, the cultures pack tightly to form a regular packed polygonal array of cells, characteristic of immortalized PT cells (fig. 1a and b, respectively).

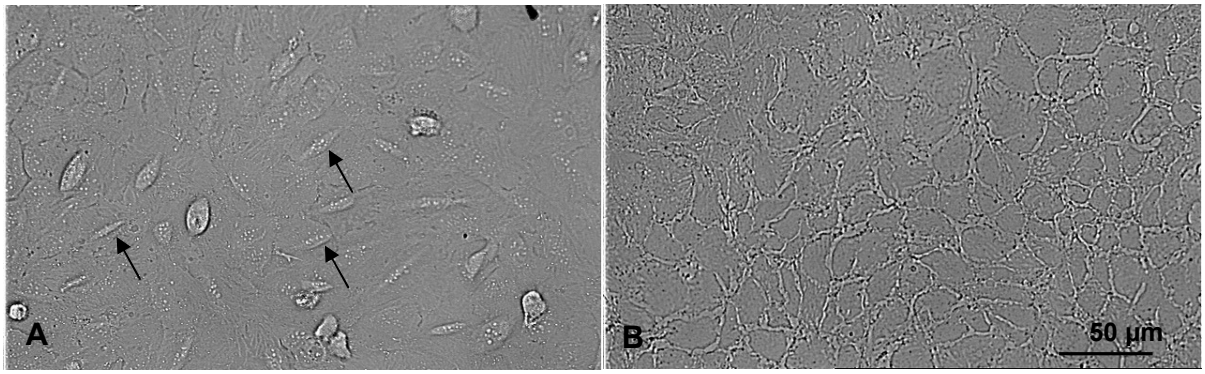

Figure 1. Morphology of Caki-1 cells. Caki-1 cells 4 days post-plating are elongated and characteristic of healthy PT cells (arrows) (A) and after 8 days post-plating a confluent monolayer of Caki-1 cells is formed with typical polygonal shaped cells, a characteristic of immortalized PT cells (B). Caki-1 cells were grown in 75 cm^2 tissue culture flasks and observed via phase contrast microscopy.

Cells grown in transwell plates on PC filters were analyzed by TEM. The ultrastructure of Caki-1 cells revealed an organized polarized epithelium, with apically located microvilli, multiple mitochondria and tight junctions were observed between adjacent cells (fig 2a-c).

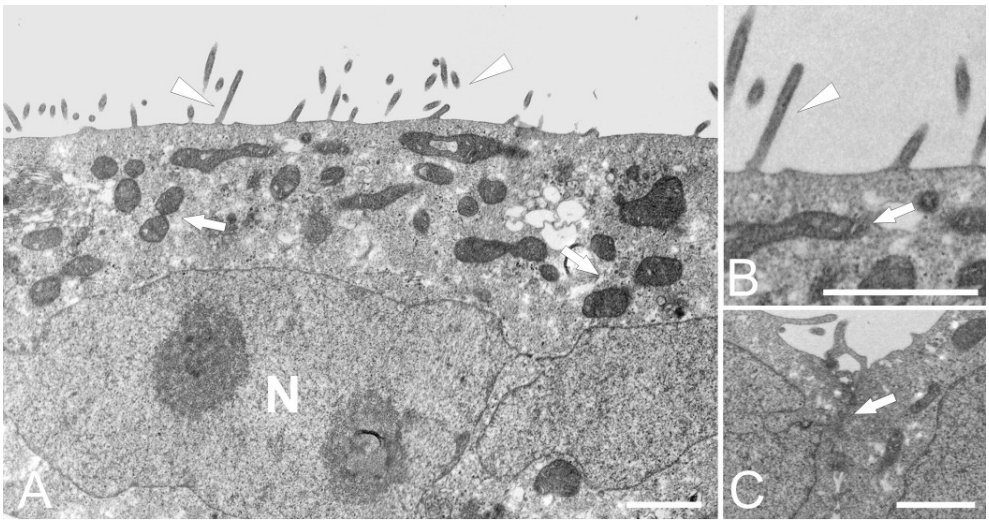

Figure 2. Transmission electron microscopic analysis of the ultrastructure of Caki-1 cells. Upon confluent monolayer formation on polycarbonate filters, which imparts cell polarity and differentiation, cells were fixed and sectioned for TEM analysis (see methods section). Caki-1 cells showed typical epithelial ultrastructure, including the cell nucleus (N), apically located microvilli (arrowheads) and multiple mitochondria (arrows) (A), an enlargement of the microvilli (arrowheads) and multiple mitochondira per cell (arrows) is depicted (B) and the apically located tight junctions with a looser association below the junctional areas (arrow) is shown (C). All scale bars represent 1 µm.

Identification of Marker Molecules. Marker molecules specific to epithelial cells and/or the human PT epithelium were selected for the characterization and confirmation of the epithelial and PT origin of Caki-1 cells. NHE3, GGT, APM, DPPIV and AP are characterized to be selective human PT cell markers. Shown in figure 3 are the amplification products of NHE3 detected in Caki-1 and Caco-2 cells; an expected band size of 245 bp was observed for both cell types. Caco-2 cells were used as a positive control since it has been previously confirmed to express the NHE3 isoform.

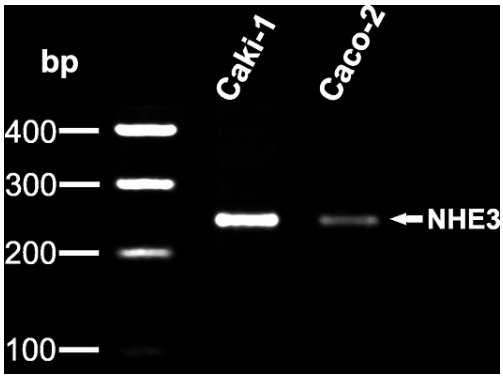

Figure 3. RT-PCR demonstrating NHE3 isoform mRNA expression in Caki-1 and Caco-2 cells. PCR products were electrophoresed on 2% agarose gel and stained with ethidium bromide. Lane 1 and 2 represent the 245 bp product determined for Caki-1 and Caco-2 cells respectively; Caco-2 cells were used as a positive control. Molecular weight markers were a 100-bp molecular ladder.

The presence of GGT in Caki-1 cells was indicated by the bright red staining profile (fig. 4a). No staining was observed with the negative control containing serine borate (fig. 4b).

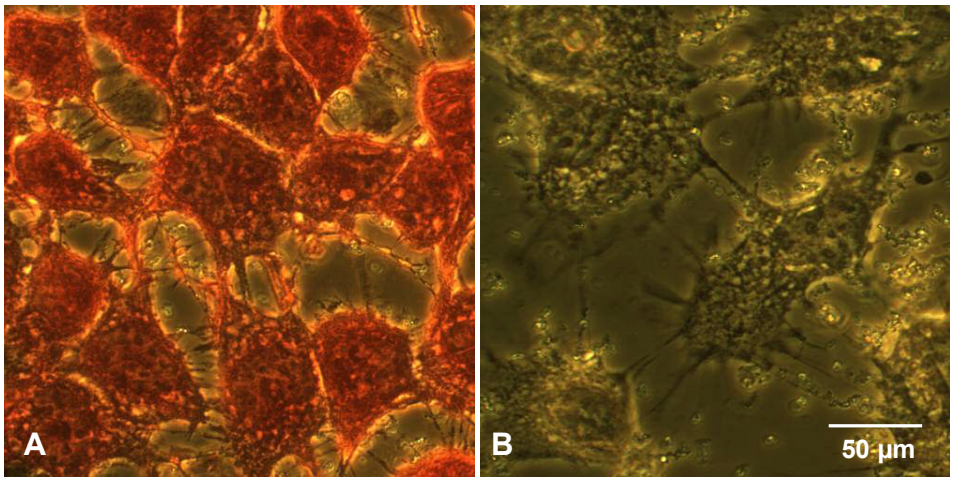

Figure 4. Cytochemical staining of GGT in Caki-1 cells. Cells grown in 24 multi-well plates were stained for GGT expression as described in the methods section. Positive GGT labeling was observed as a brilliant brownish-red staining (A) and negative controls containing serine borate, ran in parallel, were unstained (B).

The enzyme activity of APM and DPP IV were analyzed by the application of specific enzyme substrates (see methods section) and the representative end product (p-nitroaniline) accumulation. A linear increase in p-nitroaniline was observed over a time span of 30 min, with an end concentration of approximately 175 µM being produced by both enzymes/per well (fig. 5).

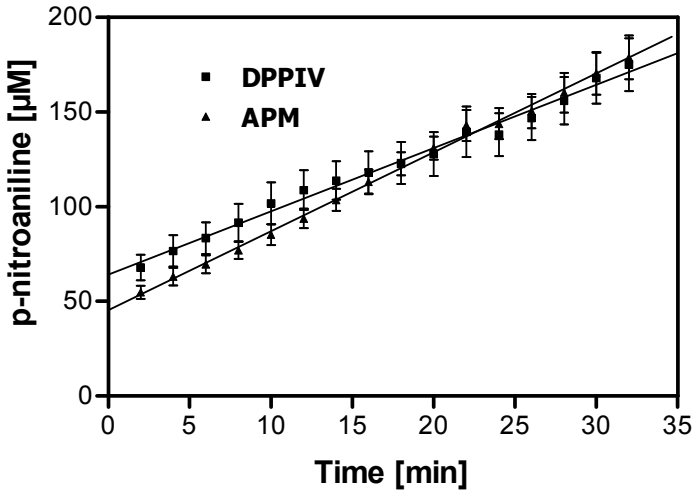

Figure 5. Determination of DPPIV and APM activity as assessed by p-nitroaniline accumulation. DPP IV or APM enzyme activity was measured spectrophotometrically using Gly-Pro-pNA and L-alanine-nitroanilide hydrochloride as DPP IV and APM substrates respectively over a time span of 30 minutes. All enzymatic activity determinations represent the mean of n=12 ± SEM.

AP expression was visualized as a deep purple staining along the cell membranes in Caki-1 cultures (fig. 6).

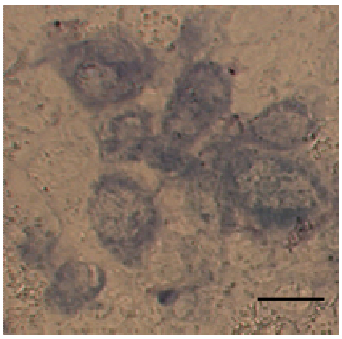

Figure 6. Cytochemical staining of AP in Caki-1 cells. Caki-1 cells were stained for AP using BCIP/NBT as a precipitating substrate. AP was located to the cell membranes in Caki-1 cells. Negative controls, without BCIP/NBT were run in parallel, no staining was observed (not depicted here). Scale bar represents 50 µm

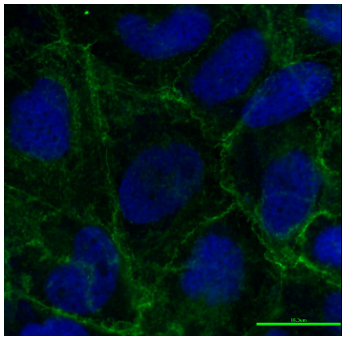

Figure 7. Expression pattern of E-cadherin in Caki-1 cells. Confluent monolayers of Caki-1 cells on glass coverlsips were processed for the visualization of E-cadherin (see methods section). E-cadherin was located to the cell-cell adhesions of Caki-1 cells.

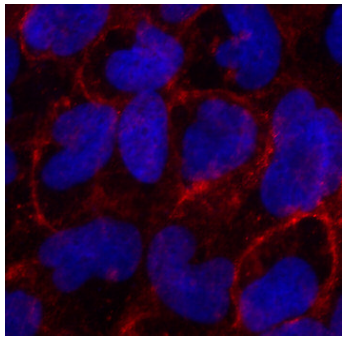

Figure 8. Expression pattern of the Na^+/K^+-ATPase in Caki-1 cells. Confluent monolayers of Caki-1 cells grown on glass coverslips were processed for the visualization of Na^+/K^+-ATPase (see materials and methods section). Na^+/K^+-ATPase was located to the cell membranes of Caki-1 cells.

Epithelial marker molecules included the epithelial barrier protein E-cadherin, known to be the most predominant cadherin protein found in vitro, in PT cells. Accordingly, immunostainings of E-cadherin in Caki-1 cultures indicated a sharp staining pattern located to the cell-cell contacts as observed in Figure 7. Na^+/K^+-ATPase known to be present in cells of epithelial origin was located to the cell membranes of Caki-1 cells via immunocytochemical studies (fig. 8).

Transepithelial Electrical Resistance (TER) Experiments. To determine the effect of passage number, the time course and extent of tight junction formation and the influence of substrata on tight junction formation in Caki-1 cells, TER values were measured. Several passages of Caki-1 cells between passage 8 and 71 were routinely cultivated (see materials and methods section) and were observed for changes in TER; no significant changes were observed with passaging, TER values consistently remained representative of a 'leaky' epithelium (fig. 9a). In a second experiment, Caki-1 cell monolayers were grown for 10 d on semi-permeable PC filters. A steady increase in TER until day 9 (32.55 ± 1.70 Ω cm^2) was observed and a significant decrease in TER was noticed on day 10 (13.5 ± 2.9 Ω cm^2), as can be seen in Figure 9b. In a series of TER measurements, the influence of substrata on tight junction formation was investigated. PC filters revealed the highest TER after 7 d in culture and collagen coated PC filters resulted in the leakiest monolayer

formation. Polyethylene (PE) and matrix-gel filters revealed similar TER values of moderate proportion in comparison to the PC and collagen coated PC filter TER values.

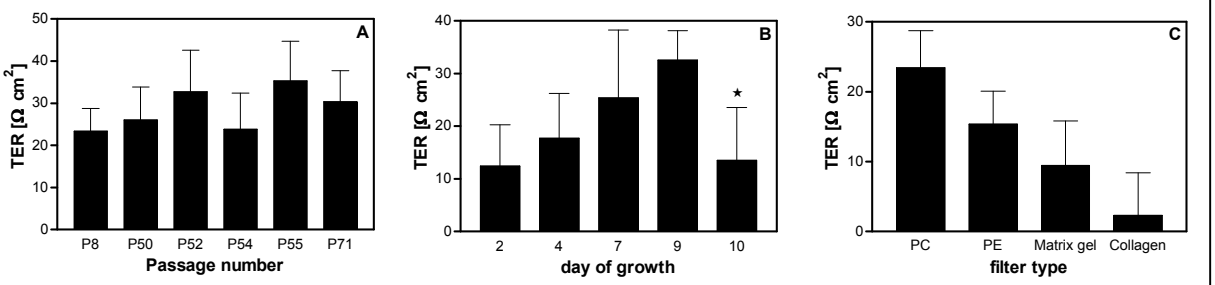

Figure 9. TER measurements of Caki-1 cells grown under varying conditions. Caki-1 cells were cultivated on polycarbonate (PC) filters between passages 8 and 71 and TER values were measured every second day for a duration of 7 days, no significant differences were observed (A), the development of TER in Caki-1 cells was observed for 10 days when cells were grown on PC filters, a significant decrease in TER was observed after day 9 as indicated by an * ($p<0.0001$) (B) and in a last series of experiments Caki-1 cells were cultivated on filters of polyethylene (PE), PC, collagen coated PC or matrix-gel, for a duration of 7 days and the changes in TER were observed (C). All values are expressed as the mean ± SEM of a minimum of 6 independent determinations.

TER values of Caki-1 and HPT cells, after routine cultivation were compared, with no significant differences observable (Table 1). Successful completion of monolayers was confirmed via light microscopy.

Table 1. The TER values were determined with cell monolayers 7 days post-plating. Caki-1 and HPT cells were grown on polycarbonate permeable inserts, at a seeding density of $1x10^5$ cells per 1.13 cm^2 filter support. TER measurements were taken every second day using a Millicell-ERS device. The results represent the mean ± SEM of a minimum of 6 independent determinations.

Cell Type	TER [Ω cm^2]
Caki-1	23.42 ± 1.54
HPT	19.04 ± 4.88

3.5 Discussion

Cell cultures provide researchers with a simplified system to focus directly on the epithelium, uninfluenced by higher order regulatory systems. The handling of primary cell cultures is tedious, time-consuming, costly and limited in regards to cell life span and cell number, should fresh human kidney tissue not be readily available. Therefore, the introduction of new PT cell lines with their unlimited life span would provide valuable tools

to the field of PT research. Unfortunately, many of the cell lines used for these purposes thus far, have not been critically evaluated and standardized or have not met the necessary requirements. As an example, the human PT cell line HK-2, is the most commonly used human PT cell model system, however, it has been discovered that it expresses abnormalities in the expression pattern of many molecules. It was confirmed to be an inadequate representation of the intact PT barrier [75]. Secondly, HEK cells of embryonic origin, are commonly used as an in vitro system for studying the human kidney, however, it may not adequately represent the mechanisms of the adult tissue. Furthermore, LLC-PK1 cells form moderately tight monolayers, reaching TER values greater than 213 Ω cm^2, indicating that they no longer reflect the 'leaky' epithelial characteristics of the human PT [75]. The majority of the information regarding the expression of such marker molecules is scattered among various cell systems originating from different species; therefore, the characterization of a single PT cell line of human origin, covering all aspects would be beneficial. Important to note here, finding an in vitro cell system which completely mimics the in vivo situation is also expecting the impossible, since it is common knowledge that functional differentiation is maintained only to a limited degree when cells of malignant tissue are cultured in vitro as monolayers [87].

The key objectives of this study were to confirm the PT origin of Caki-1 cells and to determine how closely this model system reflects in vivo PT epithelium, based on the following three points: (a) morphological characterization, (b) expression and function of epithelial barrier proteins and (c) the presence or functional activity of a palette of marker PT molecules.

Morphologically, PT cells isolated from healthy tissue, grow in the form of a single layer of elongated cells [90]. PT cells of immortalized and cancerous origin have been previously described to grow as a monolayer of polygonal shaped cells. Caki-1 cells, during the initial phases of growth take on this typical healthy PT cell form and revert to a polygonal shape (cobblestone-like pattern) after a dense monolayer has been established, after approximately 7 days in culture. Reversion to the latter form may be a result of competition for space (contact inhibition) and should serve as an indicator that these cells should be allowed to grow to a maximum of 90-95% confluence in order to retain their healthy PT cell shape. Furthermore, a significant decrease in TER of Caki-1 cells was observed after 9 days in cultivation, indicating that deteriorations to the monolayer is occurring, perhaps as a direct result of the detachment of cells from the filter surface. In regards to the cell structure of polarized PT cells they are often characterized via two observations: the presence of multiple mitochondria per cell and the apically located microvilli; both were observable in Caki-1 cells demonstrating that these tubular cells are able to grow in a functional polarized fashion in vitro [91].

Physiologically, the PT epithelium forms a 'leaky' barrier in vivo, with a TER ranging from 5-12 Ω cm^2 [75, 86] . This TER value is representative of the interactions of many adhesion molecules of the tight and adherent junctions located between epithelial cells. These adhesion junctions were visible in Caki-1 cells. Nephrotoxic injury often involves the disruption of barrier function and hence alterations in the permeability and/or polarity of the PT epithelium [75]. The TER values of the Caki-1 and HPT cell systems were

approximately 23 and 19 Ω cm^2 respectively, when grown on PC filter supports. Both values are slightly higher than the expected in vivo TER but nevertheless representative of a 'leaky' epithelium. It should be noted that in the current study, a relatively simple volt-ohm meter was used to determine TER measurements. This is a valid and commonly used technique for evaluating changes in relative TER, however its sensitivity and reliability in determining absolute electrical resistances have been questioned [75]. Nevertheless, the validity of the results can be confirmed by observing the consistency in data across the series of experiments which were conducted in the Caki-1 cells. Significant to the current studies on TER was the demonstration that Caki-1 cells adequately represent a 'leaky' epithelium with resistances in the range of what is expected in vivo. To put the leakiness of the PT epithelium into perspective, one should consider that tight epithelia such as the human bladder, can reach TER values up to 300 000 Ω cm^2 [86]. E-cadherin was selected as a marker molecule of the epithelial barrier proteins since it has been classified to be one of the most significant AJ molecules involved in the maintenance of the epithelial barrier [75, 92, 93]. In vitro it has been proven to be the most common cadherin found in PT cells, and was strongly visible in the Caki-1 cells via immunocytochemical techniques. However, in vivo it has been suggested that N-cadherin is the most prominent cadherin located in the PTs, indicating that cadherin expression may be altered during in vitro cultivation of immortal PT cell lines [93]. This de-differentiation of Caki-1 cells should be taken into consideration when in vitro research studies are concerned with tight junction formation and/or activity.

The selective isolation of PTs from kidney tissue is most often confirmed by the expression of specific marker molecules [90]. The most commonly used and characterized marker molecules of human PTs are NHE3, GGT, APM, DPPIV and AP [2, 72]. Expression and or activity of all of these proteins, using previously published techniques, was confirmed for Caki-1 cells, verifying the PT nature of this cell line. The origin and purity of the commercially available HPT cells were as well partially confirmed by the aforementioned techniques. Previous studies have stated that a full expression and sorting of apical and basolateral proteins to their respective domains requires a high degree of differentiation [94, 95]. The basolaterally positioned Na^+/K^{+-}ATPase was located to the basolateral membrane in Caki-1 cells and other proteins to the apical membrane, providing further proof that the Caki-1 cells retain an extended well differentiated growth pattern in vitro [49, 86, 92, 96].

The Caki-1 cell line has been previously used for various fields of research including nephrotoxicity, general cytotoxicity, physiology, and carrier-mediated drug transport studies. In respect to drug transport, PTs are the major site of active drug reabsorption and secretion in the kidney, therefore, as one example, this data provides support for previous studies, which employed Caki-1 cells as a representative model for kidney drug transport [49, 97, 98]. However, to our knowledge no single comprehensive study confirming the PT nature of Caki-1 cells via morphological, physiological and biochemical characterization, has been previously published. Caki-1 cells show considerable potential as a PT cell model system for the following reasons: (1) Caki-1 cells possess the typical membrane proteins NHE3, GGT, APM, DPPIV and AP suggesting a normal PT phenotype is retained, (2) Caki-1 cells react to antibodies specific to E-cadherin and Na^+/K^+-ATPase, specific to in vitro PT

and/or epithelial cells, (3) Caki-1 cells retain functional activity in respect to specific proteins of the PT, including drug transport proteins and metabolizing enzymes, (4) Caki-1 cells represent a polarized adherent epithelium, (5) Caki-1 cells are classified BS1 and (6) Caki-1 cells were derived from human adult kidney tissue. Important to note here, certain results present in this work are in contradiction to previous results published regarding Caki-1 cells. In a previous study, it has been stated that Caki-1 cells do not express AP and do not form discrete monolayers [99]. Based on these criteria Caki-1 cells were not considered to be an accurate representation of the PT epithelium. In contrast, our work indicated a membrane localization of AP, the presence of E-cadherin at the tight junctions, the constant formation of a 'leaky' monolayer independent of passage number and substrata and additionally formed polarized monolayers as observed via TEM.

In light of these findings, the Caki-1 cell line appears to be a suitable, convenient and reliable new tool for PT epithelium research, which is commercially available and represents the first characterized human PT cell line classified to be BS1. Caki-1 cells have already shown promising results in many fields of research. Important to note is that this cell line has been proven to phenotypically resemble the PT epithelium in many ways but a full characterization does require further studies and is currently underway. Immortalization may also lead to the acquisition of atypical properties; therefore methods should be tailored to fit the immediate purpose. The investment into in depth characterizations of other commercially available cell lines could unveil further interesting and useful information.

3.6 Acknowledgements

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4. mRNA expression profiles of ATP-binding Cassette, Solute Carrier and Organic Anion Transporting Polypeptide transporters and Cytochrome P450 metabolizing enzymes in Caki-1 and primary human proximal tubule cells

(Running chapter head: Gene expression of transporters and drug metabolizing enzymes)

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4.1 Abstract

The mRNA expression level of 42 transport proteins and drug metabolizing enzymes (DMEs) belonging to the ATP-binding cassette (ABC), solute carrier (SLC), organic anion transporting polypeptide (SLCO) and cytochrome P450 (CYP P450) superfamilies were determined and compared between a human proximal tubule (PT) cell line, Caki-1, and primary human proximal tubule (HPT) cells. The mRNA expression level of each target protein or enzyme was analyzed from total RNA by real-time reverse transcription PCR (RT-PCR), using an ABI PRISM 7900ht Sequence Detection System and 384 microfluidic cards. Twenty-three mRNA transcripts were present in both Caki-1 and HPT cells, 19 commonly expressed between the two cell types. These initial results support the use of Caki-1 cells as a valid screening system for drug-drug interactions and drug metabolism studies, by-passing the difficulties of working with primary cell culture systems.

4.2 Introduction

In the recent years it has become clear that drug disposition is not solely based on physiochemical properties such as lipophilicity and molecular weight. Carrier-mediated transporters play a vital role in the absorption, distribution and elimination of drugs, and are a partial determinant of the pharmacodynamic and pharmacokinetic properties of many therapeutic agents [100]. In order for drugs to exert their pharmacological effects they must reach the target site at sufficient concentrations and in order to accomplish this, certain drugs must traverse the biological membranes via carrier-mediated proteins (active and facilitated transport processes). In the brain, liver, intestine and kidney drug transporters are generally classified into the ATP-binding cassette (ABC), solute carrier (SLC) and organic anion transporting polypeptide (SLCO) transporter superfamilies and are primarily involved in the secretory barrier function of these organs. These transporters may therefore be promising candidates for targeted drug delivery in many tissues.

The kidney has a crucial function in regulating whole body homeostasis. It is involved in the excretion of endogenous metabolic waste products and xenobiotics. The functional unit of the kidney is the nephron, consisting of the glomerulus and the cortical and medullary tubular systems. It has become clear that certain transporters belonging to the aforementioned superfamilies play crucial roles in the active and facilitated reabsorption and secretion of endogenous and exogenous compounds in the PTs [101]. For example, it is known that SLC22A2 and SLC22A3 subtypes are responsible for the carrier-mediated secretion of cations, SLC22A5 for the reabsorption of l-carnitine, and many ABCs such as, ABCB1, ABCC4 and ABCC3, are involved in the last step of anion secretion in the kidney.

The ABC transporter superfamily consists of ATP-dependent efflux transporters with broad substrate specificities; a total of 49 genes belonging to this family have been identified [102]. The superfamily is divided into seven subfamilies; the ABCB, ABCC and ABCG subfamilies are considered to be the main families involved in the distribution of xenobiotics [100]. SLC transporters are equally wide ranging in tissue distribution and substrate specificity, with the SLC6A, 10A, 15A, 16A, 17A, 22A and 29A subfamilies playing

significant roles in drug disposition [100]. The SLCO superfamily, formerly part of the SLC family, consists of eleven sodium-independent large organic anion transporters. SLCO subtypes are expressed in a variety of different tissues and are responsible for the transport of a broad group of substrates [103]. Reviews containing the specifics of these three superfamilies can be accessed elsewhere and will not be discussed in further detail within the current work [104-107].

DMEs also play a central role in metabolism, elimination and detoxification of xenobiotics and drugs entering the human body. Various DMEs are well distributed throughout the tissues, including phase I, phase II and ancillary metabolizing enzymes. The kidney plays a limited albeit significant role in drug metabolism. Several phase I and II, and ancillary DMEs are active in the human kidney [108]. Phase I DMEs consist primarily of isozymes belonging to the cytochrome P450 (CYP 450) superfamily of microsomal enzymes. In the kidney CYP 450 isoforms are responsible for the metabolism of endogenous substances including vitamins, steroids and fatty acids and are additionally involved in the detoxification of xenobiotics [109]. For the kidney, it is believed that xenobiotics and drugs are metabolized by the CYP subfamilies CYP1, CYP2 and CYP3 [110].

In vitro research of transport proteins and DMEs commonly involves the use of established cell lines. Cell lines provide researchers with convenient tools for studying toxicological effects in vitro. Such methods allow the investigator to reduce the number of variables and work under defined conditions with relative ease, in comparison to working in vivo or with primary cell cultures. Many in vitro cell systems exist for studying drug transport and metabolism. However, the number of characterized human systems at the cell level is minimal. Transport and metabolism studies are often complicated by species differences and selective tissue distribution. The purpose of this study was to investigate the expression of relevant xenobiotic transport proteins and DMEs in the commercially available renal clear cell (RCC) carcinoma cell line Caki-1 and to introduce an in vitro assay system for predicting toxicology via transport proteins and DMEs. Caki-1 cells were obtained from a 54-year old Caucasian patient suffering from RCC carcinoma. Caki-1 cells are frequently used for toxicology research but little information is available concerning the capacity of these cells to functionally transport and metabolize probe substrates. We and others have previously used Caki-1 cells to functionally characterize the carrier-mediated transport processes of two known carrier proteins, OCT3 and OCTN2 [49].

The data available to date on transporter/enzyme function and expression is a compilation of results from many researchers using various approaches. Expression profiling can be accomplished by a wide array of techniques including RT-PCR, northern blotting, western blotting and immunohistochemical methods. Therefore, the goal of the current work was to determine the relative expression of transport proteins and DMEs present in the commercially available Caki-1 and HPT cells, under consistent and identical experimental conditions via a high-throughput screening method. The rapid estimation of 42 genes from the ABC, SLC, SLCO and CYP superfamilies were examined using a real-time RT-PCR technique and TaqMan analysis.

4.3 Materials and Methods

Growth of Cells in Culture

Caki-1 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ, Braunschweig, Germany). Caki-1 cells were grown in McCoy's 5A medium (Biochrom AG, Berlin, Germany) supplemented with FBS 10%, (Biochrom AG), penicillin/streptomycin 1.14% (Biochrom AG), non-essential amino acid solution 1.14% (Biochrom AG), and stable L-glutamine 0.7% (Biochrom AG). Three individual lots of primary human proximal tubule cells (HPT) were used at passage 0 without further subcultivation. HPT cells were grown in *Invitro*GRO PT complete medium (In vitro Technologies, Baltimore, MD, USA). Both cell cultures were maintained at 37°C, 5% CO_2 and 95% relative humidity.

RNA Isolation and Reverse Transcription to cDNA

RNA was isolated using RNA STAT-60™ (Tel-Test Inc., Friendswood, TX, USA) and performed according to the products manual. RNA preparations were treated with DNase DNA-free™ (Ambion Ltd., Huntingdon, Cambridgeshire, UK) and the quantity and purity of the RNA samples was determined using a GeneQuant pro RNA/DNA calculator. The RNA integrity was assessed by visualizing the sharpness of ribosomal RNA bands on a 1% agarose gel.

cDNA was prepared from total RNA using Superscript™ First-Strand Synthesis System for real-time RT-PCR (Invitrogen Ltd., Paisley, UK) according to the product manual. The two step reaction mixture contained 2 µg RNA, 100 ng random hexamers, 0.5 mM dNTP mix (dATP, dCTP, dGTP, dTTP), 10 mM Tris-HCl (pH 8.4), 25 mM KCl, 5 mM $MgCl_2$ 10 mM DTT, 40 units RNAseOUT™ recombinant ribonuclease inhibitor. Incubation was performed at 65°C for 5 min and 25°C for 2 min, with subsequent incubation after the addition of Superscript II, for 10 min at 25°C, followed by 42°C for 50 min. The reaction was terminated by heating at 70°C for 15 min.

TaqMan RT-PCR Conditions

TaqMan® real-time PCR was carried out in 384-well reaction cards and an ABI PRISM® 7900ht Sequence Detection System. Genes were selected based on their potential roles in drug disposition. Assay-on-Demand™ probe products were used and probe IDs and sequences can be found in Table 1. All TaqMan® equipment was obtained from Applied Biosystems (Foster City, CA, USA). TaqMan® analysis was performed in a 1µl reaction mixture containing 2 ng RNA converted to cDNA, 1x TaqMan® Universal PCR Master Mix (containing AmpliTaq Gold DNA polymerase, dNTPs with dUTP, passive reference and optimised buffer), 900 nM each of custom designed forward and reverse primer and 250 nM of custom-designed probe. Cycling conditions were as follows: 2 min at 50°C, 10 min polymerase activation, at 95°C and 40 cycles at 95°C for 15 s and 60°C for 1 min.

Table 1. Probes used for RT-PCR analysis

Gene Symbol	Common Name	Assay-on-Demand™ ID	Probe Sequence (5' FAM → 3' NFQ)
ABCB1	MDR1	Hs00184500_m1	GGAAGACATGACCAGGTATGCCTAT
ABCB11	BSEP	Hs00184824_m1	GTTCAAGGGGCTGCCGGCTCTCAGA
ABCB4	MDR3	Hs00240956_m1	CATCAGCAGCAAACAAAAAAGGAAA
ABCC1	MRP1	Hs00219905_m1	GGAAGACATGACCAGGTATGCCTAT
ABCC2	MRP2	Hs00166123_m1	ACCTCCAACAGGTGGCTTGCAATTC
ABCC3	MRP3	Hs00358656_m1	CCACAGCTGCTCAGCATCCTGATCA
ABCC4	MRP4	Hs00195260_m1	ATCACAAGAAAAGGTTGGCATTGTG
ABCC5	MRP5	Hs00194701_m1	GAGCAGGGGCGCAGGAATTCTGATG
ABCC6	MRP6	Hs00184566_m1	GGGAGCAGGGCATGAATCTCTCCGG
ABCG2	BCRP	Hs00184979_m1	TCCAAGGTTGGAACTCAGTTTATCC
SLC10A1	NTCP	Hs00161820_m1	CATGAACCTCAGCATTGTGATGACC
SLC10A2	IBAT	Hs00166561_m1	ACCCTGGTACAGGTGCCGAACGGTT
SLC15A1	PEPT1	Hs00192639_m1	GGCTGGGAAAGTTCAAGACCATTGT
SLC15A2	PEPT2	Hs00221539_m1	GTGGTTGGGAAAATTCAAGACAATC
SLC16A1	MCT1	Hs00161826_m1	CCACCACTTTTAGGTCGGCTCAATG
SLC16A4	MCT4	Hs00190794_m1	ACCACCTATAGCAGGCTGGTTATAT
SLC22A1	OCT1	Hs00427550_m1	GCTCTACTACTGGTGTGTGCCGGAG
SLC22A2	OCT2	Hs00533907_m1	CATAGCAGACAGGTTTGGCCGTAAG
SLC22A3	OCT3	Hs00222691_m1	TCTGATCATCTTTGGTATCCTGGCA
SLC22A4	OCTN1	Hs00268200_m1	TTGCTGCTATGGATGCTGACCTCAG
SLC22A5	OCTN2	Hs00161895_m1	CAGACAGGTTTGGCCGGAAGAATGT
SLC22A6	OAT1	Hs00537914_m1	CTCCATGCTGTGGTTTGCCACTAGC
SLC22A7	OAT2	Hs00198527_m1	CTCCATGCTGTGGTTTGCCACTAGC
SLC22A8	OAT3	Hs00199599_m1	TCTGACAGGTTTGGCCGCAGGCCCA
SLC22A9	OAT4	Hs00375768_m1	CCCTAACCCTGGAGATTTTGAAATC
SLC22A11	OAT4/UST3	Hs00218486_m1	GTGGCCAAGTGGGACCTGGTGTGCA
SLC28A3	CNT1	Hs00223220_m1	GGCTGAAGTGGGTGATCTGGAGCTC
SLC29A1	ENT1	Hs00191940_m1	TCAGCAGGCCCCTGAGGGAGGGAGC
SLCO1A2	OATPA	Hs00366488_m1	CTTGAGGTGTATGAAATCTGAAGAG
SLCO1B1	OATPC	Hs00272374_m1	ATAATTCCACATCATTTTCAAGGGT
SLCO1B3	OATP8	Hs00251986_m1	TGCTAACTTTTTGTTGGGAATCATA
SLCO1C1	OATPF	Hs00213714_m1	TACCAAGGAACCAAACCTGTCTCTT
SLCO2B1	OATPB	Hs00200670_m1	CCTGCCAGGAAGGGCAAGGACTCTC
SLCO3A1	OATPD	Hs00203184_m1	TCCTCGCTCTATATAGGAATCCTGT
SLCO4A1	OATPE	Hs00249583_m1	TCAGAGACCTGCCTCTCTCCATCTG
SLCO4C1	OATPH	Hs00698884_m1	TGCTATGGGAGAAAGCACTGATGTC
CYP1A1		Hs00153120_m1	TCAGTACCTCAGCCACCTCCAAGAT
CYP1A2		Hs00167927_m1	TATCAGGACTTTGACAAGAACAGTG
CYP2C9		Hs00426397_m1	CCCAGAGGTCACAGCTAAAGTCCAG
CYP2D6		Hs00164385_m1	GCGTTCCCAAGGGGTGTTCCTGGCG
CYP3A4		Hs00604506_m1	GTCCTACCATAAGGGCTTTTGTATG
CYP3A5		Hs00241417_m1	AAAATGTGGGGAACGTATGAAGGTC
RPLPO	Ribosomal phosphoprotein large P0	Hs99999902_m1	ATGTTTCATTGTGGGAGCAGACAAT

Data Analysis

Samples were deemed positive at any given cycle when the value of the emitted fluorescence was greater than the threshold value calculated by the instruments software. The threshold cycle (C_T) indicates the cycle at which the amplified target reaches a fixed threshold (approximately 15-fold greater than the standard deviation of the baseline) and is defined as the mean of a minimum of three replicates. Relative expression was calculated using the ΔC_T method (ΔC_T is obtained by subtracting the C_T value of the endogenous control, RPLP0, from the C_T value of the target mRNA), as employed in previous studies. Specifically, the amount of target mRNA relative to RPLP0 is expressed as $2^{-(\Delta C_T)}$. RPLP0 was selected as the endogenous control to normalize the PCRs for the amount of RNA added to the reverse transcription reactions.

4.4 Results

The mRNA expression levels of 42 human transport proteins and DMEs in Caki-1 and HPT cells are summarized in Tables 2-5. Analysis was conducted by real-time RT-PCR using and ABI PRISM 7900ht Sequence Detector System in combination with TaqMan® analysis. Genes were selected based on their potential roles in drug disposition. Assay-on-Demands™ probe products were used and probe IDs and sequences can be found in Table 1.

Initial experimental design involved the selection of an appropriate endogenous control. Several house-keeping genes were tested and the large ribosomal protein (RPLP0) mRNA showed the lowest variability (between samples) among the house-keeping genes tested and was therefore selected as the endogenous control for all future experiments (data not shown).

Table 2. Relative expression of ABC transporter mRNAs in Caki-1 and HPT cells. All values are expressed as $2^{-(\Delta C_T)}$ relative to the endogenous control RPLP0.
BLQ = below the level of quantification.

Gene symbol	Common name	Caki-1	HPT
ABCB1	**MDR1**	0.049801981	0.009568212
ABCB11	**BSEP**	BLQ	BLQ
ABCB4	**MDR3**	0.000196566	0.006120338
ABCC1	**MRP1**	0.065171678	0.014465737
ABCC2	**MRP2**	0.000497121	0.007347632
ABCC3	**MRP3**	0.155253742	0.056751951
ABCC4	**MRP4**	0.192441386	0.053525264
ABCC5	**MRP5**	0.003003082	0.00394342
ABCC6	**MRP6**	0.000187391	0.001551584
ABCG2	**BCRP**	0.001577501	BLQ

The mRNA expression of ABC transporters in Caki-1 and HPT cells is shown in Table 2. MRP3 and MRP4 (ABCC3 and 4, respectively) were found to be the highest expressed ABC transporters among the two cell types. Relative expression was generally higher for Caki-1 cells as compared to HPT cells with the exception of MRP2 and MRP6. MDR1 and MRP1 (ABCB1 and C1, respectively) were moderately expressed in both cell types. A lower expression or expression below the limit of quantification (BLQ) was determined for the remaining ABC transporters.

Table 3. Relative expression of SLC transporter mRNAs in Caki-1 and HPT cells. All values are expressed as $2^{-(\Delta C_T)}$ relative to the endogenous control RPLP0.
BLQ = below the level of quantification.

Gene symbol	Common name	Caki-1	HPT
SLC10A1	**NTCP**	BLQ	BLQ
SLC10A2	**IBAT**	BLQ	BLQ
SLC15A1	**PEPT1**	BLQ	0.013366976
SLC15A2	**PEPT2**	BLQ	0.001939629
SLC16A1	**MCT1**	0.125911901	0.026039313
SLC16A4	**MCT4**	0.129776826	0.038436514
SLC22A1	**OCT1**	0.000139123	BLQ
SLC22A11	**OAT4**	0.001193632	0.001820209
SLC22A2	**OCT2**	BLQ	0.013336423
SLC22A3	**OCT3**	0.028952525	0.006320223
SLC22A4	**OCTN1**	0.000735523	BLQ
SLC22A5	**OCTN2**	0.044186101	0.026707592
SLC22A6	**OAT1**	BLQ	BLQ
SLC22A7	**OAT2**	BLQ	BLQ
SLC22A8	**OAT3**	BLQ	BLQ
SLC22A9	**OAT4**	BLQ	BLQ
SLC28A3	**CNT1**	BLQ	BLQ
SLC29A1	**ENT1**	0.002247954	0.028048291

The mRNA expression levels of 18 SLC transporters are summarized in Table 3. Highest expression among the SLCs in Caki-1 cells was observed for MCT1 and MCT4 (SLC16A1 and 4, respectively), with moderate expression for OCT3 and OCTN2 (SLC22A3 and 5, respectively). HPT exhibited moderate expression for PEPT1 (SLC15A1), MCT1, MCT4, OCT2 (SLC22A2), OCTN2 and ENT1 (SLC29A1). All other SLCs were either expressed to a low level or BLQ for both cell types. Expression was overall higher in Caki-1 cells except for ENT1.

The mRNA expression levels of 8 SLCO transport proteins are summarized in Table 4. OATPE and OATPH (SLCO4A1 and SLCO4C1, respectively) were found to have a relatively high expression in Caki-1 cells. HPT cells expressed moderate levels of OATPE and OATPH. All other SLCO proteins were expressed to low levels in Caki-1 and HPT cells or were BLQ.

Table 4. Relative expression of SLCO transporter mRNAs in Caki-1 and HPT cells. All values are expressed as $2^{-(\Delta C_T)}$ relative to the endogenous control RPLP0. BLQ = below the level of quantification.

Gene symbol	Common name	Caki-1	HPT
SLCO1A2	**OATPA**	BLQ	BLQ
SLCO1B1	**OATPC**	BLQ	BLQ
SLCO1B3	**OATP8**	0.000226416	BLQ
SLCO1C1	**OATPF**	BLQ	BLQ
SLCO2B1	**OATPB**	BLQ	0.000956071
SLCO3A1	**OATPD**	0.002808919	0.006122112
SLCO4A1	**OATPE**	0.122609974	0.042178085
SLCO4C1	**OATPH**	0.524654428	0.058463443

The mRNA expression level of 6 CYP 450 isoforms is displayed in Table 5 for Caki-1 and HPT cells. The CYP 1A1 and CYP 3A5 isoforms were expressed to similar extents in Caki-1 and HPT cells. All other isoforms were BLQ.

Table 5. Relative expression of CYP 450 drug metabolizing enzyme mRNAs in Caki-1 and HPT cells. All values are expressed as $2^{-(\Delta C_T)}$ relative to the endogenous control RPLP0.
BLQ = below the level of quantification.

Gene Symbol	Caki-1	HPT
CYP1A1	0.001753076	0.004042285
CYP1A2	BLQ	BLQ
CYP2C9	BLQ	BLQ
CYP2D6	BLQ	BLQ
CYP3A4	BLQ	BLQ
CYP3A5	0.001593795	0.000785912

4.5 Discussion

Drug transporters and DMEs are major determinants of the bioavailability and disposition of endogenous and exogenous compounds introduced into the human body. The kidney and liver are two major organs involved in drug disposition, therefore, the knowledge of transporter and DME expression is very valuable for drug development and targeting. In vitro experiments are standard precursors to clinical studies and the availability of established systems would contribute to pre-clinical applications.

The current work provides a comprehensive overview of the expression of relevant transport protein and DME genes in a commercially available kidney cell line and commercially available HPT cells. The protein and enzyme products are important to the pharmaco- and toxicokinetics of many commonly administered drugs and perhaps for those in the developmental stages. Investigations in these studies concentrated on subtypes of the ABC, SLC and SLCO transporter superfamilies known to be expressed in the human kidney and involved in xenobiotic disposition [107]. DMEs of the CYP 450 superfamily, belonging to the CYP1, CYP2, and CYP3 subfamilies were examined, since they are considered to be most active in the metabolism of xenobiotics and drugs [111].

The ABC transporter superfamily is one of the broadest expressed transporter superfamilies known and is extremely well characterized. ABC transporters are responsible for the active transport of a variety of compounds across biological membranes and can actively confer multi-drug resistance. The most well known, ABCB1, is more commonly referred to as MDR1 or P-glycoprotein (P-gp). MDR1 has been shown to be significantly expressed in the proximal tubular epithelium of the kidney; therefore the expression of MDR1 in Caki-1 and HPT cells was in accordance [12]. BSEP (ABCB11), more commonly known as the bile salt export pump, MDR3 (ABCB4) and MRP6 (ABCC6) belong to the major hepatobiliary membrane transporters and function together with subtypes of the SLC and SLCO superfamilies in the process of bile acid transport in the liver [112]. BSEP is almost exclusively expressed in the liver which explains its absence in Caki-1 and HPT cells [113]. This is additionally consistent with what has been previously reported for the lack of BSEP expression in the porcine and canine proximal tubule cell lines LLC-PK1 and MDCKII respectively [114]. The expression of MDR3 and MRP6 has been reported for the human kidney, explaining the expression in Caki-1 and HPT cells [115]. MRP1, 3 and 5 (ABCC1, 3 and 5 respectively), also partially involved in the transport of bile acids, have been found to have a wide tissue distribution, including relatively high expression in the kidney (basolateral membrane of proximal tubule cells), confirming the expression determined for MRP1, 3 and 5 in Caki-1 and HPT cells [102]. MRP2 (ABCC2) is responsible for the transport of conjugated drug metabolites and toxins into the bile and urine, which is consistent with its relatively high expression levels in liver canalicular membranes and apical membranes of proximal tubules and expression in Caki-1 and HPT cells [116]. MRP2 has as well been detected in renal cancers [116]. MRP4 (ABCC4) reported to be highly enriched in the proximal tubule of the human kidney was the highest expressed MRP subtype in Caki-1 and HPT cells [117]. BCRP (ABCG2) expression has been confirmed in humans, being predominantly expressed in the liver and brain of humans, explaining the low levels and/or absence of expression in Caki-1 and HPT cells.

BCRP has been detected in the kidney where it is most likely involved in the secretion of xenobiotics into the lumen. BCRP has as well been previously detected in patients suffering from RCC: the origin of Caki-1 cells [118]. Overall, ABCs are expressed in malignant and healthy tissue and are involved in the protection of tissues from xenobiotic accumulation and resulting toxicity. Multi-drug resistance in malignant tissue to multiple anticancer agents is however, a major impediment to the success of many treatment regiments.

In regards to the expression of SLC members, they exhibit a wide tissue distribution in many species. SLC22A subtypes appear to be the most significantly expressed transporters in the kidney, involved in the renal clearance of small organic cations and anions. OCT2, OCTN1, OAT1, OAT3 and OAT4 (SLC22A2, A4, A6, A8 and A9) have been confirmed in many studies to have a relatively high expression in the kidney but Caki-1 and HPT cells showed low levels of expression, indicating that expression is apparently lost during in vitro culturing of these cells [101]. Only OCTN1 was expressed to a relatively low extent in Caki-1 cells and OCT2 was moderately expressed in HPT cells. In agreement with the literature OCTN2 was expressed in Caki-1 and HPT cells [100]. The relative expression of OCT3 and OCTN2 in Caki-1 cells correlates with data obtained from patients with RCC [101]. It is necessary to take into consideration that a different expression pattern between human kidney and the cell systems investigated may as well be the consequence of the probe designs where no detection for both cell types was observed (e.g. OAT1-4). OCT1 (SLC22A1) is strongly expressed in the liver and weakly in the kidney, as seen by the weak or absence of expression in Caki-1 and HPT cells. A second transport protein also represented by the abbreviation OAT4 (SLC22A11) is primarily expressed in the placenta and kidney, where it is responsible for the reabsorption of organic anions, driven by an outwardly directed dicarboxylate gradient and was expressed to the same extent in Caki-1 and HPT cells [119]. The last OAT expression pattern investigated was that of OAT2, which is said to be the most distantly related OAT and to be expressed to a higher extent in the liver as kidney, perhaps explaining its absence in the Caki-1 and HPT cells [120]. PEPT 1 and 2 were absent in Caki-1 cells, which is in contrast to what was expected but was present in HPT cells. It has been previously stated that PEPT1 and 2 are enriched in the kidney, where they function in the renal reabsorption of di- and tripeptides and related drugs [121]. MCT1 and 4 (SLC16A1 and A4) are members of the monocarboxylic acid transporter subfamily and are responsible for the transport of endogenous monocarboxylic acids such as lactic and pyruvic acid but also drugs such as benzoic and salicylic acid [122]. MCT1 and 4 had a relatively high expression in Caki-1 cells and were moderately expressed in HPT cells. NTCP, sodium-taurocholate co-transporting polypeptide (SLC10A1) and IBAT (SLC10A2) also known as the apical sodium-dependent bile acid transporter are located in the liver and intestine to the apical membranes and IBAT is as well located in renal proximal tubular cells [123]. They are involved in the gastrointestinal absorption and renal excretion of bile acids respectively: expression was absent in Caki-1 and HPT cells which was expected for NTCP [107, 121]. CNT1 (concentrative) and ENT1 (equilibrative) belonging to the SLC28 and 29 nucleoside specific transporter families respectively, are involved in the salvage pathways of nucleotide synthesis and additionally have the capacity to transport nucleoside analogues implemented in the treatment of cancers and viral diseases [124]. CNT1 was not

expressed in Caki-1 nor HPT cells and ENT1 was slightly and moderately expressed in Caki-1 and HPT cells respectively. Expression has been previously shown for the kidney, where it is proposed to play a role in the renal disposition of endogenous and therapeutic nucleosides [125].

The current results compliment previous data which detected various SLCO transcripts in the kidney. The information regarding the expression of SLCO transporters is rather limited in comparison to the vast knowledge regarding ABC and SLC transport proteins. However, recent reviews have detailed the SLCO expression in a variety of human tissues [100, 105, 126]. In general, OATPs mediate the transport of a wide range of organic substrates of endogenous and exogenous origin [126]. Previous studies have indicated that OATPA (SLCO1A2) shows either highest expression or is limited to the brain, testis and retina tissues explaining the lack of expression in Caki-1 and HPT cells [126]. OATPC and OATP8 (SLCO1B1 and B3) were found to be liver specific, also confirming the lack of expression in Caki-1 and HPT cells [100] [127]. OATPB (SLCO2B1), OATPD (SLCO3A1) and OATPE (SLCO4A1) are reported to be ubiquitously expressed in all tissues investigated so far [103, 128, 129]. There was a comparatively very low expression of OATPB in Caki-1 and HPT cells and OATPD and E exhibited moderate to high expression in these cells. OATPF, a relatively new OATP subtype is selectively located to the brain and testis, explaining its absence in Caki-1 and HPT cells [130]. OATPH exhibited high and moderate expression in Caki-1 and HPT cells respectively and is in accordance with the high level observed in kidney tissue from previous studies [100, 131]. OATPH was the highest expressed gene of the 42 genes screened for in Caki-1 and HPT cells.

Metabolism in the kidney is accomplished by a wide range of enzymes including isoforms of the oxidative Phase I superfamily CYP 450 [108]. CYP 1A1 was expressed in Caki-1 cells at a level consistent to what has been previously published for whole kidney tissue [111]. CYP 3A5 was expressed in Caki-1 cells but to a lower extent than that previously published for the kidney and expression was even lower in the HPT cells [111]. CYP 1A2, 2C9, 2D6, and 3A4 typically expressed in the kidney were not detected in Caki-1 or HPT cells. This may due to the fact that CYP enzymes are not stable during the passaging and/or cryopreservation of cells in vitro. Previous results obtained with LLC-PK1 cells have as well indicated the loss of CYP enzymes in vitro [132].

In conclusion, transport proteins and DMEs possess distinct but broad and overlapping substrate specificities; therefore, the knowledge of which transporters and DMEs are present and to what extent, in the test system, would be advantageous. Uptake and efflux transporters and DMEs are increasingly recognized for their pharmacological significances but for many transport proteins, the physiological role and substrate specificity has not yet been completely characterized. Additional investigations to understand precisely the localization and function of each protein and enzyme are required to utilize transporters and enzymes for drug delivery. Successful studies investigating the functional activity and localisation of ‘moderately’ expressed transport proteins in Caki-1 cells has been previously reported [49]. Lending insight that the Caki-1 cells may prove to be a model system for other transporters and enzymes expressed to the same or greater extent (e.g. MDR1, MRP1, MRP4, MCT1, MCT4, OATPE, and OATPH) as determined in the current studies.

Relatively low expressed transporters may also be expressed to an adequate extent to enable functional detection but remains to be tested. It is important to take into consideration that a direct correlation can not be made between mRNA expression and membrane protein content. However, expression at the gene level can be used as a stepping stone. Divergence in mRNA expression between studies may be interpreted as differences in methodology.

The use of cell culture systems is an increasingly popular tool for in vitro studies, due to the ability to screen a large number of compounds in a high-throughput fashion. Caki-1 cells represent an easy to cultivate cell line and could represent a good choice for such basic studies; bypassing the time-consuming and tedious work involved in cultivating primary HPT cells. A foundation has been laid for future studies with Caki-1 cells, involving various transport proteins and perhaps DMEs. Real-time RT-PCR has become a powerful tool to quantify gene expression and has allowed in the current work, a detailed characterization and comparison of a commercially available renal cell line and HPT cells under constant and identical parameters and represents the first human kidney cell line to be characterized in this manner.

5. OCTN2 mediated carnitine uptake in a newly discovered human proximal tubule cell line (Caki-1)

(Running chapter head: OCTN2 activity in Caki-1 cells)

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5.1 Abstract

The proximal tubular reabsorption of carnitine in the human kidney is significant because more than 95% of the carnitine filtered in the kidney is reabsorbed by the proximal tubules therefore maintaining the homeostatic balance of carnitine in the body. Objectives of this study include the characterization of OCTN2 function in the Caki-1 cell line and the potential interactions of carnitine uptake with renally secreted drugs, including drugs of quaternary ammonium structure. Caki-1 cells were additionally characterized to be of proximal tubule nature and an apical membrane expression pattern of OCTN2, in Caki-1 cells was discovered. Uptake studies with radiolabeled L-carnitine in Caki-1 cells revealed a Na^+ and temperature dependent carrier mediated process (K_m = 15.90 µM) which was unaffected by pH in a range from 6.5-8.5. All drugs tested were able to inhibit the carnitine uptake process to different degrees. The quaternary ammonium compounds ciclotropium bromide and ipratropium bromide were strong inhibitors with IC_{50} values of 30 µM and 95 µM respectively. The observed kinetics, immunohistolocalization and inhibition studies indicate that the high-affinity uptake of carnitine in the Caki-1 cell line is most likely mediated by OCTN2. The interaction of drugs at the renal level with OCTN2, indicates a possible pathway for the final step of cationic secretion into the urine.

* Full text available at: http://pubs.acs.org/cgi-bin/abstract.cgi/mpohbp/2007/4/i01/abs/mp060073a.html

5.2 Figures and Tables from Mol Pharm. 2007 Jan-Feb;4(1):160-8

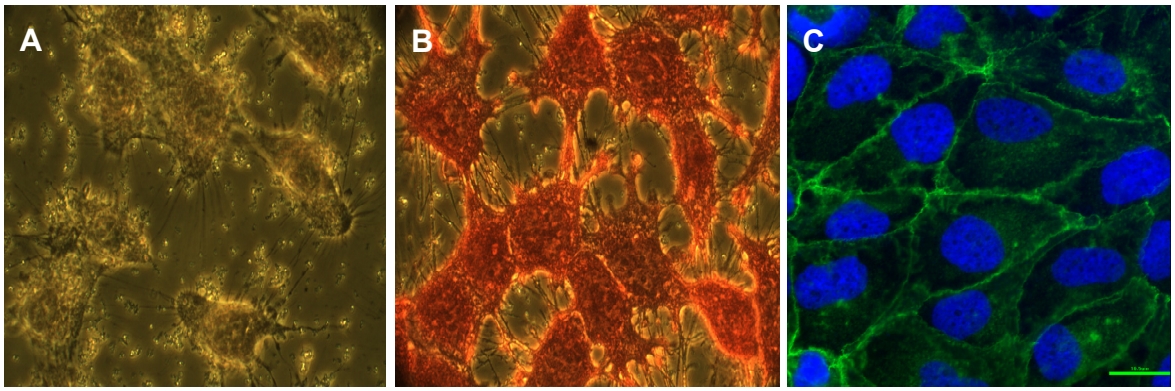


Figure 1. Proximal tubule staining and expression of E-cadherin in Caki-1 cells. For proximal tubule staining, Caki-1 cells were grown in 24 multi-well plates and maintained at 37°C, 5% CO_2 and 95% humidified atmosphere. Cells were stained for the presence of γ-glutamyl transferase, a characteristic marker of human proximal tubule cells. Figure 1A is a negative control of Caki-1 cells (solution containing serine borate) (*yellowish brown*) and Figure 1B is a positive staining of the Caki-1 cells (*red*). Figure 1C illustrates the organized expression of E-cadherin (*green*) and the cell nuclei (*blue*).

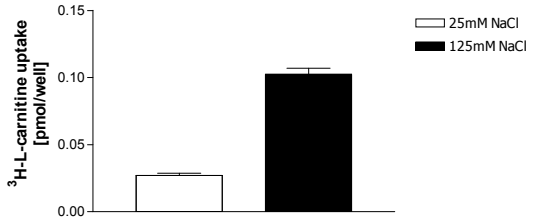


Figure 2. Na^+ dependence of Caki-1 cell L-carnitine uptake. Caki-1 cells were grown in 24 multi-well plates and maintained at 37°C, 5% CO_2 and 95% humidified atmosphere. methyl-L-[^{3}H] carnitine uptake was measured in buffer containing either 25 or 125 mM NaCl. The data represents the mean ± SEM of three separate determinations.

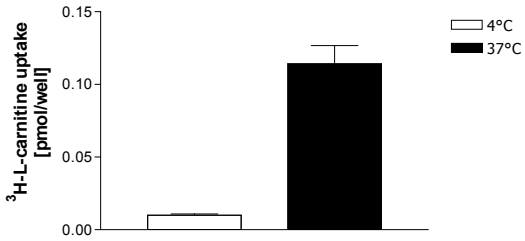


Figure 3. Temperature dependence of L-carnitine uptake into Caki-1 cells. Cells were grown in 24 multi-well plates as previously descibed. methyl-L-[^{3}H] carnitine uptake was measured in buffered HBSS at pH 7.4 at 4 and 37°C for 10 minutes. The data represents the mean ± SEM of three separate determinations.

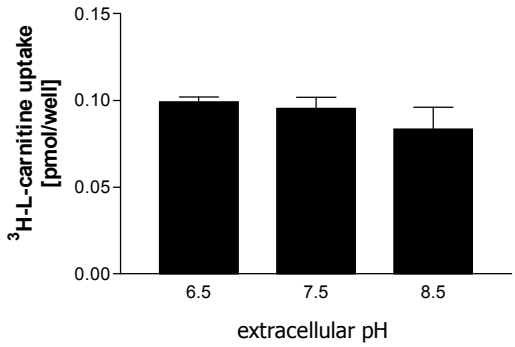


Figure 4. pH-independence of methyl-L-[^{3}H] carnitine uptake by Caki-1 cell monolayers. Uptake was measured at 37°C for 10 minutes in buffered HBSS; each data point and error bar represents the average and SEM for three individual determinations.

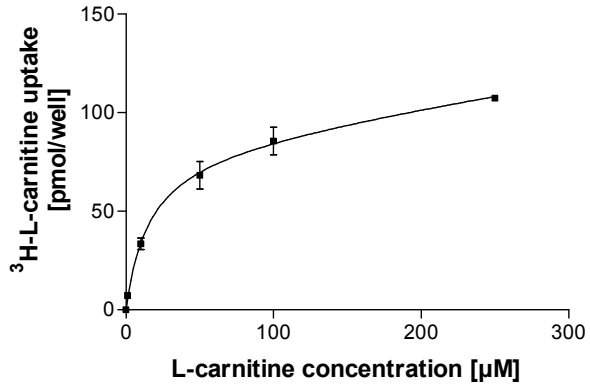


Figure 5. Concentration-dependent uptake of L-carnitine by Caki-1 cells. Cells were grown in 24 multi-well plates and maintained at 37°C, 5% CO_2 and 95% humidified atmosphere. Uptake of L-carnitine was measured at 37°C for 10 minutes in buffered HBSS, in the presence of different concentrations of L-carnitine. Each point represents the mean ± SEM of three separate determinations.

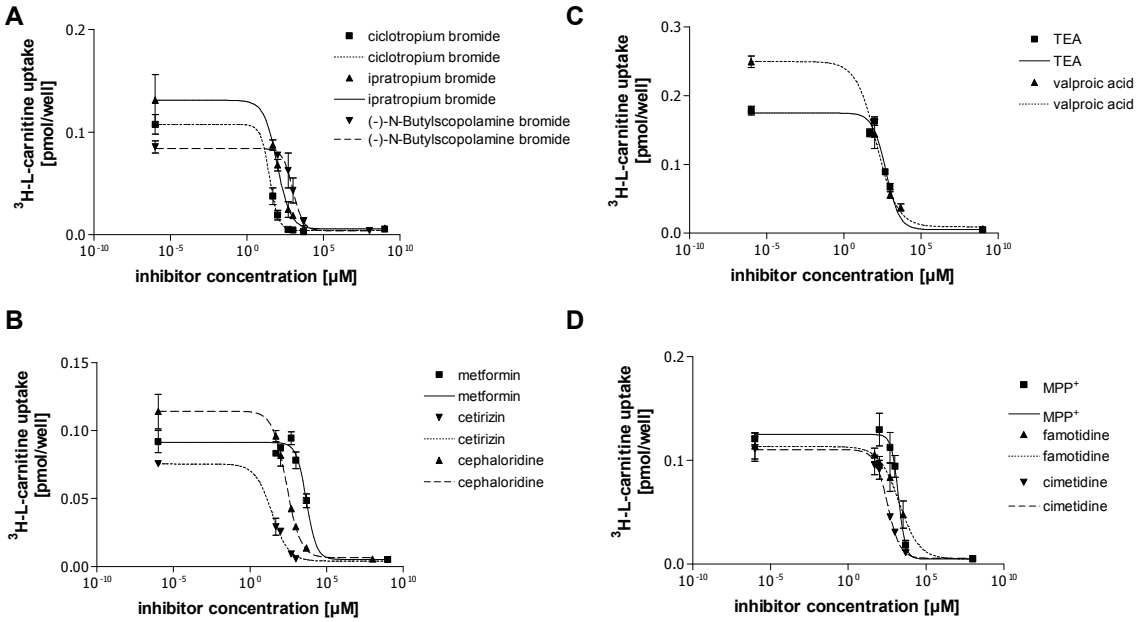


Figure 6. Inhibition of methyl-L-[^{3}H] carnitine uptake in Caki-1 cells by various concentrations of inhibitors: (A) ciclotropium bromide, ipratropium bromide, and (-)-N-Butylscopolamine (quaternary compounds), (B) metformin, cetirizin, and cephaloridine, (C) tetraethylammonium (TEA) bromide and valproic acid and (D) MPP^+, famotidine and cimetidine. Each point and error bar represents the the average ± SEM for three separate determinations of uptake. Fitted lines reflect the IC_{50} values displayed in Table 1.

Table 1. Inhibitory effects of various compounds on L-[^{3}H] carnitine uptake by Caki-1 cells

Inhibitor	IC_{50} [µM]	95% confidence intervals
valproic acid	139.06	88.07 to 221.2
famotidine	1920	890.8 to 4138
cimetidine	336.7	243.1 to 466.3
MPP^+	1713	1231 to 2384
TEA	551.1	395.8 to 767.3
cephaloridine	248	187.4 to 328.1
cetirizin	27.08	16.04 to 45.70
metformin	4963	3541 to 6956
ipratropium bromide	95.84	67.43 to 136.2
ciclotropium bromide	30.8	22.37 to 42.42
(−)-N-butylscopolamine bromide	1007	615.7 to 1648

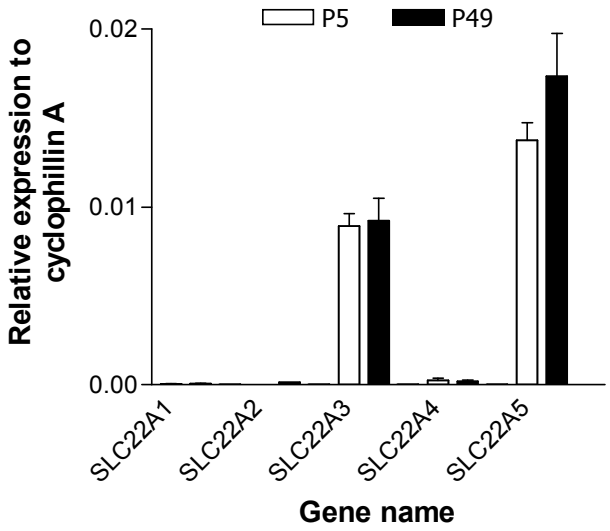


Figure 7. Relative gene expression levels ($2^{-\Delta C}{}_{T}$) of OCT1, OCT2, OCT3, OCTN1, and OCTN2 in two passages (P5 and 49) of Caki-1 cells. Each value represents the mean ± SEM for three separate determinations.

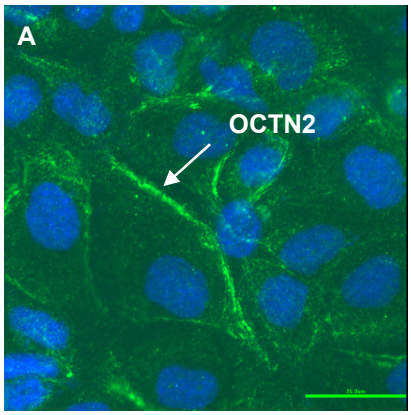
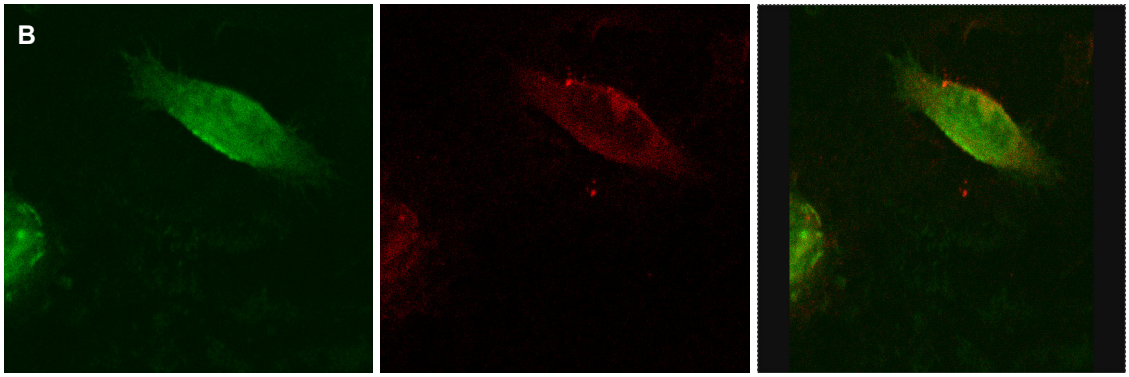


Figure 8. Detection of OCTN2 by immunofluoresence in Caki-1 cells: (A) Caki-1 cells were grown on glass coverslips and maintained in a 5% CO_2 and 95% humidified atmosphere at 37°C. Cells were used between 5-7 days (70-90% confluence). They were fixed in -20°C methanol, immunostained for OCTN2 and viewed by fluorescence microscopy. Nuclei were counterstained with 4, 6-diamidino-2-phenylindole (DAPI) (*blue*) and OCTN2 with the anti-OCTN2 polyclonal antibody (*green*), (B) localization of OCTN2 (red) to the apical membrane and Na^+K^+-ATPase (green) to the basolateral membrane. Scale bar represents 26.0 µm.



5.3 Correlation of data between Caki-1 and human primary proximal tubule cells (HPT)

It has been previously determined that transport proteins are concentrated to the PTs and that OCTN2 is specifically expressed in vivo in human PTs. Therefore, HPT cells (various lots) were selected as the simplest possible control system to confirm the expression pattern and functional activity observed in the Caki-1 test system. As detailed in Table 3 of section 4.4, the mRNA expression of OCTN2 in HPT cells was approximately one half that of the Caki-1 cells.

5.3.1 OCTN2 protein expression in HPT cells

OCTN2 was localized to the cellular membranes in HPT cells (lot TUV) via previously described methods [49]. An exact subcellular localization was not determined for the HPT cells based on two criteria: (1) it has been previously confirmed that OCTN2 is localized to the apical membrane of human PT cells and (2) due to the cumbersome and costly cultivation of HPT cells. Figure 9 illustrates the localization of OCTN2 to a single membrane of the HPT cells; presumably the BBM.

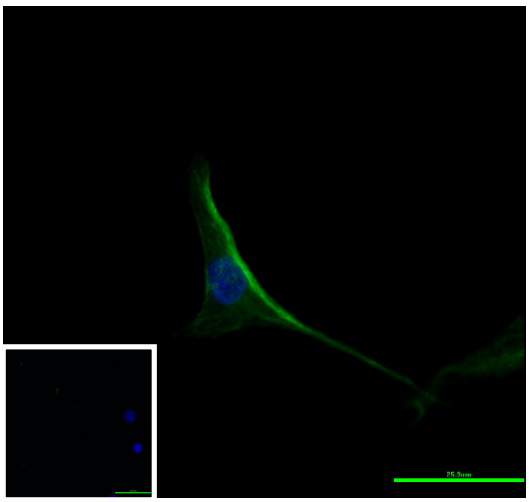


Figure 9. Membrane localization of OCTN2 in HPT cells. According to the methods described in section 5.3, the localization of OCTN2 to a single side of the cell membrane was possible. The insert represents a PBS control without OCTN2-specific antibody (negative control). Scale bar represents 25.9 µm.

5.3.2 Functional activity of OCTN2 in HPT cells and comparison to Caki-1 cells

The uptake of radiolabeled l-carnitine into HPT cells (lot CGS) was investigated according to methods previously published [49]. Betaine was selected as a specific inhibitor for the uptake of l-carnitine via OCTN2. Once again, due to the cumbersomeness and costs of cultivating HPT cells, it was not possible to determine the inhibition affinities (IC_{50} values) of any drug substances tested in the case of the Caki-1 cells. As indicated in Figure 10A, the uptake of [^{3}H]-L-carnitine into HPT cells (K_m ≈ 58.38 µM) followed a similar process as was determined for the Caki-cells 1 (K_m ≈ 11.90 µM). In figure 10B the inhibition of l-carnitine uptake into HPT and Caki-1 cells was determined; increased inhibition was observed at higher concentrations of betaine for both HPT and Caki-1 cells, indicative of competitive inhibition.

5.3.3 Conclusions in respect to the correlation of data between HPT and Caki-1 cells

HPT cells are of healthy human PT origin and were selected to determine the validity of the data obtained from the Caki-1 cells. At the gene level, HPT cells expressed OCTN2, however, to approximately 50% the extent of that in the Caki-1 cells. This may be attributed to the fact that primary cells are difficult to cultivate in vitro and are known to de-differentiate rather rapidly. At the protein level a specific membrane localization of OCTN2 could be made which correlates well with what has been previously published in respect to OCTN2 and the data obtained for the Caki-1 cells. At the functional level saturable and inhibitable uptake of l-carnitine could be observed in the HPT cells. Comparing the kinetics between the HPT and Caki-1 cells one can make a few clear observations: (1) the uptake of l-carnitine by Caki-1 cells is significantly higher under experimental conditions, (2) the affinity of l-carnitine for the transport process (assumed to be OCTN2) is higher in Caki-1 cells and (3) a stronger inhibition of l-carnitine uptake by betaine was observed in the Caki-1 cells. It is difficult to make a clear conclusion as to whether the differences observed are significant. For example, the level of uptake may be directly correlated to the level of gene/protein expression and secondly one must additionally keep in mind that the same protein will function differently when expressed in two different systems, regardless if it is endogenously expressed or transfected. Last but not least, different lots of HPT cells were used for the various experiments and variances in expression and activity must be taken into consideration; the lack of material and consistency is a typical downfall to the use of primary cultures.

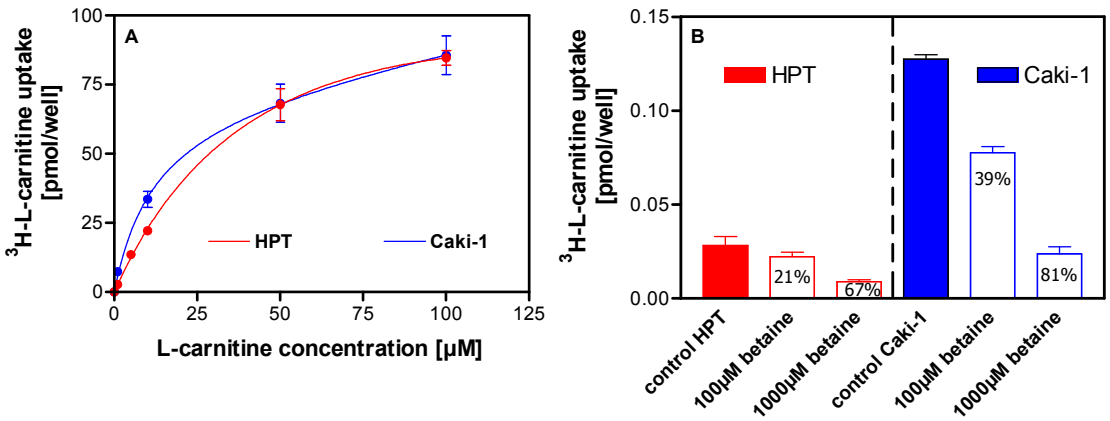


Figure 10. Comparison of l-carnitine uptake kinetics in HPT and Caki-1 cells. Figure A details the saturability of l-carnitine uptake into HPT and Caki-1 cells and Figure B the inhibition of l-carnitine uptake by betaine.

6. Caki-1 cells as a model system for the interaction of renally secreted drugs with OCT3

(Running chapter head: OCT3 activity in Caki-1 cells)

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Nephron Experimental Nephrology (submitted July 2007)

6.1 Abstract

Background/Aims: Organic cation transporters (OCT) in the proximal tubules (PTs) participate in the renal secretion of several therapeutic agents. The exact role of OCT3 in renal secretion remains undetermined, partially due to the lack of an appropriate in vitro model system. The current work introduces the PT representative cell line, Caki-1, as a model system for studying the involvement of OCT3 in renal secretion.

Methods: Caki-1 cells were characterized for OCT3 expression via real-time RT-PCR and immunocytochemical staining techniques. Uptake kinetics of OCT3 in Caki-1 cells was determined using prototypical substrates and inhibitors. Inhibition of OCT3 mediated uptake via several renally secreted drugs and those specifically of quaternary ammonium structure were determined.

Results: OCT3 expression was confirmed at the gene level and subcellular localization to the basolateral membrane (BLM) was illustrated for the first time. Caki-1 cells exhibited trademark kinetics of OCT3 and interacted with all therapeutic agents tested with varying affinities. The apparent IC_{50} values for cimetidine and trimethoprim were pharmacologically relevant.

Conclusion: Confirmation for the usefulness of Caki-1 cells as a PT model system for investigations of OCT3 was obtained, a novel BLM localization of OCT3 was possible and relevant interactions between OCT3 and renally secreted drugs were shown.

6.2 Introduction

The mammalian kidney plays a critical role in the elimination of a variety of structurally diverse xenobiotics from the blood. Within the kidney, specific transport mechanisms exist in the basolateral and apical membrane of tubular cells (highly concentrated to the proximal tubule (PT)), which function together in a concerted effort to mediate the vectorial transport of positively charged xenobiotics from the blood into the tubular lumen [23]. The majority of drugs for therapeutic uses, belonging to a wide array of clinical classes (including skeletal muscle relaxants, antiarrhythmics, antihistamines and ß-adrenoreceptor blocking agents), are also organic cations (OCs), therefore the kidney is in regular contact with these substrates. Furthermore, many of the aforementioned OCs are polar and positively charged at physiological pH, therefore, their transport across cell membranes is largely determined by the activity of membrane-bound transport systems. Organic cation transporters (OCTs) are thus critical in the absorption, targeting and disposition of many clinically administered drugs. During the last years it has become increasingly clear that multiple mechanisms are responsible for OC transport in various tissues including the kidney.

Generalized, the renal secretion of organic cationic xenobiotics occurs via a two-step process: substrates are believed to enter the PT cells from the blood via potential-sensitive OCTs located in the basolateral membrane (BLM) and are subsequently effluxed across

the luminal (apical) membrane by the electroneutral exchange of intracellular OCs for intraluminal H^+ (via an OC/H^+ antiporter) [14]. Similar mechanisms are believed to function in the hepatocytes and intestinal epithelium, as well as the placental syncytiotrophoblast [45, 133].

Physiological evidence has been consistent with the activity of a single mediated process for the transport of several OCs at the BLM of PT cells; however, it is evident that multiple transporters with overlapping functional properties are expressed in human PT cells [134]. Therefore, it is necessary to clarify the interactions of the individual variants. To date three different potential-sensitive OCTs have been cloned and characterized, all of which are expressed in human PT cells. OCT1 originally cloned from rat kidney was followed up by the isolation of mouse, human and rabbit homologs [15]. OCT2 was cloned from the rat kidney by homology screening. Subsequently the porcine and human OCT2 homologs were isolated and characterized. Northern blot analysis has shown that OCT1 and 2 are mainly expressed in the liver and kidney of humans, respectively, and to a smaller extent in the human intestine. A subcellular localization of OCT2 to the BLM of human PT cells has been determined [135]. OCT1 and 2 recognize a multitude of endogenous and exogenous OCs, many of which are commonly administered therapeutic agents. Not surprisingly, due to their close ancestral origin and chromosome location, these drug transporters exhibit a considerable overlap in substrate specificity. The third OCT, OCT3, was first cloned from the rat placenta and its orthologs have been cloned from mice and humans [21, 22]. It is expressed most abundantly in the placenta but exhibits a broad tissue distribution, additionally expressed in the kidney, small intestine and several other tissues. Due to the high affinity of OCT3 to monoamines, it was also termed the extraneuronal monoamine transporter (EMT).

There is a general consensus that basolateral OC transport in the kidney is dominated by OCT2, however, OCT1 may additionally play a significant role. Furthermore, OCT3 which has a 10-fold or greater expression in the kidney as compared to OCT1, appears to play a minor albeit unclarified role in the renal secretion of certain OCs [134].

OCT3 has received limited attention in comparison to OCT1 and 2. The majority of studies involving OCT3 transport mechanisms have been concerned with its interactions with monoamines and steroid hormones, the latter to which OCT3 exhibits high affinity [21, 136]. However, its interaction with renally secreted drugs has not been studied intensively enough, perhaps due to the lack of an adequate in vitro test system or the until now, unknown subcellular localization of this transport protein in human PT cells.

Some of the first in vitro cell culture studies on OCT3 date back to the 1990's with the employment of the renal clonal cell line Caki-1 [82]. These cells were used as a model system for studying EMT activity; OCT3 was additionally cloned from Caki-1 cells. However, since then these cells have been rarely implemented in the field of drug transport. A few groups have employed these cells for studying what is now more commonly referred to as OCT3 but in respect to monoamine transport. These cells were not considered for their potential as a model for the renal secretion of OCs via OCT3. A recent work by Glube et al. has confirmed this cell line to be a representative model system

of the human PT epithelium (Nephron Experimental Neph 2007; (in press)). Therefore, this cell line could provide an excellent tool for investigations regarding the involvement of OCT3 in renal secretion based on several points: (1) OCT3 is endogenously expressed in Caki-1 cells, (2) Caki-1 cells represent the PT epithelium, the major site of renal secretion and (3) functional evidence of OCT3 in Caki-1 cells has been previously confirmed. In addition, the current work has illustrated the absence of expression of OCT2, which is opposite of what may be expected in vivo but excellent for an in vitro system intended for studying OCT3 activities (due to the overlapping substrate and inhibitor profiles between OCT variants).

Therefore, the objectives of the current investigations were to determine the usefulness of Caki-1 cells as a renal model system for studying OCT3 activity and to clarify within a single system, the interaction of OCT3 with renally secreted OC drugs such as cimetidine, famotidine, metformin, procainamide, ranitidine, triamterene and trimethoprim. The mRNA expression level of OCT1-3 in Caki-1 cells was determined and furthermore the subcellular protein expression pattern of OCT3 in a human renal cell culture system was determined for the first time using Caki-1 cells. Last but not least, MPP^+, a quaternary ammonium derivative, is the preferred substrate of choice for OCT3. Therefore, a second objective of the current work was to determine the potential of xenobiotics of quaternary ammonium structure to interact with OCT3. A series of commonly or previously administered therapeutic agents were selected, which included: ciclotropium bromide, ipratropium bromide, (−)-N-Butylscopolamine bromide and trospium hydrochloride.

6.3 Materials and Methods

Preparation of cDNA and Real Time Reverse Transcriptase PCR (RT-PCR). RNA was isolated from confluent cultures after 6-8 d in culture (passage 5), using RNA STAT-60™ (Tel-Test Inc.; Friendswood, TX, USA) and performed according to the products manual. RNA preparations were treated with DNA-free™ (Ambion Ltd.; Huntingdon, Cambridgeshire, UK) and the quantity and purity of the RNA samples was determined using a GeneQuant pro RNA/DNA calculator. The RNA integrity was assessed by visualizing the sharpness of ribosomal RNA bands on a 1% agarose gel. cDNA was prepared from total RNA using the Superscript™ First-Strand Synthesis System for real-time PCR (Invitrogen Ltd.; Paisley, UK) according to the product manual. The two step reaction mixture contained 2 µg RNA, 100 ng random hexamers, 0.5 mM dNTP mix (dATP, dCTP, dGTP, dTTP), 10 mM Tris-HCl (pH 8.4), 25 mM KCl, 5 mM $MgCl_2$, 10 mM DTT, and 40 units RNAseOUT™ recombinant ribonuclease inhibitor. Incubation was performed at 65°C for 5 min and 25°C for 2 min, with subsequent incubation after the addition of Superscript II, for 10 min at 25°C, followed by 42°C for 50 min. The reaction was terminated by heating at 70°C for 15 min.

TaqMan® real-time PCR was carried out in 384 well reaction cards and an ABI PRISM® 7900ht Sequence Detection System. Assay-on-Demand™ Gene Expression Products were used for all genes tested. All TaqMan® equipment was obtained from Applied Biosystems (Foster City, CA, USA). TaqMan® analysis was performed in a 1 µl reaction mixture containing 2 ng RNA converted to cDNA, 1x TaqMan® Universal PCR Master Mix

(containing AmpliTaq Gold DNA polymerase, dNTPs with dUTP, passive reference and optimised buffer), 900 nM each of custom designed forward and reverse primer and 250 nM of custom-designed probe. Cycling conditions were as follows: 2 min at 50°C, 10 min polymerase activation at 95°C and 40 cycles at 95°C for 15 s and 60°C for 1 min.

For normalisation of gene levels, RPLP0 (ribosomal protein) was used to correct for minor variations in the input RNA or inefficiencies in reverse transcription. The cycle number and the threshold cycle (C_T) were used to quantify the PCR product. The relative expression of the target gene, normalised to the endogenous control was calculated as follows:

$\Delta C_T = C_T$ target gene - C_T control gene

and were converted according to Applied Biosystems (1997) to:

$2^{-\Delta C_T}$

Immunofluorescence/Membrane Localization of OCT3. Caki-1 cells were grown in Petri dishes, harvested after 7 d, pelleted by mild centrifugation in 1.5 ml epi-caps and shock frozen in liquid nitrogen. Double-stainings were performed with pelleted cells, from which 10 µM thick cryosections were prepared. Cryosections were fixed in isopropanol and incubated with 50% horse serum in 0.05 M PBS for 1 h. Primary antibodies OCT3 (kindly provided by Prof. Koepsell and described in [37]), and Na^+/K^+-ATPase (SantaCruz; Heidelberg, Germany), diluted in PBS 1:500 and 1:100 respectively were applied overnight. Secondary antibodies were applied for 1 h at RT: fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse (Dianova; Hamburg, Germany), and Cy3-conjugated donkey anti-rabbit IgG (Chemicon; CA, USA). Cells were PBS-rinsed, fixed (4% PFA) and transferred to cover slips with carbonated-glycerol (pH 8.6). Samples were evaluated with an epifluorescence microscope.

Cell Culture. Caki-1 cells were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ; Braunschweig, Germany). Cells between passages 29-41 were used for uptake studies. Cells were routinely seeded out at a density of 80 000 cells into 75 cm^2 tissue culture flasks (Greiner Bio-One GmbH; Frickenhausen, Germany) and cultivated in McCoy's 5A (Biochrom AG; Berlin, Germany), supplemented with 10% FBS, 1.14% penicillin/streptomycin, 1.14% non-essential amino acids and 0.7% stabile L-glutamine (Biochrom AG). The cells were maintained at 37°C, 5% CO_2 and 95% relative humidity. After confluence (95-100%), cells were harvested using 0.25% trypsin-EDTA solution (Biochrom AG).

MPP^+ Uptake. MPP^+ uptake studies were carried out as described previously with minor modification [137]. Caki-1 cells (between passages 29 and 41) were plated (12 000 cells/well) onto 24 multi-well plates (Nunc™; Wiesbaden, Germany) in McCoy's 5A complete medium. The cells were used for uptake studies at confluence (6-8 d) and all experiments were conducted at 37°C with the exception of temperature dependence studies which were carried out at 4°C. The cells were washed twice with 1.5 ml buffered Hanks Balanced Salt Solution (HBSS) (5 mM HEPES, pH 7.4) before experiment begin.

The confluent cells were incubated at 37°C with a final volume of 0.5 ml of buffered HBSS containing [^{3}H]-1-methyl-4-phenylpyridinium (MPP^+) (specific radioactivity of 80.0 Ci/mmol, Hartmann Analytic GmbH; Braunschweig, Germany) at a concentration of 0.2 µM with (or without) inhibitors. At the end of the standard incubation period (5 min), the solutions were aspirated off and the monolayers were immediately washed five times with 1 ml of ice-cold buffered HBSS and thereafter dissolved at RT with 0.5 ml of 1 N NaOH. The cells were incubated for approximately three hours with shaking (75 rpm) at RT. The complete volume of dissolved cells was collected, 4 ml of Rotiszint® Ecoplus scintillation fluid (Carl Roth GmbH + Co. KG; Karlsruhe, Germany) was added and radioactivity was quantified with a liquid scintillation counter. In another series of experiments the temperature and pH dependence on [^{3}H]-MPP^+ uptake was determined. Temperature dependence studies were carried out as described above at either 37 or 4°C. For pH dependence studies, buffered HBSS in a pH range from 5.5-7.5 was employed (HBSS/10 mM MES (pH 5.5-6.5) and HBSS/5 mM HEPES (pH 7.5); pH values were adjusted with 1 M HCl and 1 M NaOH). All experiments were carried out in triplicates (n=3).

Assay with Xenobiotic Inhibitors. For the inhibitor experiments, confluent Caki-1 monolayers in 24-well plates, were incubated with 0.5 ml of buffered HBSS (pH 7.4) containing 0.2 µM [^{3}H]-MPP^+ plus varying concentrations of decynium22 (OCT3 specific inhibitor), cimetidine, famotidine, metformin, procainamide, triamterene, trimethoprim, ciclotropium bromide, ipratropium bromide, (-)-N-Butylscopolamine bromide or trospium hydrochloride. All substances were purchased from Sigma Aldrich with the exception of procainamide (Fagron GmbH & Co. KG; Barsbüttel, Germany) and ciclotropium bromide and trospium chloride which were kindly provided by Prof. Hildegard Spahn-Langguth.

Cell Viability Assay. Cell viability was determined using the colorimetric XTT based TOX 2 in vitro toxicology assay kit (Sigma Aldrich). Experiments were carried out on confluent Caki-1 cell monolayers grown in 96-well tissue culture plates (seeding density of 2000 cells/well) for 7 d. In brief, xenobiotic test solutions were prepared in buffered HBSS at the highest concentration tested in xenobiotic inhibition assays. Cells were then incubated for 5 min (standard duration of uptake/inhibition experiments) at 37°C with 200 µl of the respective xenobiotic solutions; solutions were subsequently aspirated off and the monolayers were washed 1x with DMEM medium without phenol red (Biochrom AG). XTT test solution, reconstituted in DMEM medium was applied to the cells (100 µl) and incubated for 3 h at 37°C. A microplate reader equipped with a 450 nm optical filter and 690 nm reference filter was utilized to measure the absorbance and respective cell viability in each well. Untreated cells were taken as 100% cell viability. All toxicity assays were carried out at a minimum of n=3.

6.4 Results

mRNA Expression of OCT Variants in Caki-1 Cells. The expression of OCT1-3 was investigated via real-time RT-PCR technology for passage 5 and 49 of Caki-1 cells (Figure 1). Various potential endogenous controls were tested and RPLP0 was chosen as the endogenous control, since it showed C_T values closest in range to those of the Caki-1 samples (data not shown). A definite expression of OCT3 was demonstrated, with relatively

constant expression over an extended passaging period (passages 5-49). Minimal detection of OCT1 was observed and OCT2 expression was absent in both passages.

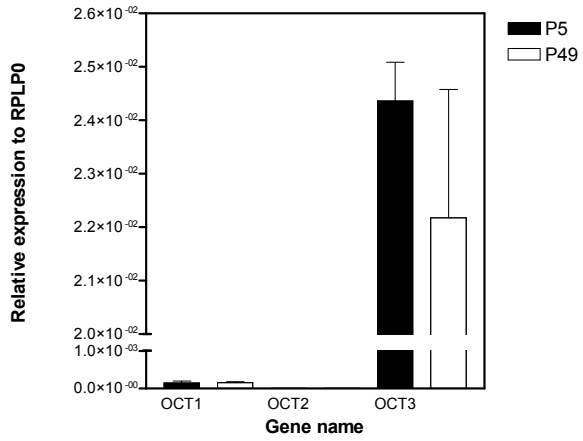

Figure 1. Relative gene expression levels ($2^{-\Delta C_T}$) of OCT1, OCT2 and OCT3 in two passages (P5 and P49) of Caki-1 cells. Each value represents the mean ± SEM for three separate determinations.

Immunolocalization of OCT3 in Caki-1 Cells. Immunocytochemical staining with an anti-OCT3 specific antibody revealed a specific positive fluorescence of OCT3 protein in Caki-1 cells (Figure 2a). Double-stainings with the basolaterally located Na^+/K^+-ATPase resulted in a partial colocalization of OCT3 and Na^+/K^+-ATPase, suggesting a basolateral location of OCT3 in Caki-1 cells (Figure 2b). Preabsorption and PBS control experiments indicated the specificity of the fluorescence observed (not shown).

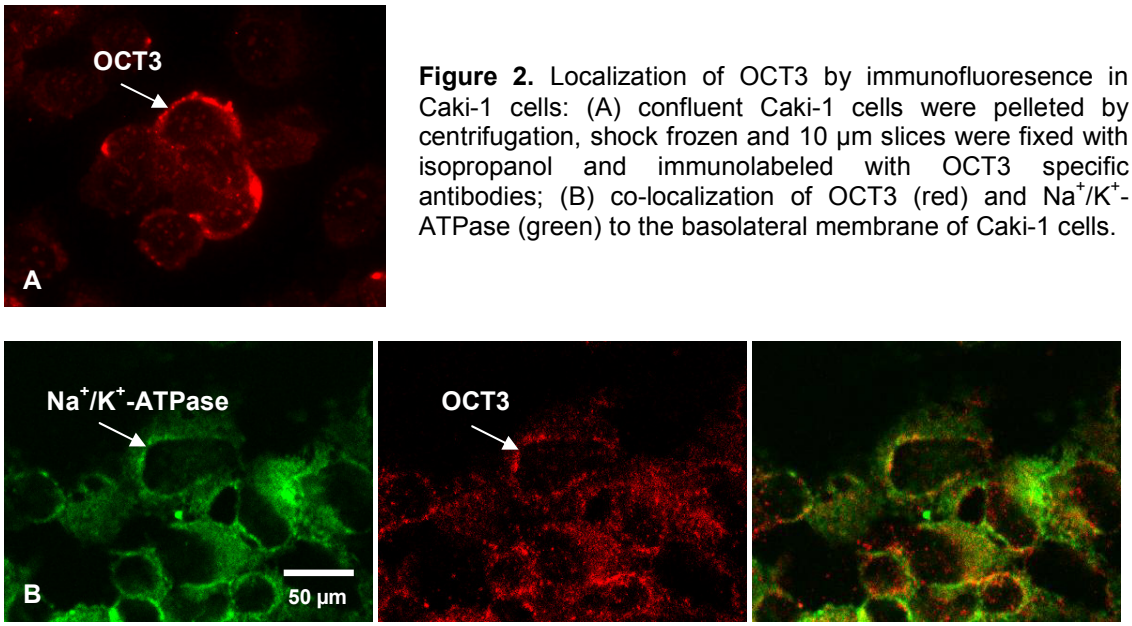

Figure 2. Localization of OCT3 by immunofluoresence in Caki-1 cells: (A) confluent Caki-1 cells were pelleted by centrifugation, shock frozen and 10 µm slices were fixed with isopropanol and immunolabeled with OCT3 specific antibodies; (B) co-localization of OCT3 (red) and Na^+/K^+-ATPase (green) to the basolateral membrane of Caki-1 cells.

[^{3}H]-MPP$^+$ Uptake Kinetics. The uptake of [^{3}H]-MPP$^+$ (0.2 µM) into confluent Caki-1 monolayers grown on 24 multi-well plates was investigated. [^{3}H]-MPP$^+$ uptake remained in the linear phase for approximately 10 min (Figure 3a) and in order to perform experiments within the linear uptake phase, an incubation period of 5 min was chosen for all subsequent experiments. This incubation additionally allowed for a reproducible level of DPM counts.

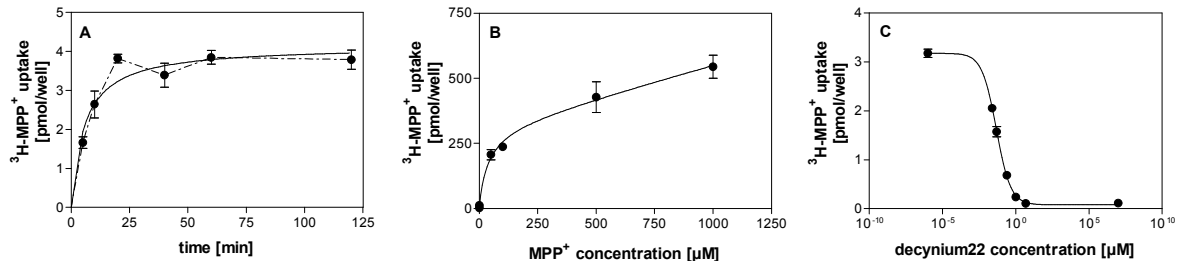

Figure 3. Characterization of [^{3}H]-MPP$^+$ uptake into Caki-1 cells as a function of time, concentration and specific inhibition (cells were used at confluency in 24-well plate format): (a) uptake of [^{3}H]-MPP$^+$ (0.2 µM) was determined at the indicated time points; (b) concentration dependent uptake of [^{3}H]-MPP$^+$ (0.2 µM, 5 min, 37°C) was measured over the concentration range indicated; (c) inhibition capacity of the OCT3 specific inhibitor, decynium22 was determined for [^{3}H]-MPP$^+$ uptake into Caki-1 cells at 37°C and 5 min. Each point represents the mean ± SEM of a minimum of three separate determinations.

The uptake of [^{3}H]-MPP$^+$ was measured at increasing concentrations ranging between 0.2 and 1000 µM in order to prove the saturability of the uptake process and to determine kinetic parameters. Figure 3b illustrates the saturable uptake process of [^{3}H]-MPP$^+$ with a calculated K_m of 36.65 ± 17.42 µM. Decynium22, a known specific inhibitor of OCT3 was chosen to confirm that the [^{3}H]-MPP$^+$ uptake observed was mediated by OCT3. An IC_{50} of approximately 0.05 µM was determined, correlating well with what has been previously published in the literature [138].

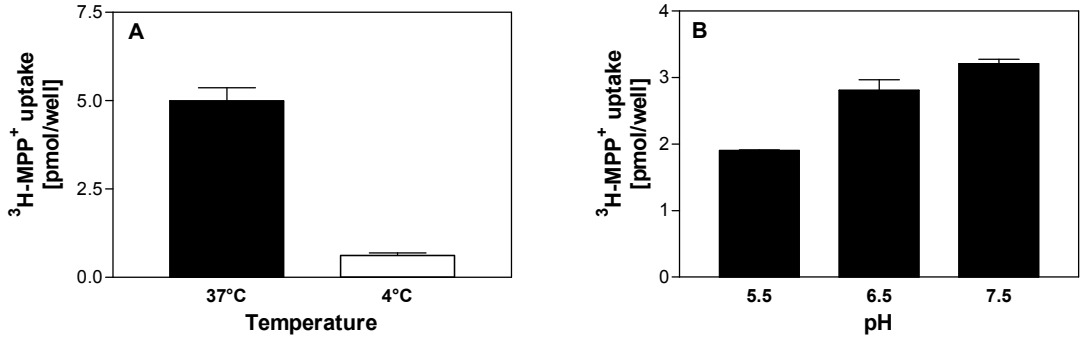

Figure 4. Temperature and pH dependence of [^{3}H]-MPP$^+$ uptake into Caki-1 cells (cells were used at confluency in 24-well plate format): (A) Caki-1 cells were incubated with [^{3}H]-MPP$^+$ (0.2 µM, 5 min, 4 or 37°C); (B) [^{3}H]-MPP$^+$ uptake (0.2 µM, 5 min) was observed over a pH range of 5.5-7.5. Data represents the mean ± SEM of a minimum of three separate determinations.

The influence of temperature on [^{3}H]-MPP$^+$ uptake was tested, where a significant decrease in the uptake was observed at 4°C (Figure 4a), a factor indicative of carrier-mediated transport. Varying the pH between 5.5 and 7.5 resulted in significant differences of [^{3}H]-MPP$^+$ uptake into Caki-1 cells; uptake was highest at a physiological pH of 7.5 (Figure 4b). This data is in accordance with what has been previously reported in the literature for [^{3}H]-MPP$^+$ uptake via OCT3.

Effect of Renally Secreted Drugs on [^{3}H]-MPP$^+$ Uptake into Caki-1 Cells. The uptake of [^{3}H]-MPP$^+$ by Caki-1 cells was characterized in the presence of various renally secreted drugs known to inhibit members of the OCT system. Caki-1 cells were incubated with 0.2 µM [^{3}H]-MPP$^+$ together with varying concentrations of cimetidine, famotidine, metformin, procainamide, triamterene or trimethoprim (Figure 5a and b). The approximate potency rank order of these inhibitors for [^{3}H]-MPP$^+$ uptake were (given as the mean IC_{50} values in µM of a minimum of 3 determinations): famotidine (7.5) > triamterene (9.0) > trimethoprim (14.5) > cimetidine (24.8) > ranitidine (124.3) > procainamide (213.5). Metformin at a concentration of 5 mM was a weak inhibitor, exhibiting less than 50% inhibition of [^{3}H]-MPP$^+$ uptake, therefore, an IC_{50} value was not determined.

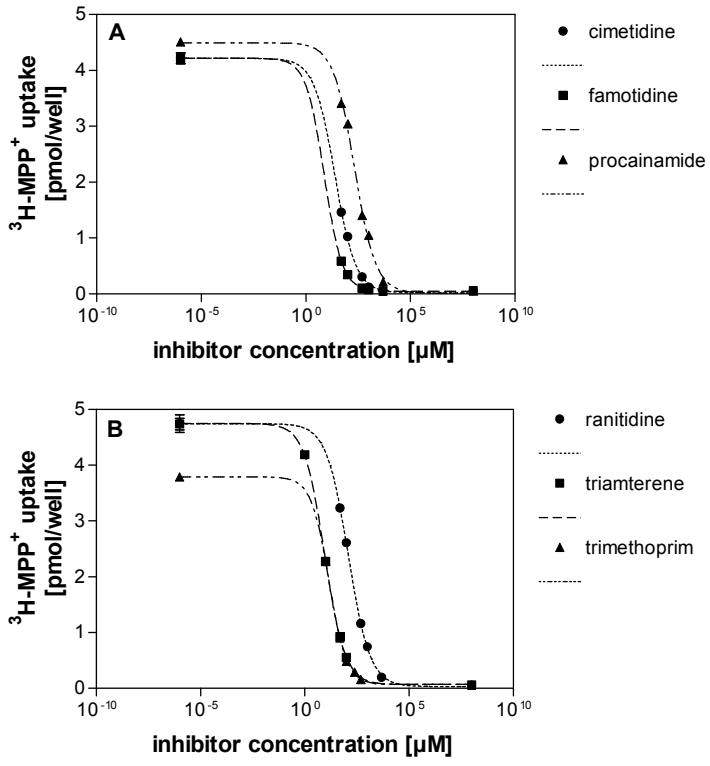

Figure 5. Concentration dependent inhibition of [^{3}H]-MPP$^+$ uptake (0.2 µM, 5 min, 37°C) into Caki-1 cells via various therapeutic agents (cells were used at confluency in 24-well plate format): (a) cimetidine, famotidine and procainamide and (b) ranitidine, triamterene and trimethoprim. Each point represents the mean ± SEM of a minimum of three separate determinations. Fitted lines represent the IC_{50} values displayed in Table 2.

Effect of Quaternary Ammonium Derivatives on [^{3}H]-MPP$^+$ Uptake into Caki-1 Cells. Caki-1 cells were additionally incubated with drugs of quaternary ammonium structure: ciclotropium bromide, ipratropium bromide, (−)-N-Butylscopolamine bromide or trospium hydrochloride, to determine the influence these substances have on the uptake of [^{3}H]-MPP$^+$ into Caki-1 cells. In all cases, the [^{3}H]-MPP$^+$ uptake was inhibited in a concentration-dependent manner, however with varying intensities as summarized in Figure 6. The approximate potency rank order of these derivatives for [^{3}H]-MPP$^+$ uptake were (given as the mean IC_{50} values in µM of three individual determinations): ipratropium bromide (83.4) > ciclotropium bromide (163.3) >> trospium hydrochloride (1423). (−)-N-Butylscopolamine bromide at a concentration of 5 mM was a weak inhibitor, exhibiting less than 50% inhibition of [^{3}H]-MPP$^+$ uptake therefore, an IC_{50} value was not determined.

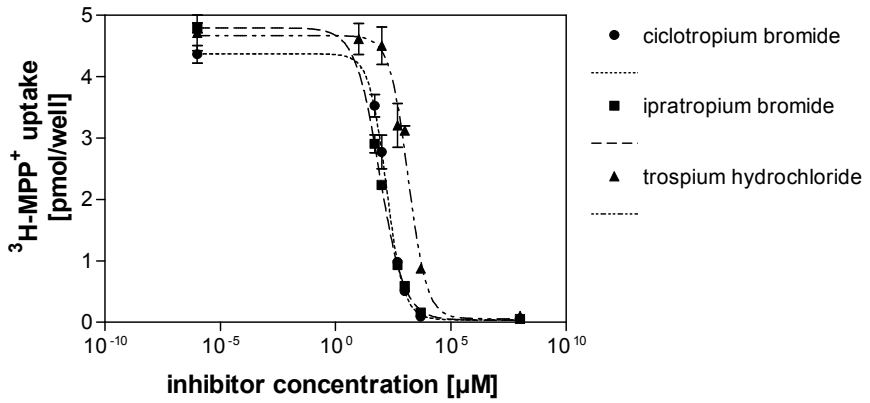

Figure 6. Concentration dependent inhibition of [^{3}H]-MPP$^+$ uptake (0.2 µM, 5 min, 37°C) into Caki-1 cells via therapeutic agents of quaternary ammonium structure (cells were used in at confluency in 24-well plate format): uptake of [^{3}H]-MPP$^+$ (0.2 µM, 5 min, 37°C) in the presence of ciclotropium bromide, ipratropium bromide and trospium hydrochloride. Each point represents the mean ± SEM of a minimum of three separate determinations. Fitted lines represent the IC_{50} values displayed in Table 2.

Cell Viability Assay. Cell toxicity assays were carried out with the highest concentration of all test substances under typical experimental conditions (5 min/ 37°C). Cells remained ≥ 85% viable in the presence of all substances tested (Table 1).

6.5 Discussion

The involvement of OCT system(s) in the renal secretion of numerous therapeutic agents was recognized more than 40 years ago [139]. With advancements in molecular technologies, researchers have identified and cloned 3 OCT variants and 1 OC/H$^+$ exchanger, with assumed locations at the basolateral and apical membranes of PT cells, respectively [14]. However, the contribution of the individual OCT variants to the overall process remains to be clarified. Of the 3 OCT variants, OCT2 is assumed to play the major role in human OC secretion in the kidney [134]. Therefore, the bulk research on renal OC

secretion has focused on this single transport protein. Nevertheless, the function of OCT1 and OCT3, which are additionally expressed in PT cells of the human kidney, require due attention. Additionally, many research groups have suggested that OCT3 due to its sequence similarity to OCT2, may be involved in some aspect of the first step of renal secretion. Therefore, it has been assumed without concrete evidence, that OCT3 is located to the BLM of human PT cells. Accordingly, the aim of the current work was to present the Caki-1 cell line as a renal model system for studying the interaction of OCs with OCT3 by: (1) determining the levels of expression of OCT1-3 in Caki-1 cells, (2) determining the protein expression pattern of OCT3 in Caki-1 cells, (3) characterizing the interaction of OCT3 with renally secreted OC drugs and those specifically of quaternary ammonium structure and (4) to determine the correlation and perhaps significance of the in vitro inhibition data, with that of the maximum plasma concentrations (C_{max}) of the respective drugs found in vivo after oral administration (data acquired from literature).

Table 1. Effect of the various test compounds on the viability of confluent Caki-1 cell monolayers (cells were grown in 96-well plate format and incubated with the highest concentration of test substances tested for 5 min at 37°C): (a) renally secreted drugs, (b) drugs specifically of quaternary ammonium structure and (c) OCT3 prototypical substrate and specific inhibitor. Untreated cells were assumed 100% viable. Data represents the mean ± SEM of a minimum of three separate determinations.

	Substance	***Concentration [mM]***	***% Toxicity***
A	cimetidine	5	12.85
	famotidine	5	13.93
	metformin	5	15.13
	procainamide	5	13.45
	ranitidine	5	12.46
	triamterene	0.25	4.88
	trimethoprim	0.5	2.04
B	(-)-n-Butylscopolamine bromide	5	8.88
	ciclotropium bromide	5	3.84
	ipratropium bromide	5	1.50
	trospium hydrochloride	5	-8.97
C	decynium22	0.005	11.09
	MPP^+	5	4.71

Important to note here is that the Caki-1 cells have been previously used for the characterization of EMT/OCT3 interactions with monoamines and steroid hormones [138]. For these purposes, MPP^+ and decynium22 were selected as prototypical substrate and specific inhibitor, respectively; the kinetic parameters K_m and IC_{50} were determined in initial experiments to prove the functionality of OCT3 in the Caki-1 cells. Furthermore, the selection of the Caki-1 cells as a model system was based on a recent work confirming that Caki-1 cells are a representative model of the human PT epithelium (Nephron Experimental Neph 2007; (in press)).

Due to the close ancestral origin of the OCTs, it is not surprising that these transporters exhibit overlapping profiles in regards to substrate and inhibitor affinities. Therefore, in order to study a specific variant, one requires a system where a protein of interest can be investigated without confusion of a second protein with similar transport/inhibition profiles. Transfected *Xenopus oocytes* or mammalian cell lines are routinely employed for such studies. However, notable downsides to such models include the modified functional activity of the transport proteins due to expression in an 'artificial' environment [67]. An ideal system is represented by the endogenous expression of a protein in the tissue of interest.

Hence, based on the aforementioned information, the expression of OCT1-3 was determined via a state-of-the-art real-time RT-PCR detection system; which confirmed the previously reported expression of OCT3 in Caki-1 cells and additionally the minimal level and/or absence of OCT1 and 2, respectively. Furthermore, the level of expression remained stable over extended passaging (no significant differences), indicating the robustness of the system. The absence of OCT2 in Caki-1 cells is opposite of the in vivo situation but an in vitro necessity when wishing to study OCT3 activity in isolation, proving the suitability of the system.

Immunocytochemical staining of the Caki-1 cells for OCT3 allowed for the first time, the detection of OCT3 at the protein level in human renal tissue. Furthermore, a partial colocalization between Na^+/K^+-ATPase, (a basolateral specific maker of PT and epithelial tissue) and OCT3 could be made, which provides the first visual evidence of a probable BLM localization of OCT3 in the human kidney. An elaboration on the subcellular localization of OCT3 is currently underway, involving healthy human renal tissue and confocal microscopy methodologies, to confirm the initial results presented in the current work.

Kinetic studies confirmed an uptake of MPP^+ which was saturable with time (~ 10 min) and concentration (K_m = 36.65 µM) and which could be inhibited with decynium22 (specific inhibitor of OCT3), with the expected affinity (IC_{50} = 0.05 µM). All kinetic data correlated well with what has been previously reported for OCT3 in Caki-1 cells and other model systems for OCT3 [138, 140]. The uptake was further characterized to be carrier-mediated by the significant decrease in the uptake observed at 4°C. An influence of pH on uptake was observed, with highest activity at a physiological pH of 7.5, correlating well with what has been previously published for OCT3 [23, 133, 141].

In order to determine whether OCT3 in Caki-1 cells can interact with renally secreted OCs, several drugs which are known to be renally secreted by the OCT system were selected. OCT3 interacted with all drugs to varying intensities, as was the case with drugs of quaternary ammonium structure (IC_{50} values for renally secreted drugs are summarized in Table 2). It has been previously proven that OCT3 in other tissues or systems such as the placenta or transfected systems can interact with some of the drugs tested in the current work. The IC_{50} values determined in the current work lie in a range similar to what has been previously published, further exemplifying the accuracy of OCT3 function in Caki-1

cells [45, 142]. Exclusion of toxic effects was determined via a XTT based in vitro toxicity assay.

Very few research papers exist where the authors have attempted to make a correlation of the in vitro data (e.g. IC_{50} values) to the physiological concentrations expected. In order to determine the pharmacological significance of the in vitro kinetic data determined here, the IC_{50} values were compared with the expected C_{max} values of these drugs after oral administration. The concentration of a drug required to significantly interfere with a transport process in vitro is often so high that the chances of a significant clinical interaction is negligible. Table 2 summarizes the expected C_{max} values of the various drugs tested (after oral dosing) and the correlation of these values with the IC_{50} values determined in the current work.

Table 2. Summary and comparison of the maximum plasma concentrations (C_{max}) after oral dosing of the renally secreted drugs and the in vitro IC_{50} values determined for the inhibition of ^{3}H-[MPP^{+}] uptake into Caki-1 cells. In vivo data was obtained from the literature or drug data banks as indicated by the references. IC_{50} values are indicated as the average of 3 individual determinations and the 95% confidence intervals.

Drug	approximate C_{max} [µg/ml] (oral dose)	C_{max} [µM]*	IC_{50} [µM]
cimetidine	4.0 (800mg)	15.8	24.8 (20.47 to 30.04)
famotidine	0.1 (40mg)	0.3	7.5 (3.22 to 17.35)
procainamide	6.0 (1000mg)	22.1	213.5 (186.5 to 244.5)
ranitidine	0.8 (300mg)	2.3	124.3 (108.0 to 143.1)
triamterene	0.04 (100 mg)	0.1	9.0 (7.830 to 10.29)
trimethoprim	2.2 (150 mg)	7.6	14.5 (13.65 to 15.42)

* C_{max} values were obtained from: www.roteliste.de and/or ABDA-Datenbank

As indicated in Table 2 the concentration required for 50% inhibition of OCT3 transport activity for most drugs, is generally much higher than the expected plasma concentrations, indicating that a clinically significant interaction under these conditions would be unlikely to occur in the kidney. However, the IC_{50} values determined for cimetidine and trimethoprim are pharmacologically relevant. Additionally, the potential for interactions to occur in other tissues where OCT3 is expressed and higher concentrations of these drugs may accumulate (e.g. intestine, lung) must not be excluded. For certain drugs tested as is the case with cimetidine and famotidine, it has been confirmed or contradictory information is existing, that these drugs are not substrates of OCT3, however, it remains to be

determined whether the other drugs are substrates and this may potentiate the chance of an in vivo interaction. The low IC_{50} values of triamterene and trimethoprim may be seen as an indicator for their possible transport by OCT3. Furthermore, two important pieces of information should be considered to understand the potential physiological role of OCT3: (1) OCTs are not evenly distributed along the proximal tubule segments and (2) the expression of OCT variants is effected during disease states such as diabetes [143]. Therefore, there are specific circumstances where OCT3 may have a more significant and pronounced role in comparison to OCT2 than previously thought. Increased drug levels can as well be expected in disease states and geriatric patients possibly leading to pronounced interactions.

In conclusion, the current work has illustrated the effectiveness of using the clonal cell line, Caki-1, as a renal model system for studying the interaction of various therapeutic agents with the OCT3 variant. Interactions of OCT3 with renally secreted drugs known to have clinically significant interactions (due to their carrier-mediated renal secretion) were systematically shown. Additionally, the inhibition of OCT3 transport processes via quaternary ammonium derivatives was demonstrated for the first time. A highlight of the current work was the first-time detection of OCT3 in kidney cells of human origin, with probable localization to the BLM. Further studies focusing on the substrate specificity of this transporter will enlighten its role in drug transport in the kidney.

6.6 Acknowledgements

The authors would like to thank Dr. Constanze Hilgendorf for her assistance with the real-time RT-PCR experiments and Prof. Hermann Koepsell for providing the OCT3 specific antibody.

Summary

One of the major roles of the human kidney is to purify blood by extracting the waste products of metabolism, including endogenous compounds such as creatinine, urea and uric acid. Exogenous compounds, encompassing drugs and their derivatives, are additionally excreted by the kidneys. Furthermore, the kidney plays a limited albeit important role in drug metabolism. Structurally, the nephron is the functional unit of the kidney and can be divided into several distinct segments. The proximal tubule (PT) segment, located mainly in the cortex of the kidney is the site at which maximum carrier-mediated secretion and reabsorption occurs, along with drug metabolism. Solutes are transported across the PT epithelium via transcellular and/or paracellular routes. Transport proteins involved in the transcellular transport of xenobiotics play a major role in drug interactions occurring in the kidney. Transport proteins can be subdivided into various classes based on substrate selectivity. Organic cation transport (OCT) proteins, belonging to the solute carrier 22 (SLC22) subfamily play a significant role in the carrier-mediated secretion and reabsorption of cationic drugs in the kidney.

Various in vitro systems currently exist for studying drug interactions in the human kidney, including whole kidney tissue, isolated proximal tubules, primary cell cultures and immortalized cell lines. Cell lines represent one of the most convenient in vitro tools to date in the field of drug studies, due to their unlimited availability, lack of interfering hormonal factors, constant conditions and easy handling. However, to date an intensively characterized cell line of human PT origin for implication in such studies does not exist. Until now, researchers have relied on the use of more cumbersome methods and/or uncharacterized cell lines of human and non-human origin.

In the current work, a commercially available human renal clear cell carcinoma cell line, Caki-1, was selected for characterization. Three goals were set: (1) the morphological, biochemical and physiological characterization to determine the possible PT origin, (2) determination of the level of expression of 42 relevant drug transport genes and metabolizing enzymes and immunohistochemical localization of relevant OCTs and (3) functional characterization of the relevant OCTs via drug uptake and inhibition studies.

Morphologically, Caki-1 cells were characterized to grow as a polarized epithelium. Electron microscopy allowed the visualization of apically located microvilli and multiple mitochondria per cell, both typical characteristics of the polarized PT epithelium in vivo. The basolateral location of the Na^+-K^+/ATPase in Caki-1 cells gave further proof to the polarized nature of these cells. PT cells are often isolated via determination of specific PT marker molecules including the protein/enzymes: NHE3, DPP IV, AP, APM and GGT. Enzymatic activity, gene detection and/or cytochemical staining confirmed the presence of all aforementioned enzymes in Caki-1 cells. In vivo, PT epithelium forms a ‘leaky’ low resistance barrier, with a reported transepithelial electrical resistance (TER) of 5-12 Ω cm^2. TER was measured in Caki-1 and human primary proximal tubule (HPT) cells using the ‘chopstick’ electrode technique. TER values below 25 Ω cm^2 were determined for both cell types. E-cadherin is known to be one of the most significant molecules involved in the formation of tight junction

barriers in PT epithelium in vitro and therefore, its expression pattern was determined in Caki-1 cells. As expected, E-cadherin expression was located to the cell-cell contacts of Caki-1 monolayers.

42 transport proteins and metabolizing enzymes were selected for analysis on the transcriptional level based on their contributions to the transport and metabolism of xenobiotics. Screening was carried out via real-time RT-PCR for Caki-1 and HPT cells. Of the 42 transcripts tested 23 were present in Caki-1 and HPT cells, 19 of these being commonly expressed. Caki-1 cells showed the highest expression for ABCC3, ABCC4, SLC16A1, SLC16A4, SLCO4A1, and SLCO4C1. Moderate expression was observed for ABCB1, ABCC1, SLC22A3 and SLC22A5. The HPT cells exhibited high expression for ABCC3, ABCC4, SLC16A1, SLC16A4, SLCO4A1 and SLCO4C1. Moderate expression of ABCC1, ABCB1, SLC16A1, SLC22A2, SLC22A5, and SLC29A1 was observed in the HPT cells. Low expression was observed for all CYP isoforms investigated.

The initial focus of this project was the design of an in vitro tool for studying cationic drug interactions, therefore, further work focused on OCT3 and OCTN2, the protein products of SLC22A3 and SLC22A5 respectively. Previously published results have confirmed the presence of OCT3 and OCTN2 in the human kidney. Additionally, it is known that OCTN2 is located to the apical membrane of human PT cells but a subcellular localization of OCT3 in human PT cells remained until now unknown. The apical location of OCTN2 was confirmed for Caki-1 cells via confocal microscopy using Na^+-K^+/ATPase as a marker for the basolateral membrane. An epifluorescence microscope allowed for the first time, the detection of OCT3 in human kidney cells and its probable localization to the basolateral membrane of Caki-1 cells (co-localization to Na^+-K^+/ATPase). OCTN2 protein expression was also confirmed in HPT cells.

In order to determine the functional activity of OCT3 and OCTN2 in Caki-1 cells, uptake studies employing the prototypical radiolabeled substrates MPP^+ and l-carnitine, respectively, were performed. Saturation constants (K_m) of approximately 36 and 16 µM were determined for MPP^+ and l-carnitine for OCT3 and OCTN2 respectively. Decynium22 and valproic acid were selected as known and selective inhibitors of OCT3 and OCTN2. Inhibition constants (IC_{50}) of approximately 0.05 and 140 µM were determined for the inhibition of MPP^+ and l-carnitine uptake via OCT3 and OCTN2 respectively. All data correlated well with previously published information regarding the kinetics of these transporters and with uptake studies carried out in HPT cells (control system). Several partially non-metabolized renally secreted drugs, known to have clinical interactions were chosen as potential inhibitors of OCT3 and OCTN2 uptake processes. Cimetidine, famotidine, procainamide, ranitidine, triamterene and trimethoprim, among many other drugs were able to inhibit both OCT3 and OCTN2 uptake processes with distinct potencies, several at pharmacologically relevant concentrations.

In conclusion, the current work offers the following significant contributions to the fields of PT and drug transport research: (1) confirmation and introduction of the first commercially available PT cell line, which is categorized under the biosafety class 1, (2) introduction of an in vitro tool for testing/simulating potential PT drug interactions via OCT3 and OCTN2, (3)

the novel detection and localization of OCT3 to the basolateral membrane of human PT cells and (4) the systematic evaluation of several possible drug interactions via OCT3 and OCTN2 in a single characterized system.

Caki-1 cells represent the first human PT cell line to be characterized at various levels with partial comparison to HPT cell cultures. They show great potential as a screening tool for currently administered drugs and those in their developmental stages, for interactions with OCT3 and/or OCTN2. Further studies analyzing the functionality of other transport proteins expressed in Caki-1 cells could unveil useful information and extended advantages to this cell culture system.

Zusammenfassung

Eine der Hauptfunktionen der menschlichen Niere ist die Reinigung des Blutes durch Filterung der Stoffwechselprodukte, endogene Verbindungen wie Kreatinin, Harnstoff und Harnsäure eingeschlossen. Exogene Verbindungen, Wirkstoffe und ihre Metabolite werden ebenfalls durch die Niere ausgeschieden. Darüber hinaus spielt die Niere eine begrenzte aber wichtige Rolle im Wirkstoffmetabolismus.

Die Struktur betreffend ist das Nephron die funktionelle Einheit der Niere und kann in unterschiedliche Bereiche unterteilt werden. Der proximale Tubulus (PT), der sich zum größten Teil in der Nierenrinde befindet, ist der Ort der stärksten tubulären Sekretion und Reabsorption, sowie des Wirkstoffmetabolismus. In Lösung befindliche Stoffe werden durch das Epithel des PT auf transzellulärem und/oder parazellulärem Weg transportiert. Die in den transzellulären Xenobiotikatransport involvierten Transportproteine spielen eine tragende Rolle bei Wirkstoffinteraktionen, die in der Niere stattfinden können. Transportproteine können hinsichtlich ihrer Substratselektivität in verschiedene Klassen eingeteilt werden. Organische Kationtransportproteine, die zur Solute Carrier 22-Unterfamilie gehören, spielen eine signifikante Rolle in der Carrier-vermittelten Sekretion und Reabsorption kationischer Wirkstoffe in der Niere.

Zurzeit existieren einige in vitro-Systeme um Wirkstoffinteraktionen in der menschlichen Niere zu untersuchen. Eingesetzt werden intaktes Nierengewebe, isolierte Tubuli, primäre Zellkulturen und Krebszelllinien. Zelllinien sind dank unbegrenzter Verfügbarkeit, bislang des Ausbleibens störender hormoneller Faktoren, der konstanten Bedingungen und der einfachen Handhabung eine der gebräuchlichsten in vitro-Systeme im Bereich von Wirkstoffstudien.

Derzeit existiert keine ausreichend charakterisierte Zelllinie menschlichen PT-Ursprungs, um derartige Studien durchführen zu können. Bis zum heutigen Tage verwenden Forscher aufwendige Methoden und/oder unzureichend charakterisierte Zelllinien menschlichen oder tierischen Ursprungs.

In der vorliegenden Arbeit wurde eine kommerziell verfügbare menschliche, renale clear cell carcinoma' Zelllinie, Caki-1, zur Charakterisierung ausgewählt. Auf vier Ziele wurde hingearbeitet: (1) die morphologische, biochemische und elektrophysiologische Charakterisierung zur Bestimmung des möglichen PT-Ursprungs, (2) Untersuchung der Genexpression von 42 relevanten Wirkstofftransportproteinen und metabolisierenden Enzymen, (3) immunohistochemische Lokalisierung relevanter OCT-Proteine (OCT3 und OCTN2 wurden in STEP 2 (siehe ‚Aims of thesis') detektiert) und (4) die funktionelle Charakterisierung von OCT3 und OCTN2 durch Wirkstoffaufnahme und Hemmstudien mit renal ausgeschiedenen Xenobiotika.

Die Ausbildung eines polarisierten Monolayers der Caki-1 Zellen konnte gezeigt werden. Mittels elektronenmikroskopischer Untersuchungen wurden apikal lokalisierte Microvilli und zahlreiche Mitochondrien in den Zellen sichtbar gemacht, jeweils typische Charakteristika

des polarisierten PT-Epithels in-vivo. Die basolaterale Lokalisierung der Na^+/K^+-ATPase in Caki-1 Zellen erbrachte eine weitere Bestätigung der polarisierten Natur dieser Zellen. PT-Zellen werden oft durch Bestimmung spezifischer PT-Marker Enzyme wie NHE3, AP, APM, DPP IV und GGT charakterisiert. Enzymatische Aktivität, Gennachweis und/oder cytochemische Färbung bestätigten die Anwesenheit aller zuvor genannten Markerproteine in Caki-1 Zellen. Das in-vivo PT-Epithel bildet eine ‚löchrige' Barriere mit einem niedrigen transepithelialen elektrischen Widerstand (TER) von 5-12 Ω cm^2. TER wurde in Caki-1 und menschlichen primären PT-Zellen mittels ‚Chopstick' Elektrodentechnik bestimmt. TER-Werte unter 25 Ω cm^2 wurden für beide Zelltypen gemessen. Als eines der wichtigsten Moleküle, welches an der Bildung der „Tight Junctions"- Barrieren im PT-Epithel in vitro beteiligt ist, wurde die Exprimierung von E-cadherin an den Zell-Zell-Kontakten der Caki-1-Monolayer nachgewiesen. „Tight Junctions" wurden zusätzlich durch Transelektronenmikroskopie-Analyse detektiert.

Im zweiten Teil dieser Arbeit wurden, aufgrund ihrer Beiträge zum Transport und Metabolismus von Xenobiotika, 42 Transportproteine und metabolisierende Enzyme für die Analyse auf Transkriptionsebene ausgewählt. Die Prüfung wurde mittels real-time RT-PCR für Caki-1 und HPT Zellen durchgeführt. Von 42 getesteten Transkripten wurden 23 in Caki-1 und HPT Zellen gefunden, wovon 19 in beiden exprimiert werden. Caki-1 Zellen zeigten die höchste Expression für ABCC3, ABCC4, ABC16A1, SLC16A4, SLCO4A1 and SLCO4C1. Moderate Exprimierung wurde für ABCB1, ABCC1, SLC22A3 and SLC22A5 nachgewiesen. Die HPT Zellen wiesen hohe Exprimierung für ABCC3, ABCC4, SLC16A1, SLC16A4, SLCO4A1 und SLCO4C1 auf hingegen wurden ABCB1, ABCC1, SLC16A1, SLC22A5 und SLC29A1 hier lediglich moderat exprimiert. Keine oder sehr geringe Exprimierung wurde für alle untersuchten CYP Isoformen ermittelt.

Das ursprüngliche Konzept des Projektes beinhaltete die Entwicklung eines in vitro Systems zur Untersuchung kationischer Wirkstoffinteraktionen. Daher wurde besonderer Wert auf OCT3 und OCTN2, die in den Teilen 2 und 3 (siehe ‚Aims of the thesis) detektiert wurden, gelegt. Zuvor publizierte Arbeiten liefern bereits Hinweise auf die Anwesenheit von OCT3 und OCTN2 in der menschlichen Niere. Außerdem ist bekannt, dass OCTN2 in der apikalen Membran lokalisiert ist, eine subzellulare Lokalisierung von OCT3 war bisher für das menschliche PT-Epithel unbekannt. Die apikale Position von OCTN2 wurde für Caki-1 Zellen durch konfokale Mikroskopie untermauert. Hierfür wurde die Na^+/K^+-ATPase als Marker für die basolaterale Membran gewählt. Erstmalig erlaubte eine Epifluoreszenzmikroskop-Aufnahme den Nachweis von OCT3 in menschlichen Nierenzellen und seine wahrscheinliche Lokalisierung in der basolateralen Membran der Caki-1 Zellen (Colokalisierung mit der Na^+/K^+-ATPase). Eine Proteinexprimierung von OCTN2 wurde ebenso in HPT-Zellen nachgewiesen.

Zur Bestimmung der funktionellen Aktivität von OCT3 und OCTN2 in Caki-1 Zellen wurden Uptake-Studien durchgeführt, in denen die prototypisch radioaktiv markierten Substrate MPP^+ beziehungsweise L-Carnitin verwendet wurden. Sättigungskonstanten von 36 und 16 µM wurden für MPP^+ und L-Carnitin und somit OCT3 beziehungsweise OCTN2 Aktivität bestimmt. Decynium22 und Valproinsäure wurden als bekannt selektive Inhibitoren von OCT3 und OCTN2 eingesetzt. Hemmkonstanten (K_m) von 0,05 und 140 µM wurden für die

Hemmung der MPP^+- und L-Carnitin-Aufnahmen durch OCT3 beziehungsweise OCTN2 bestimmt. Die Ergebnisse stimmen mit bislang publizierten Daten über die Kinetik dieser Transporter und mit bei HPT-Zellen durchgeführten Uptake-Studien überein.

Einige vorwiegend renal unverändert ausgeschiedene Wirkstoffe, die für klinische Interaktionen bekannt sind, wurden als potentielle Inhibitoren bei OCT3- und OCTN2-Uptake-Studien ausgewählt. Cimetidin, Famotidin, Procainamid, Ranitidin, Triamteren und Trimethoprim zeigten neben zahlreichen weiteren Wirkstoffen einen hemmenden Einfluss auf die Transporteraktivität sowohl von OCT3- als auch von OCTN2. Dem pharmakologisch relevanten Konzentrationsbereich wurde dabei Beachtung geschenkt.

Zusammenfassend leistet die vorliegende Arbeit folgende Beiträge zur Methodenentwicklung von Transportuntersuchungen im Nierenepithel und der Wirkstoffprofilierung allgemein: (1) Nachweis und Einführung der ersten kommerziell erwerblichen PT-Zelllinie, die in die Biosicherheitsklasse 1 eingeordnet wird, (2) Einführung eines in vitro-Systems zur Überprüfung/Simulierung möglicher PT-Wirkstoffinteraktionen über OCT3 und OCTN2, (3) Nachweis von OCT3 auf der basolateralen Seite des PT und (4) systematische Analyse von möglichen Wirkstoffinteraktionen via OCT3 und OCTN2. Caki-1 Zellen stellen die erste menschliche PT-Zelllinie dar, die auf unterschiedlichen Ebenen charakterisiert und teilweise mit HPT-Zellkulturen verglichen wurde. Das Modell zeigt großes Potential zur Prüfung derzeit verabreichter Wirkstoffe und solcher in Entwicklungsphasen auf Interaktionen bezüglich OCT3 und OCTN2. Weitere Studien, welche die Funktionalität in Caki-1 Zellen exprimierter weiterer Transportproteine analysieren, könnten nützliche Information und weitere Vorteile dieses Zellkultursystems liefern.

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Appendix

Appendix A: OCT3-uptake inhibition assay

Table A. Inhibition of OCT3 mediated uptake of ^{3}H-MPP+ into Caki-1 cells. IC_{50} values of selected compounds are listed together with the 95% confidence interval of the IC_{50} value, the hill slope of the respective curve, the coefficient of correlation of the fit and the maximum concentration tested.

Compound	IC_{50} [µM]	95% confidence interval		Hill Slope	Coefficient of correlation [R^2]	Concentration tested [µM]
		upper level	lower level			
cetirizin	638.1	553.9	735.1	-1.293	0.9913	1000
cefazolin	4782	3404	6720	-1	0.971	5000
ciclotropium bromide	163.3	141.9	187.8	-1.165	0.9951	5000
cimetidine	24.8	20.47	30.04	-0.8756	0.9973	5000
decynium22	0.05	0.04	0.05	-0,8895	0,9980	5
famotidine	7.5	3.23	17.35	-1.004	0.9985	5000
ipratropium bromide	83.4	77.1	90.21	-0.8228	0.9987	5000
MPP^+	13	8.17	20.8	-0,7260	0,9872	5000
procainamide	213.5	186.5	244.5	-0.8559	0.9966	5000
ranitidine	124.3	108	143.1	-0.8349	0.9961	5000
talinolol	178.2	146.5	216.8	-0.9917	0.9885	1000
triamterene	8.97	7.83	10.29	-0.8916	0.9978	100
trimethoprim	14.5	13.65	15.42	-1.021	0.9991	500
trospium HCl	1423	1048	1931	-1.079	0.9762	5000
zidovudine	234.4	191.7	286.7	-0.875	0.9956	5000

Appendix B: OCTN2-uptake inhibition assay

Table B. Inhibition of OCTN2 mediated uptake of methyl-L-[^{3}H] carnitine into Caki-1 cells. IC_{50} values of selected compounds are listed together with the 95% confidence interval of the IC_{50} value, the hill slope of the respective curve, the coefficient of correlation of the fit and the maximum concentration tested.

Compound	IC_{50} [µM]	95% confidence interval		Hill Slope	Coefficient of correlation [R^2]	Maximum Concentration tested [µM]
		upper level	lower level			
betaine	129.9	102.8	164.2	-0.8071	0.9898	5000
(-)-N-Butylscopolamine bromide	1007	615.7	1648	-1.287	0.9363	5000
cetirizin	27.08	16.04	45.7	0.7895	0.9718	1000
cephaloridine	248	187.4	328.1	-0.9487	0.9846	5000
ciclotropium bromide	30.8	22.37	42.42	-1.504	0.9852	5000
cimetidine	336,7	243.1	466.3	-1.082	0.9776	5000
famotidine	1920	890.8	4138	-0.6716	0.9452	5000
ipratropium bromide	95.84	67.43	136.2	-1	0.9649	5000
metformin	4963	3451	6956	1.342	0.9678	5000
MPP^+	1713	1231	2384	-1.928	0.9736	5000
procainamide	2436	1712	3465	-1.676	0.9601	5000
ranitidine	83.95	63.71	110.6	-0.9324	0.9808	5000
talinolol	434.1	401.4	469.5	-2.337	0.9945	1000
TEA	551.1	395.8	767.3	-0.9986	0.9754	5000
trospium HCl	112.6	80.39	57.7	-0.8483	0.9846	5000
valproic acid	139.6	88.07	221.2	-0.6517	0.9857	5000
zidovudine	3504	2486	4941	-1.069	0.9394	5000