THE STUDY OF FUNCTION AND REGULATION OF RENAL DRUG TRANSPORTERS IN HUMAN PROXIMAL TUBULE EPITHELIAL CELLS

Pedro Miguel Caetano Pinto

The research presented in this thesis was performed at the Department of Pharmacology and Toxicology, Radboud Institute for Molecular Life Sciences, Nijmegen and the Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University. Financial support was provided by the Nephrotools consortium, a Marie Skłodowska-Curie Innovative Training Network of the European Union Seventh Framework programme (grant no. 289754)

Financial support by the Utrecht Institute for Pharmaceutical Sciences and the Dutch Kidney Foundation for the publication of this thesis is gratefully acknowledged

ISBN: 978-94-6182-766-1

Cover design, layout & printing: Off Page, Amsterdam

About the cover: Fluorescent image of a developing kidney *ex vivo* (Pedro Pinto).

Copyright © 2017 Pedro Caetano-Pinto
All rights reserved. No parts of this book may be reproduced by any means, without written permission of the author.

THE STUDY OF FUNCTION AND REGULATION OF RENAL DRUG TRANSPORTERS IN HUMAN PROXIMAL TUBULE EPITHELIAL CELLS

DE STUDIE NAAR DE FUNCTIE EN REGULATIE VAN FARMACONTRANSPORTERS IN HUMANE PROXIMALE TUBULUSEPITHEELCELLEN VAN DE NIER

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof. dr. G.J. van der Zwaan,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen
op woensdag 15 maart 2017 des middags te 4.15 uur

door

Pedro Miguel Caetano Pinto
geboren op 10 Mei 1982 te Vidigueira,
Portugal

PROMOTOR

Prof. dr. R. Masereeuw

COPROMOTOR

Dr. M. Wilmer

TABLE OF CONTENTS

The good thing about science is that it's true
whether or not you believe in it
Neil DeGrasse Tyson

CHAPTER 1

General introduction and thesis outline

Partially published as content of:
Chapter 5: Membranes for bioartificial kidney.Natalia Chevtchik, Pedro Caetano-Pinto, Rosalinde Masereeuw, Dimitrios Stamatialis Book "Biomedical membranes and bioartificial organs", in preparation (2017)
Editor: D. F. Stamatialis,
Publisher: World Scientific.

INTRODUCTION

The kidneys play a fundamental role in maintaining whole body homeostasis. Renal physiological and regulatory functions include hormone production, the regulation of blood pressure by controlling extracellular fluid volume, safeguarding physiological pH, keeping appropriate electrolyte balance and systemic waste and xenobiotics removal [1, 2]. For these purposes, the kidneys evolved into complex organs in which the nephron is the structural and functional unit with a complex architecture, where blood vessels converge into a filtering compartment, the glomerulus, and adjacent tubular structures produce and collect urine [3]. The breakdown of normal renal functions, leading to kidney disease has an immense impact on the life of those affected and is a major healthcare concern. The overall global prevalence of kidney diseases is now estimated to be 11-13% of the population [4, 5]. Improving and developing novel strategies to cope with kidney disease is a recognized priority with a central role for fundamental research in drug development and regenerative medicine. This chapter offers an overview on kidney disease and on kidney tubular function in particular. Furthermore, details on cellular models available for kidney-related research are described [6, 7].

KIDNEY DISEASE AND RENAL FUNCTION

Kidney diseases often disrupt kidney function and structure [8]. The primary causes of kidney disease are diabetes and hypertension, where blood vessel damage (*e.g.* atherosclerosis) prevents proper blood filtering, leading to systemic waste and fluid accumulation, increasing blood pressure, in a spiral leading to the disease progression. Other factors include inflammation, infections and disruptions of the urinary and urogenital apparatus, autoimmune diseases, nephrotoxicity and genetic pre-disposition, although less common [9, 10].

Chronic kidney disease (CKD) is a progressive loss of kidney function over a prolonged period of time. There are five recognized stages of CKD, based on the traditional method of measuring glomerular filtration rate (GFR), accounting for age, gender, race and creatinine blood levels. Stages one and two represent a slight and mild renal damage, stage three and four account for moderate to severe damage and stage five implies kidney failure or end stage renal disease (ESRD) [11]. Early stages of CKD can be therapeutically managed when diagnosed on time, however current treatment options for patients with ESRD are limited, and replacement therapy is inevitable [12].

A kidney transplant can offer a permanent solution for kidney failure; nonetheless it carries risks, depends on organ availability and requires permanent monitoring. As an end of the line solution, transplants are preceded by dialysis, most often hemodialysis [13]. Developed and introduced in the 1940's by Willem Kolff, dialysis is based on the physical processes of diffusion and ultrafiltration, and can remove fluid and solutes within a range of molecular weights from the blood. Despite a breakthrough in ESRD treatment, traditional dialysis only covers a fraction of the physiological renal function and its efficiency in waste removal is incomplete, only offering a temporary solution [14].

In recent years, kidney disease prevalence has increased worldwide. Arguably, this trend can be the reflection of an aging population in developed countries or the result of better diagnosis and screening of renal diseases in human populations [15]. Surveys pointed out that 1 out of 10 people suffer from CKD (http://www.worldkidneyday.org/) and that half of the population above seventy-five years of age manifests some form of renal disease. With such numbers, kidney disease is a major healthcare concern [5]. Treatment options are limited at advanced stages, while early diagnosis and subsequent treatments result in a better prognosis. CKD diminishes the quality of life, and despite its obvious consequences for patients with increased morbidity and mortality, it is also a burden for society with rising costs for healthcare systems and caregivers [16]. As renal disease is now recognized as a major and increasing healthcare concern, research towards a better understanding of the molecular mechanisms behind kidney function, physiology and disease progression in order to improve the efficacy of current treatments and to develop new or alternative therapies to tackle kidney disease is warranted.

RENAL TUBULAR FUNCTION

After glomerular filtration, the proximal tubules are responsible for reabsorbing essential components from the ultra-filtrate, but also for additional removal of a great variety of solutes and wastes from the blood. Its lumen is convoluted where the apical side of proximal tubule epithelial cells (PTEC) is organized into dense microvilli forming a brush border, with an increased surface area to enhance the reabsorption process. Active membrane transport at the basolateral side also facilitates the removal of compounds potentially bound to plasma proteins in the blood. A great variety of endogenous solutes, their metabolites and exogenous compounds and their metabolites, such as drugs, are transported in the blood stream coupled to plasma proteins such as albumin, and can only be removed from the circulation by active secretion, shifting the protein-bound to the free fraction.

To this end, PTEC are equipped with a highly specialized molecular machinery to meet this requirement (Figure 1). These polarized cells act as a barrier for compounds in the basolateral (capillary side) and apical side (pre-urine). To a great extent, the functional characteristics of PTEC are associated with the presence of multiple energy dependent membrane transporters that mediate the transport of ions, small molecules, nucleotides, xenobiotics and other substances. These carrier proteins can move solutes against steep concentration gradients, and give the cells their barrier-specific selectivity and high excretion capacity. Transporters in PTEC membranes can be unidirectional efflux pumps, co-transporters, facilitated diffusion carriers and exchangers, belonging to either the Adenosine triphosphate-ATP binding cassette (ABC) [17] superfamily or the Solute carrier family (SLC; www.slc.bioparadigms.org) of proteins [18]. ABC transporter function requires ATP hydrolysis, a feature that needs an increased mitochondrial activity to meet the energy demand. Activity of SLC transporters involves co-transport driven

by a membrane potential, established by the basolaterally expressed Na,K-ATPase. Basolateral and apical transporters are usually complementary in substrate specificities to enable compounds that are taken up by the cells to be excreted subsequently [19].

The most prominent uptake transporters are the organic anion transporter 1 (OAT1/*SLC22A6*), organic anion transporter 3 (OAT3/*SLC22A8*)[20] and the organic cation transporter 2 (OCT2/*SLC22A2*) [21]. The predominant efflux transporters are breast cancer resistance protein (BCRP/*ABCG2*) [22], P-glycoprotein (P-gp/*ABCB1*) and the multidrug resistance proteins 2 and 4 (MRP2/4; *ABCC2/4*) [23]. Other carriers, such as the transporter organic anion transporter polypeptide (OATP4C1/*SLCO4C1*) and the multidrug and toxin extrusion 1 and 2 transporters (MATE1/2K; *SLC47A1/2K*) are also present at PTEC [24]. These transporters can share common regulatory pathways that modulate their activity. A comprehensive depiction of all major PTEC transporters is presented in figure 1. Intracellularly, PTEC can also metabolize drugs and compounds via phase I and II enzymes, in a process that can increase the excretory efficacy of certain compounds that undergo hydrolysis or glucuronidation [25]. Furthermore, both transcriptional and post-transcriptional regulation of all transporters have been described [26, 27].

Multiple SLC and ABC carriers (among others) show affinity for the same solutes, leading to promiscuity in substrate specificity [28]. This phenomenon can be explained by the high degree of homology within transporters families. Further, certain cytochrome P450 enzymes (CYP) share similar substrate profiles with P-gp and in a similar fashion, substrate glucuronidation, by the action of UDP-glucuronosyltransferases (UGT), increases affinity for MRP transporters [29, 30][17]. This transporter-enzyme crosstalk is an example of co-evolution, where separate cellular mechanisms articulate to enhance xenobiotic metabolism and excretion. Both enzyme systems are present in PTEC, which adds another layer of complexity to drug clearance, since the process involves uptake, metabolism and efflux.

In addition to xenobiotic excretion, PTEC also play an extensive role in the reabsorption of nutrients and ions back from the glomerular filtrate into the blood stream, a process taking place at the apical (luminal) side (Figure 1). An important active reabsorption mechanism is receptor-mediated endocytosis, in which proteins with different sizes (e.g. albumin, transferrin, ferritin) that initially pass the glomerular membranes, are carried from the filtrate to the circulation. This transport is mediated by three receptors expressed at the apical membrane: megalin, cubilin and amnionless [31]. A number of key ions is also reabsorbed, although this process is not exclusive to PTEC, taking place along other segments of the nephron, and can occur passively with the enormous amount of water that diffuses back to the circulation or via a number of membrane carriers that handle *e.g.* potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), phosphate (PO_4^{3-}) [32, 33]. In addition, glucose is reabsorbed exclusively by PTEC via both Na^{2+}-dependent co-transporters (*SLC5A* family) and facilitated glucose transporters (*SLC2A* family) [34, 35]. Aminoacids are also recovered via a Na^{2+}-dependent transporters belonging to the SLC6A family [36].

Throughout the nephron, water reabsorption is facilitated by selective water channels known as aquaporins (AQP), with different channels expressed in the proximal tubules, loop of Henle and collecting duct [37]. Though renal function derives from the actions of the different nephron segments, PTEC are key in drug elimination and its activity accounts for a significant part of the renal function in terms of xenobiotic excretion.

As a comprehensive picture of the compounds handled by PTEC came to light, it became evident that a variety of small protein-bound molecules are not being removed by dialysis [38]. A class of substances tied to the progression of kidney diseases is the so-called uremic retention solutes, also known as uremic toxins. Composed of different solutes with various physical and chemical properties [39], uremic solutes share that they accumulate in patients with CKD as a result of deficient renal clearance. The systemic build-up of these substances, that are often bi-products of metabolic processes, is known to cause pathological effects in multiple organs, lead to disease progression and further aggravate kidney failure [40, 41]. In health, the solutes are efficiently cleared by the proximal tubules [42] and, although traditional dialysis is able to remove small water-soluble toxins, the protein-bound solutes can only be eliminated via the kidney through active biological processes inherent to PTEC [38]. This illustrates the limitations of current therapies/ technologies available as renal replacement therapy, and it also highlights the importance of our knowledge on renal physiology in order to improve and develop new treatments.

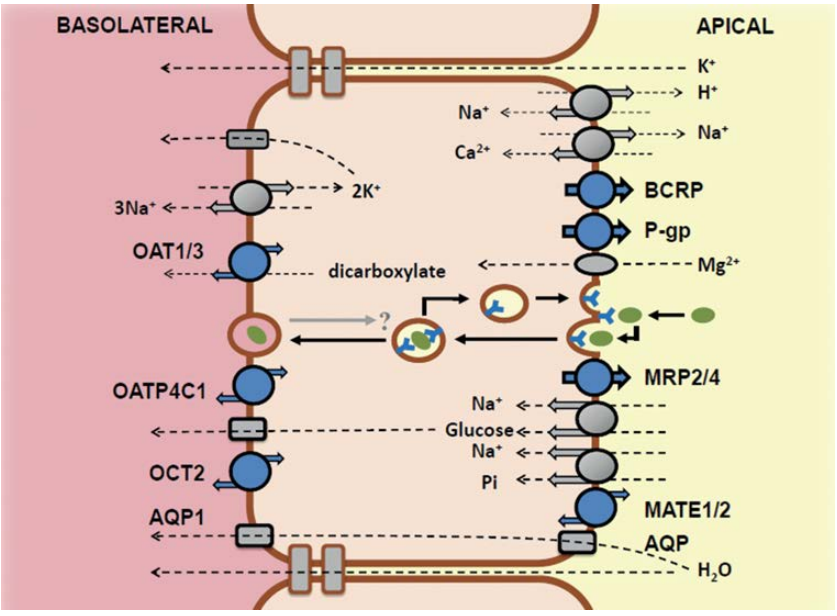


Figure 1. Schematic presentation of the major basolateral and apical membrane transport systems in renal proximal tubule cells. Reabsorption mechanisms are represented in grey and drug transporters are represented in dark blue.

RENAL TUBULE CELL MODELS IN RESEARCH

Renal cells can perform and regulate, with extreme efficiency, highly complex and specific chemical and physical processes simultaneously. Harnessing and exploiting cells to study renal physiology is central to kidney research. Nowadays a variety of renal-derived cell types are available, from different sources and used in fundamental bioengineered kidney research. A fundamental aspect of culturing cells *in vitro* is assuring that the cells retain a phenotype that closely resembles the *in vivo* situation. Cells can drastically change their properties while being in culture, due to the artificial environment. Cellular plasticity allows cells to change their gene and protein expressions as well as their metabolic activity when confronted with an artificial environment. Traditionally, cells are grown on flat plastic surfaces, fed with a cocktail of nutrients and factors (culture medium) and maintained in a humidified environment at physiological temperature, while *in vivo* cells are arranged in three-dimensional structures often containing multiple cell types that cross-talk and are nourished from the blood stream. Culture medium composition can be tailored to maintain tissue specific phenotypes, however, the addition of growth factor and serum, often required to maintain proliferation, can influence chromosome stability and can lead to gene mutations, affecting the phenotype of the cells [43]. To monitor that cells maintain their phenotype, an array of assays and techniques has to be performed.

When growing PTEC *in vitro* it is key to determine whether the specific molecular machinery of this cell type is expressed at the gene and protein levels. It is also important to determine the proper morphology, capability of the cells to polarize, tight monolayer formation, generation of the appropriate membrane potential and selective barrier function. Subsequently, it is crucial to evaluate the functional activity of the cells, ensuring recapitulation of the activities of native PTEC.

Primary cells

Primary cells are directly derived from renal tissue or urine, collected from healthy donors (either through a biopsy or from a discarded kidney transplant). The renal tissue is then disaggregated into a heterogeneous cell suspension that is purified further via flow cytometry or magnetic beads, making use of membrane markers in order to isolate PTEC from other cell types. Afterwards, cells can be cultured and characterized to confirm the cell phenotype. These primary cells retain only temporarily the PTEC phenotype, losing their epithelial characteristics with each population doubling in culture, and their use is limited to the availability of donors [44]. Due to these limitations, primary cells are not a preferred source for long-term applications and are mostly used for cellular and molecular research into the inner works of PTEC, as well as for drug efficacy and safety testing. An alternative source of PTEC cells also explored is urine [45, 46]. Being easily accessible, urine is an abundant source of cells and incidentally PTEC are shed in reasonable numbers.

Stem cells

Another primary cell source are stem or progenitor cells for which cells can be derived also from other tissues than the kidney. Stem cells are undifferentiated cells that can, in one hand, self-regenerate and, on the other, give rise to various terminally differentiated cell types [47]. These cells are found in developing embryos, being pluripotent (able to generate any lineage) at earlier stages. As embryonic development progresses, stem cells differentiate into particular tissue lineages and gradually occupy specific niches. Stem cells can be isolated using specific membrane markers and cultured under defined conditions, they can be expanded without losing their properties, or differentiate upon specific inducers [48]. As with primary cells, stem cells are also limited by donor availability. They can be collected from embryos, which is directly associated with the additional challenge of being a highly controversial ethical issue and quite limited source. Alternatively, stem cells can be derived from adults, mainly from blood, bone marrow and adipose tissue. The latter source is the least invasive and relatively abundant. However, the biggest bottleneck in the use of these cells is the differentiation *in vitro*, which is a time and resource consuming process for which adequate PTEC phenotype still needs to be demonstrated. Nonetheless, stem cells have the promise of providing an autologous cell source for biomedical research applications and can potentially be expanded in large quantities.

Induced pluripotent stem cells

A cell type that was introduced less than a decade ago are induced pluripotent stem cells (iPS), that now make their way to the spotlight of cellular research. The technique to produce this new type of cells, bypasses the issues with limited sources since they can be derived from somatic cells [49]. Furthermore, the cells can differentiate into virtually any cell type in the body, hence subscribing their pluripotency. These iPS cells are generated by exposing adult cells (terminally differentiated) to the defined factors Klf4 (Kruppel-like factor 4) and c-Myc [50], that will trigger the cells to re-arrange their genetic program and change their phenotype into undifferentiated stem-like cells, in a process labeled trans-differentiation. New factors, Oct4 (octamer binding transpiration factors 4) and SOX2 (sex determining region Y-box2) have been shown to induce PTEC into iPS [51].

Human iPS cells have been obtained from fibroblasts and other sources, and kidney organoids grown from such cells formed functional PTEC [52]. iPS cells are a potential source of autologous cells, however their use and generation are still a laborious process. Cells trans-differentiated using viral vectors may not be appropriate for clinical use, therefore novel delivery vectors, including *piggyBac* transposons and synthetic mRNA, have been explored [53, 54]. The use of ectopic transcription factors can be potentially tumorigenic and an incomplete re-programing compromises the cells pluripotency [55, 56]. As renal derived iPS cells become a reality, comprehensive validation is needed to confirm the cells function and determine the correct phenotype [57].

Cell lines

Cell lines are also a prominent source of renal cells that usually originate from a primary cell culture that is transformed to enable prolonged culturing while maintaining the cell-type specific phenotypical properties. These cells are widely used in research and can be obtained commercially or generated in an adequate facility, both for research or commercial uses. Drug screening and fundamental research into kidney pharmacology and physiology are important applications for kidney-derived cell lines. Two commonly used kidney cell lines, and examples of early developments in this area, are the Human embryonic kidney 293 (HEK293) and the Human kidney 2 (HK-2) cells. HEK293 cells are derived from primary cultures of human embryonic kidney cells and exposed to adenovirus in order to achieve sustainable cell growth *in vitro* [58]. Although originally derived from human renal tissue, these cells show abnormal chromosomes and lack a defined phenotype (namely the transport machinery characteristic of PTEC), while culture conditions for optimal proliferation are well established [59]. HEK293 cells are easy to transfect, stimulating their use in cellular research study protein expression on a molecular level. HK-2 are derived from human primary PTEC cultures transfected with the human papilloma virus 16 E6/E7 genes (HPV) in order to obtain a stable cell line [60]. These cells express several enzymes present in primary PTEC along with certain functional aspects, such as glucose uptake. However, the HK-2 cells do not entirely resemble a PTEC phenotype and show, at most, residual active transport activity of xenobiotics [59, 61]. These early generations of renal cell lines underline the problems with generating a cell that is well-defined in terms of phenotype and that retains key features of differentiated cells.

Non-human cells lines, isolated from mammals are also widely applied. The Madin-Darby canine kidney (MDCK) cells retain a strong epithelial phenotype and are simple to culture [62]. Pig derived LLC-PK1 cells also possess a well characterized PTEC phenotype [63]. Nevertheless, these cells have contributed to elucidate the cellular and molecular mechanisms involved in renal physiology and pathophysiology and also paved the way to more advanced and complex *in vitro* models that are becoming of increasingly importance.

Conditionally immortalized cells

In recent years, the amount of cell lines generated has increased as a consequence of improved molecular techniques, the need for more representative and well-defined cells and also in an attempt for refining or replacing the use of animals in research. A hand-full of cell lines has been developed relying on immortalization tools that reduce genetic variability and thereby improve the stability of the cells, largely for purposes of renal *in vitro* pathophysiology and drug safety testing. The renal PTEC line (RPTEC) and NKi-2 cell line were generated by overexpressing the human telomerase reverse transcriptase (hTERT) via viral transfection. This transformation allows the cells to maintain their intact chromosomes after every doubling, resulting in stable lines [64-66]. Functionally, the cells

express metabolic enzymes, including esterase and glucuronidase [67], and are used as an *in vitro* model for kidney toxicity studies with emphasis on drug screening [68]. These cells were non-invasively harvested, and can be cultured *in vitro.* Subsequently, the cells are transformed to grow in a sustained way and characterized to confirm a PTEC phenotype. Conditionally immortalized human proximal tubule cells (ciPTEC) are a type of PTEC cells generated by overexpressing the simian virus 40 large T tsA58 antigen (SV40T) together with hTERT [45]. These transformations enable the cells to proliferate at a temperature of 33 °C and subsequently mature at 37 °C, inactivating the large T antigen and acquiring a differentiated PTEC phenotype. CiPTEC can be derived both from urine or adult renal tissue [45, 46] and functionally express OCT2, BCRP, P-gp and MRP4, key drug transporters that are native to PTEC. Consequently, the cells are sensitive to nephrotoxic drugs and can extrude protein-bound uremic toxins [42, 69]. These cells can be grown abundantly and are functional after high population doublings. Arguably, cells derived from urine are different from cells derived from tissue; the fact they were shed from the proximal tubule epithelium can indicate a loss in functionality. Nonetheless, ciPTEC derived from both urine and kidney biopsies, and immortalized according to the same procedure, show similar gene expressions, membrane transport functions and enzyme activities, supporting the validity of urine derived PTEC [46].

Table 1. Sources of PTEC used in kidney research

Cell	Source	Type	PTEC phenotype	Availability	References
MDCK	Dog	Cell line	Limited	High	[62, 70-72]
LCC-PK1	Pig	Primary	Limited	High	[63, 70, 73]
HK-2	Human	Cell line	Intermediate	High	[59-61]
Primary	Human	Primary	Strong	Low	[74-77]
RPTEC	Human	Cell line	Strong	High	[64, 78]
NKi-2	Human	Cell line	Strong	High	[65, 66]
ciPTEC	Human	Cell line	Strong	High	[45, 79, 80]
Stem cells	Human	Primary	Intermediate	Low	[48, 81, 82]
iPS	Human	Primary	Intermediate	Reasonable	[49, 83]

Membrane transporters kinetic studies have been conventionally conducted using overexpression systems, like HEK and MDCK cell lines, and membrane vesicles, derived from such models. Though important to determine the activity of isolated transporters, these systems lack the complexity of the interactions present under normal physiological conditions. Research into proximal tubule transport activity and physiology has benefited from the introduction of cell models as ciPTEC, cells that endogenously express both the metabolic enzymes CYP and UGT as well as the main efflux membrane transporters present in PTEC [67]. FDA guidelines for drug interaction studies require that *in vitro* assays should be used to determine drug metabolism by phase I/II metabolic enzymes

(CYPs and UGTs, respectively). Also, drugs under investigation must be studied for their affinity to OAT1, OAT3, OCT2, BCRP and P-gp, as well the role of active secretion in their renal clearance [84]. Prominent pharmacological interactions can be studied *in vitro*. The nature of the interactions can be direct, where different drugs can compete for the binding site of a transporter or enzyme. Indirect interactions occur when the actions of a drug leads to physiological changes (e.g. enzyme or transport activity inhibition) that subsequently alter the effects of other drugs. Furthermore, research into cellular regulatory mechanisms is becoming increasingly relevant in drug interaction studies, as new targets emerge and the mechanisms of action of a variety of clinically available drugs require better understanding.

SCOPE AND OUTLINE OF THIS THESIS

In recent years the research paradigms governing biomedical sciences began to shift towards what is now commonly known as translational research. The ultimate goal of this approach is to find clinical solutions based on fundamental research. Therefore, an increasing number of studies in the renal field look into the onset and progression of kidney disease. Since the recent introduction of guidelines for drug transporter interactions in the development of novel drugs by regulatory bodies, the implementation of reliable models representative of human physiology received a great incentive [18, 84]. Given the fact that proximal tubules are a transporter-rich environment and many drugs are excreted via the kidneys, PTEC have gained notoriety in renal research. Drug transporters are at the heart of renal excretion.

The aim of this thesis was to investigate the function and regulation of renal drug transporters in the proximal tubule *in vitro,* using reliable methodology in human cells representative of renal physiology. Following this introductory note, **chapter 2** reviews renal drug handling with an emphasis on the BCRP activity as this transporter is often overlooked in reviews on renal drug transporters. The impact of genetic variants, gender and cellular regulation of this transporter is discussed in depth. A detailed perspective on drug excretion is presented by analyzing drug interactions of renally cleared drugs. Furthermore, the role of BCRP in cancer drug resistance and how the effects of chemotherapeutical agents influence its activity is being discussed. In **chapter 3** we studied apical transport activity in proximal tubules, looking at the combined activities of BCRP, P-gp and MRP4, prominent efflux transporters in the human renal cell line ciPTEC. To determine the transporter function and their contribution to renal clearance, an array of high-throughput assays that exploit transporter inhibition and the retention of fluorescent substrates were developed. Building on methodology suited to determine solutes retention and transporters activity, in **chapter 4** the cellular responses to the endogenous p-cresyl metabolites was investigated. ciPTEC were exposed to the uremic solutes p-cresyl sulfate and glucuronide to determine the apical transporters responsible for their handling. Furthermore, their impact on cellular physiology, with respect to

transporters expression and activity and the integrity of the epithelial phenotype were studied. Further exploiting proximal tubule cell physiology in ciPTEC, in chapter 5 we look into the complex pharmacological interaction between anticancer drugs. Cetuximab is a monoclonal antibody that displays a synergistic effect with more conventional anticancer drugs. To determine if these interactions impact PTEC function, as well as to uncover their mechanistic root, transporter activity and toxicity were investigated in ciPTEC upon exposure to cetuximab, and methotrexate or cisplatin, both anticancer drugs with significant renal clearance.

Continuing the emphasis in drug transporters function and activity, chapter 6 describes how the transporters expression and consequent function can be used to investigate the differentiation potential of human kidney progenitor cells, derived from human fetal tissue. In a shift from adult renal cells, the activity of immature cells under different culture conditions was determined using the same experimental approach as described before. Chapter 7 offers an up to date perspective on the findings illustrated in this thesis, providing a comprehensive discussion considering the current developments and trends in the fields of drug transporters and renal research. To conclude chapter 8 presents a summary of the work in hand.

REFERENCES

1. Nigam SK, Wu W, Bush KT, Hoenig MP, Blantz RC, Bhatnagar V. Handling of Drugs, Metabolites, and Uremic Toxins by Kidney Proximal Tubule Drug Transporters. Clin J Am Soc Nephrol. 2015;10(11):2039-49. doi: 10.2215/CJN.02440314. PubMed PMID: 26490509; PubMed Central PMCID: PMCPMC4633783.
2. Konig J, Muller F, Fromm MF. Transporters and drug-drug interactions: important determinants of drug disposition and effects. Pharmacol Rev. 2013;65(3):944-66. doi: 10.1124/pr.113.007518. PubMed PMID: 23686349.
3. Hoenig MP, Zeidel ML. Homeostasis, the milieu interieur, and the wisdom of the nephron. Clin J Am Soc Nephrol. 2014;9(7):1272-81. doi: 10.2215/CJN.08860813. PubMed PMID: 24789550; PubMed Central PMCID: PMCPMC4078957.
4. Anderson J, Glynn LG. Definition of chronic kidney disease and measurement of kidney function in original research papers: a review of the literature. Nephrol Dial Transplant. 2011;26(9):2793-8. doi: 10.1093/ndt/gfq849. PubMed PMID: 21307172.
5. Hill NR, Fatoba ST, Oke JL, Hirst JA, O'Callaghan CA, Lasserson DS, et al. Global Prevalence of Chronic Kidney Disease - A Systematic Review and Meta-Analysis. PLoS One. 2016;11(7):e0158765. doi: 10.1371/journal.pone.0158765. PubMed PMID: 27383068; PubMed Central PMCID: PMCPMC4934905.
6. Zuk A, Bonventre JV. Acute Kidney Injury. Annu Rev Med. 2016;67:293-307. doi: 10.1146/annurev-med-050214-013407. PubMed PMID: 26768243.
7. Tomino Y. Pathogenesis and treatment of chronic kidney disease: a review of our recent basic and clinical data. Kidney Blood Press Res. 2014;39(5):450-89. doi: 10.1159/000368458. PubMed PMID: 25501571.
8. Levey AS, Coresh J. Chronic kidney disease. Lancet. 2012;379(9811):165-80. doi: 10.1016/S0140-6736(11)60178-5. PubMed PMID: 21840587.
9. Gansevoort RT, Correa-Rotter R, Hemmelgarn BR, Jafar TH, Heerspink HJ, Mann JF, et al. Chronic kidney disease and cardiovascular risk: epidemiology, mechanisms, and prevention. Lancet. 2013;382(9889):339-52. doi: 10.1016/S0140-6736(13)60595-4. PubMed PMID: 23727170.
10. Kurts C, Panzer U, Anders HJ, Rees AJ. The immune system and kidney disease: basic concepts and clinical implications. Nat Rev Immunol. 2013;13(10):738-53. doi: 10.1038/nri3523. PubMed PMID: 24037418.
11. Botev R, Mallie JP, Wetzels JF, Couchoud C, Schuck O. The clinician and estimation of glomerular filtration rate by creatinine-based formulas: current limitations and quo vadis. Clin J Am Soc Nephrol. 2011;6(4):937-50. doi: 10.2215/CJN.09241010. PubMed PMID: 21454722.
12. Levin A, Hemmelgarn B, Culleton B, Tobe S, McFarlane P, Ruzicka M, et al. Guidelines for the management of chronic kidney disease. CMAJ. 2008;179(11):1154-62. doi: 10.1503/cmaj.080351. PubMed PMID: 19015566; PubMed Central PMCID: PMCPMC2582781.
13. Fleming GM. Renal replacement therapy review: past, present and future. Organogenesis. 2011;7(1):2-12. PubMed PMID: 21289478; PubMed Central PMCID: PMCPMC3082028.
14. Jansen J, Fedecostante M, Wilmer MJ, van den Heuvel LP, Hoenderop JG, Masereeuw R. Biotechnological challenges of bioartificial kidney engineering. Biotechnol Adv. 2014;32(7):1317-27. doi: 10.1016/j.biotechadv.2014.08.001. PubMed PMID: 25135479.
15. Jaar BG, Khatib R, Plantinga L, Boulware LE, Powe NR. Principles of screening for chronic kidney disease. Clin J Am Soc

Nephrol. 2008;3(2):601-9. doi: 10.2215/CJN.02540607. PubMed PMID: 18032791.

16. Wang V, Vilme H, Maciejewski ML, Boulware LE. The Economic Burden of Chronic Kidney Disease and End-Stage Renal Disease. Semin Nephrol. 2016;36(4):319-30. doi: 10.1016/j.semnephrol.2016.05.008. PubMed PMID: 27475662.

17. Vasiliou V, Vasiliou K, Nebert DW. Human ATP-binding cassette (ABC) transporter family. Hum Genomics. 2009;3(3):281-90. PubMed PMID: 19403462; PubMed Central PMCID: PMCPMC2752038.

18. International Transporter C, Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, et al. Membrane transporters in drug development. Nat Rev Drug Discov. 2010;9(3):215-36. doi: 10.1038/nrd3028. PubMed PMID: 20190787; PubMed Central PMCID: PMCPMC3326076.

19. El-Sheikh AA, Greupink R, Wortelboer HM, van den Heuvel JJ, Schreurs M, Koenderink JB, et al. Interaction of immunosuppressive drugs with human organic anion transporter (OAT) 1 and OAT3, and multidrug resistance-associated protein (MRP) 2 and MRP4. Transl Res. 2013;162(6):398-409. doi: 10.1016/j.trsl.2013.08.003. PubMed PMID: 24036158.

20. Hagos Y, Wolff NA. Assessment of the role of renal organic anion transporters in drug-induced nephrotoxicity. Toxins (Basel). 2010;2(8):2055-82. doi: 10.3390/toxins2082055. PubMed PMID: 22069672; PubMed Central PMCID: PMCPMC3153278.

21. Motohashi H, Inui K. Organic cation transporter OCTs (SLC22) and MATEs (SLC47) in the human kidney. AAPS J. 2013;15(2):581-8. doi: 10.1208/s12248-013-9465-7. PubMed PMID: 23435786; PubMed Central PMCID: PMCPMC3675737.

22. Huls M, Brown CD, Windass AS, Sayer R, van den Heuvel JJ, Heemskerk S, et al. The breast cancer resistance protein transporter ABCG2 is expressed in the human kidney proximal tubule apical membrane. Kidney Int. 2008;73(2):220-5. doi: 10.1038/sj.ki.5002645. PubMed PMID: 17978814.

23. Russel FG, Koenderink JB, Masereeuw R. Multidrug resistance protein 4 (MRP4/ABCC4): a versatile efflux transporter for drugs and signalling molecules. Trends Pharmacol Sci. 2008;29(4):200-7. doi: 10.1016/j.tips.2008.01.006. PubMed PMID: 18353444.

24. Moss DM, Neary M, Owen A. The role of drug transporters in the kidney: lessons from tenofovir. Front Pharmacol. 2014;5:248. doi: 10.3389/fphar.2014.00248. PubMed PMID: 25426075; PubMed Central PMCID: PMCPMC4227492.

25. Lohr JW, Willsky GR, Acara MA. Renal drug metabolism. Pharmacol Rev. 1998;50(1):107-41. PubMed PMID: 9549760.

26. Tirona RG. Molecular mechanisms of drug transporter regulation. Handb Exp Pharmacol. 2011;(201):373-402. doi: 10.1007/978-3-642-14541-4_10. PubMed PMID: 21103976.

27. Stolarczyk EI, Reiling CJ, Paumi CM. Regulation of ABC transporter function via phosphorylation by protein kinases. Curr Pharm Biotechnol. 2011;12(4):621-35. PubMed PMID: 21118091; PubMed Central PMCID: PMCPMC3085954.

28. Morrissey KM, Stocker SL, Wittwer MB, Xu L, Giacomini KM. Renal transporters in drug development. Annu Rev Pharmacol Toxicol. 2013;53:503-29. doi: 10.1146/annurev-pharmtox-011112-140317. PubMed PMID: 23140242.

29. Gaganis P, Miners JO, Brennan JS, Thomas A, Knights KM. Human renal cortical and medullary UDP-glucuronosyltransferases (UGTs): immunohistochemical localization of UGT2B7 and UGT1A enzymes and kinetic characterization of S-naproxen glucuronidation. J Pharmacol Exp Ther. 2007;323(2):422-30. doi: 10.1124/jpet.107.128603. PubMed PMID: 17698974.

30. El-Sheikh AA, Koenderink JB, Wouterse AC, van den Broek PH, Verweij VG, Masereeuw R, et al. Renal glucuronidation and multidrug resistance protein 2-/ multidrug resistance protein 4-mediated efflux of mycophenolic acid: interaction with cyclosporine and tacrolimus. Transl Res. 2014;164(1):46-56. doi: 10.1016/j.trsl.2014.01.006. PubMed PMID: 24486136.

31. Nielsen R, Christensen EI, Birn H. Megalin and cubilin in proximal tubule protein reabsorption: from experimental models to human disease. Kidney Int. 2016;89(1):58-67. doi: 10.1016/j.kint.2015.11.007. PubMed PMID: 26759048.

32. Huang C, Miller RT. Regulation of renal ion transport by the calcium-sensing receptor: an update. Curr Opin Nephrol Hypertens. 2007;16(5):437-43. doi: 10.1097/MNH.0b013e3282b974a6. PubMed PMID: 17693759.

33. Palmer LG, Sackin H. Regulation of renal ion channels. FASEB J. 1988;2(15):3061-5. PubMed PMID: 2461326.

34. Rahmoune H, Thompson PW, Ward JM, Smith CD, Hong G, Brown J. Glucose transporters in human renal proximal tubular cells isolated from the urine of patients with non-insulin-dependent diabetes. Diabetes. 2005;54(12):3427-34. PubMed PMID: 16306358.

35. Thorens B, Mueckler M. Glucose transporters in the 21st Century. Am J Physiol Endocrinol Metab. 2010;298(2):E141-5. doi: 10.1152/ajpendo.00712.2009. PubMed PMID: 20009031; PubMed Central PMCID: PMCPMC2822486.

36. Makrides V, Camargo SM, Verrey F. Transport of amino acids in the kidney. Compr Physiol. 2014;4(1):367-403. doi: 10.1002/cphy.c130028. PubMed PMID: 24692143.

37. Xing L, Wen JG, Frokiaer J, Djurhuus JC, Norregaard R. Ontogeny of the mammalian kidney: expression of aquaporins 1, 2, 3, and 4. World J Pediatr. 2014;10(4):306-12. doi: 10.1007/s12519-014-0508-7. PubMed PMID: 25515803.

38. Vanholder RC, Eloot S, Glorieux GL. Future Avenues to Decrease Uremic Toxin Concentration. Am J Kidney Dis. 2015. doi: 10.1053/j.ajkd.2015.08.029. PubMed PMID: 26500179.

39. Vanholder R, De Smet R, Glorieux G, Argiles A, Baurmeister U, Brunet P, et al. Review on uremic toxins: classification, concentration, and interindividual variability. Kidney Int. 2003;63(5):1934-43. doi: 10.1046/j.1523-1755.2003.00924.x. PubMed PMID: 12675874.

40. Lisowska KA, Debska-Slizien A, Jasiulewicz A, Bryl E, Witkowski JM. Influence of hemodialysis on circulating CD4(low) CD25 (high) regulatory T cells in end-stage renal disease patients. Inflamm Res. 2014;63(2):99-103. doi: 10.1007/s00011-013-0679-z. PubMed PMID: 24189710; PubMed Central PMCID: PMCPMC3894426.

41. Hsu HJ, Yen CH, Wu IW, Hsu KH, Chen CK, Sun CY, et al. The association of uremic toxins and inflammation in hemodialysis patients. PLoS One. 2014;9(7):e102691. doi: 10.1371/journal.pone.0102691. PubMed PMID: 25051062; PubMed Central PMCID: PMCPMC4106871.

42. Schophuizen CM, Wilmer MJ, Jansen J, Gustavsson L, Hilgendorf C, Hoenderop JG, et al. Cationic uremic toxins affect human renal proximal tubule cell functioning through interaction with the organic cation transporter. Pflugers Arch. 2013;465(12):1701-14. doi: 10.1007/s00424-013-1307-z. PubMed PMID: 23812163.

43. Griffith LG, Swartz MA. Capturing complex 3D tissue physiology in vitro. Nat Rev Mol Cell Biol. 2006;7(3):211-24. doi: 10.1038/nrm1858. PubMed PMID: 16496023.

44. Presnell SC, Bruce AT, Wallace SM, Choudhury S, Genheimer CW, Cox B, et al. Isolation, characterization, and expansion methods for defined primary renal cell

populations from rodent, canine, and human normal and diseased kidneys. Tissue Eng Part C Methods. 2011;17(3):261-73. doi: 10.1089/ten.TEC.2010.0399. PubMed PMID: 20846053.

45. Wilmer MJ, Saleem MA, Masereeuw R, Ni L, van der Velden TJ, Russel FG, et al. Novel conditionally immortalized human proximal tubule cell line expressing functional influx and efflux transporters. Cell Tissue Res. 2010;339(2):449-57. doi: 10.1007/s00441-009-0882-y. PubMed PMID: 19902259; PubMed Central PMCID: PMCPMC2817082.

46. Jansen J, Schophuizen CM, Wilmer MJ, Lahham SH, Mutsaers HA, Wetzels JF, et al. A morphological and functional comparison of proximal tubule cell lines established from human urine and kidney tissue. Exp Cell Res. 2014;323(1):87-99. doi: 10.1016/j.yexcr.2014.02.011. PubMed PMID: 24560744.

47. Vats A, Tolley NS, Polak JM, Buttery LD. Stem cells: sources and applications. Clin Otolaryngol Allied Sci. 2002;27(4):227-32. PubMed PMID: 12169121.

48. Klingemann H, Matzilevich D, Marchand J. Mesenchymal Stem Cells - Sources and Clinical Applications. Transfus Med Hemother. 2008;35(4):272-7. doi: 10.1159/000142333. PubMed PMID: 21512642; PubMed Central PMCID: PMCPMC3076359.

49. Freedman BS, Steinman TI. iPS cell technology: Future impact on renal care. Nephrol News Issues. 2015;29(9):18, 20-1. PubMed PMID: 26454909.

50. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, et al. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell. 2007;131(5):861-72. doi: 10.1016/j.cell.2007.11.019. PubMed PMID: 18035408.

51. Montserrat N, Ramirez-Bajo MJ, Xia Y, Sancho-Martinez I, Moya-Rull D, Miquel-Serra L, et al. Generation of induced pluripotent stem cells from human renal proximal tubular cells with only two transcription factors, OCT4 and SOX2. J Biol Chem. 2012;287(29):24131-8. doi: 10.1074/jbc.M112.350413. PubMed PMID: 22613719; PubMed Central PMCID: PMCPMC3397840.

52. Takasato M, Er PX, Chiu HS, Maier B, Baillie GJ, Ferguson C, et al. Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. Nature. 2015;526(7574):564-8. doi: 10.1038/nature15695. PubMed PMID: 26444236.

53. Shao L, Wu WS. Gene-delivery systems for iPS cell generation. Expert Opin Biol Ther. 2010;10(2):231-42. doi: 10.1517/14712590903455989. PubMed PMID: 20088717; PubMed Central PMCID: PMCPMC2811526.

54. Zhou YY, Zeng F. Integration-free methods for generating induced pluripotent stem cells. Genomics Proteomics Bioinformatics. 2013;11(5):284-7. doi: 10.1016/j.gpb.2013.09.008. PubMed PMID: 24121200; PubMed Central PMCID: PMCPMC4357834.

55. Pleniceanu O, Harari-Steinberg O, Dekel B. Concise review: Kidney stem/progenitor cells: differentiate, sort out, or reprogram? Stem Cells. 2010;28(9):1649-60. doi: 10.1002/stem.486. PubMed PMID: 20652959; PubMed Central PMCID: PMCPMC2996087.

56. Medvedev SP, Shevchenko AI, Zakian SM. Induced Pluripotent Stem Cells: Problems and Advantages when Applying them in Regenerative Medicine. Acta Naturae. 2010;2(2):18-28. PubMed PMID: 22649638; PubMed Central PMCID: PMCPMC3347549.

57. Freedman BS. Modeling Kidney Disease with iPS Cells. Biomark Insights. 2015;10(Suppl 1):153-69. doi: 10.4137/BMI.S20054. PubMed PMID: 26740740; PubMed Central PMCID: PMCPMC4689367.

58. Stepanenko AA, Dmitrenko VV. HEK293 in cell biology and cancer research: phenotype, karyotype, tumorigenicity, and stress-induced genome-phenotype evolution. Gene. 2015;569(2):182-90. doi: 10.1016/j.gene.2015.05.065. PubMed PMID: 26026906.

59. Jenkinson SE, Chung GW, van Loon E, Bakar NS, Dalzell AM, Brown CD. The limitations of renal epithelial cell line HK-2 as a model of drug transporter expression and function in the proximal tubule. Pflugers Arch. 2012;464(6):601-11. doi: 10.1007/s00424-012-1163-2. PubMed PMID: 23014881.

60. Ryan MJ, Johnson G, Kirk J, Fuerstenberg SM, Zager RA, Torok-Storb B. HK-2: an immortalized proximal tubule epithelial cell line from normal adult human kidney. Kidney Int. 1994;45(1):48-57. PubMed PMID: 8127021.

61. Sun J, Wang C, Zhu B, Larsen S, Wu J, Zhao W. Construction of an erythropoietin-expressing bioartificial renal tubule assist device. Ren Fail. 2011;33(1):54-60. doi: 10.3109/0886022X.2010.536605. PubMed PMID: 21219206.

62. Dukes JD, Whitley P, Chalmers AD. The MDCK variety pack: choosing the right strain. BMC Cell Biol. 2011;12:43. doi: 10.1186/1471-2121-12-43. PubMed PMID: 21982418; PubMed Central PMCID: PMCPMC3209442.

63. Nielsen R, Birn H, Moestrup SK, Nielsen M, Verroust P, Christensen EI. Characterization of a kidney proximal tubule cell line, LLC-PK1, expressing endocytotic active megalin. J Am Soc Nephrol. 1998;9(10):1767-76. PubMed PMID: 9773777.

64. Simon-Friedt BR, Wilson MJ, Blake DA, Yu H, Eriksson Y, Wickliffe JK. The RPTEC/TERT1 Cell Line as an Improved Tool for In Vitro Nephrotoxicity Assessments. Biol Trace Elem Res. 2015;166(1):66-71. doi: 10.1007/s12011-015-0339-y. PubMed PMID: 25893367; PubMed Central PMCID: PMCPMC4470802.

65. DesRochers TM, Suter L, Roth A, Kaplan DL. Bioengineered 3D human kidney tissue, a platform for the determination of nephrotoxicity. PLoS One. 2013;8(3):e59219. doi: 10.1371/journal.pone.0059219. PubMed PMID: 23516613; PubMed Central PMCID: PMCPMC3597621.

66. Wilmer MJ, Ng CP, Lanz HL, Vulto P, Suter-Dick L, Masereeuw R. Kidney-on-a-Chip Technology for Drug-Induced Nephrotoxicity Screening. Trends Biotechnol. 2016;34(2):156-70. doi: 10.1016/j.tibtech.2015.11.001. PubMed PMID: 26708346.

67. Mutsaers HA, Wilmer MJ, Reijnders D, Jansen J, van den Broek PH, Forkink M, et al. Uremic toxins inhibit renal metabolic capacity through interference with glucuronidation and mitochondrial respiration. Biochim Biophys Acta. 2013;1832(1):142-50. doi: 10.1016/j.bbadis.2012.09.006. PubMed PMID: 23017367.

68. Wilmes A, Bielow C, Ranninger C, Bellwon P, Aschauer L, Limonciel A, et al. Mechanism of cisplatin proximal tubule toxicity revealed by integrating transcriptomics, proteomics, metabolomics and biokinetics. Toxicol In Vitro. 2015;30(1 Pt A):117-27. doi: 10.1016/j.tiv.2014.10.006. PubMed PMID: 25450742.

69. Mutsaers HA, Caetano-Pinto P, Seegers AE, Dankers AC, van den Broek PH, Wetzels JF, et al. Proximal tubular efflux transporters involved in renal excretion of p-cresyl sulfate and p-cresyl glucuronide: Implications for chronic kidney disease pathophysiology. Toxicol In Vitro. 2015;29(7):1868-77. doi: 10.1016/j.tiv.2015.07.020. PubMed PMID: 26216510.

70. Ip TK, Aebischer P, Galletti PM. Cellular control of membrane permeability. Implications for a bioartificial renal tubule. ASAIO Trans. 1988;34(3):351-5. PubMed PMID: 3196532.

71. Fey-Lamprecht F, Albrecht W, Groth T, Weigel T, Gross U. Morphological studies on the culture of kidney epithelial cells in a fiber-in-fiber bioreactor design with hollow fiber membranes. J Biomed Mater Res A. 2003;65(2):144-57. doi: 10.1002/jbm.a.10328. PubMed PMID: 12734806.

72. Aebischer P, Ip TK, Panol G, Galletti PM. The bioartificial kidney: progress towards an ultrafiltration device with renal epithelial cells processing. Life Support Syst. 1987;5(2):159-68. PubMed PMID: 3669723.

73. Sato Y, Terashima M, Kagiwada N, Tun T, Inagaki M, Kakuta T, et al. Evaluation of proliferation and functional differentiation of LLC-PK1 cells on porous polymer membranes for the development of a bioartificial renal tubule device. Tissue Eng. 2005;11(9-10):1506-15. doi: 10.1089/ten.2005.11.1506. PubMed PMID: 16259605.

74. Humes HD, Fissell WH, Weitzel WF, Buffington DA, Westover AJ, MacKay SM, et al. Metabolic replacement of kidney function in uremic animals with a bioartificial kidney containing human cells. Am J Kidney Dis. 2002;39(5):1078-87. doi: 10.1053/ajkd.2002.32792. PubMed PMID: 11979353.

75. Saito A, Sawada K, Fujimura S, Suzuki H, Hirukawa T, Tatsumi R, et al. Evaluation of bioartificial renal tubule device prepared with lifespan-extended human renal proximal tubular epithelial cells. Nephrol Dial Transplant. 2012;27(8):3091-9. doi: 10.1093/ndt/gfr755. PubMed PMID: 22467257.

76. Takahashi H, Sawada K, Kakuta T, Suga T, Hanai K, Kanai G, et al. Evaluation of bioartificial renal tubule device prepared with human renal proximal tubular epithelial cells cultured in serum-free medium. J Artif Organs. 2013;16(3):368-75. doi: 10.1007/s10047-013-0710-8. PubMed PMID: 23666226.

77. Oo ZY, Deng R, Hu M, Ni M, Kandasamy K, bin Ibrahim MS, et al. The performance of primary human renal cells in hollow fiber bioreactors for bioartificial kidneys. Biomaterials. 2011;32(34):8806-15. doi: 10.1016/j.biomaterials.2011.08.030. PubMed PMID: 21872923.

78. Sanechika N, Sawada K, Usui Y, Hanai K, Kakuta T, Suzuki H, et al. Development of bioartificial renal tubule devices with lifespan-extended human renal proximal tubular epithelial cells. Nephrol Dial Transplant. 2011;26(9):2761-9. doi: 10.1093/ndt/gfr066. PubMed PMID: 21421594.

79. Schophuizen CM, De Napoli IE, Jansen J, Teixeira S, Wilmer MJ, Hoenderop JG, et al. Development of a living membrane comprising a functional human renal proximal tubule cell monolayer on polyethersulfone polymeric membrane. Acta Biomater. 2015;14:22-32. doi: 10.1016/j.actbio.2014.12.002. PubMed PMID: 25527093.

80. Jansen J, Fedecostante M, Wilmer MJ, Peters JG, Kreuser UM, van den Broek PH, et al. Bioengineered kidney tubules efficiently excrete uremic toxins. Sci Rep. 2016;6:26715. doi: 10.1038/srep26715. PubMed PMID: 27242131; PubMed Central PMCID: PMCPMC4886219.

81. Sciancalepore AG, Sallustio F, Girardo S, Passione LG, Camposeo A, Mele E, et al. Correction: A bioartificial renal tubule device embedding human renal stem/progenitor cells. PLoS One. 2015;10(5):e0128261. doi: 10.1371/journal.pone.0128261. PubMed PMID: 25955358; PubMed Central PMCID: PMCPMC4425656.

82. Roberts I, Baila S, Rice RB, Janssens ME, Nguyen K, Moens N, et al. Scale-up of human embryonic stem cell culture using a hollow fibre bioreactor. Biotechnol Lett. 2012;34(12):2307-15. doi: 10.1007/s10529-012-1033-1. PubMed PMID: 22983716.

83. Yamanaka S, Yokoo T. Current Bioengineering Methods for Whole Kidney Regeneration. Stem Cells

Int. 2015;2015:724047. doi: 10.1155/2015/724047. PubMed PMID: 26089921; PubMed Central PMCID: PMCPMC4452081.

84. (CDER) CfDEaR. Drug Interaction Studies — Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations. 2012.

CHAPTER 2

The importance of breast cancer resistance protein to the kidneys excretory function and chemotherapeutic resistance

Pedro Caetano-Pinto[1], Jitske Jansen[1], Yehuda G. Assaraf[2] and Rosalinde Masereeuw[1,†]

[1]Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Utrecht University, The Netherlands.
[2]The Fred Wyszkowski Cancer Research Laboratory, Department of Biology, Technion-Israel Institute of Technology, Haifa, Israel.

Drug Resistance Updates.2017Jan5(30)p15-27
doi:http://dx.doi.org/10.1016/j.drup.2017.01.002

ABSTRACT

The relevance of membrane transporters gained momentum in recent years and it is now widely recognized that transporters are key players in drug disposition and chemoresistance. As such, the kidneys harbor a variety of drug transporters and are one of the main routes for xenobiotic excretion. The breast cancer resistance protein (BCRP/*ABCG2*) is widely accepted as a key mediator of anticancer drug resistance and is a prominent renal drug transporter. Here, we review the role of BCRP in both processes and present a multitude of variables that can influence its activity. An increasing number of renally cleared chemotherapeutics, including tyrosine kinase inhibitors, described as BCRP substrates can modulate its activity via transcription factors and cellular signaling pathways, such as the phosphoinositide 3-kinase (PI3K) pathway. In addition to pharmacological actions, genetic variations, as well as differences between species and gender can affect BCRP function, which are also discussed. Furthermore, the role of BCRP in light of cancer treatments and the implications for novel therapeutic interventions that take into account renal function will be discussed.

Key Words

BCRP, drug transport, renal excretion, chemoresistance

INTRODUCTION

Transporters are key players in drug disposition, with active roles in excretion, drug-drug and other pharmacologically relevant interactions, such as drug-nutrient or drug-toxicant interactions, thereby influencing the drug's concentration at active sites [1]. Our understanding of the role played by transporters and their contribution to physiological processes has gone beyond the classical view where these mechanisms were merely responsible for translocating substrates from the cytoplasm to extracellular compartments and *vice versa* [2]. Xenobiotic transporters are implicated in many processes, including intestinal absorption, maintenance of blood-organ barriers – including the brain, testis and placenta, - bile secretion in the liver, as well as in renal function, where the overwhelming majority of xenobiotics and metabolic bi-products are excreted via active membrane transport [1, 3].

The kidneys are responsible for a variety of key regulatory functions that are tightly associated with other physiological processes. These include regulation of physiological pH by maintaining appropriate acid-base homeostasis, generation of hormones responsible for stimulating the production of red blood cells and regulation of blood pressure by controlling fluid volume in the body. The kidneys are also responsible for nutrient reabsorption and the hallmark of renal function is their excretory role of endo- and xenobiotics from the peritubular capillaries into the pro-urine. [4]. The properties that enable renal transporters to mediate drug excretion are also key in chemotherapeutic resistance. Although physiologically distinct, cancer drug resistance and renal excretion both rely on active (i.e. ATP-driven) membrane transport, namely by multidrug resistance efflux transporters of the ABC superfamily.

A myriad of factors contribute to chemoresistance, as cancers are inherently heterogeneous, and their genetic instability are also [5-10]. Cell survival mechanisms can be hijacked, growth pathways permanently activated, or the DNA repair machinery enhanced, thereby reducing cellular sensitivity to DNA damage [11]. Programmed cell death cascades can become altered or silenced, rendering cancer cells resistant to toxic stress. Further, metabolic processes and enzymatic activities are upregulated to cope with high bioenergetics demands stemming from accelerated growth [8, 12]. Chemotherapy by itself can promote or enhance drug resistance phenotypes. This phenomenon arises when cancer cells alter their homeostasis by modifying gene expression or re-routing signaling pathways as a response to drugs targeting particular cellular processes. Drugs can also exert selective pressure in the cancer microenvironment, favoring the growth of subpopulations with constitutive resistance genes or that adapt to harness such mutations [13]. This plasticity, derived from their genomic instability, is a major enabler of cancer chemoresistance.

On top of these effects, multidrug efflux pumps expel a wide spectrum of structurally and mechanistically distinct chemotherapeutic agents from tumors, hence limiting their cytotoxic potential. Given the plethora of factors contributing to cancer chemoresistance

[5, 6, 8, 9, 12, 14], multidrug efflux transporters are just a piece of a fairly large array of mechanisms of chemoresistance. Nonetheless, since the original description of a multidrug resistance (MDR) protein, P-glycoprotein (P-gp, *ABCB1*), the body of evidence validating the role of membrane transporters in poor cancer drug response has markedly expanded [15]. The additional depiction of P-gp in adult organs, including the kidneys, and the discovery of a myriad of other drug transporters cemented the role of these transporter proteins in drug resistance and excretion. The breast cancer resistance protein (BCRP/ABCG2), has been increasingly implicated in the handling of renal clearance of metabolites and relevant therapeutic drugs, including many chemotherapeutic agents. In the kidneys BCRP is apically expressed along with other MDR efflux transporters, including P-gp, with great substrate promiscuity between them and consequent functional redundancy in urinary excretion. In the current review, we present the role of BCRP in both kidney function and cancer MDR.

RENAL TRANSPORTERS AND DRUG EXCRETION

In the process of removing solutes from the systemic circulation, following glomerular filtration, water, nutrients and salts are reabsorbed to prevent losses, whereas xenobiotics such as drugs and environmental toxicants, as well as metabolic bi-products, endogenous wastes, must be removed from the blood stream and concentrated in the urine. This latter process takes predominantly place at the proximal tubule epithelium (PTE), which expresses multiple membrane transporters, as well as an array of phase I and phase II metabolism enzymes. Together, these elements render the PTE cells (PTEC) crucial for disposition of xenobiotics. PTEC are highly polarized and specialized cells that consist of two surfaces, one exposed to the interstitium thus, surrounded by a network of capillaries, and another exposed to the tubular lumen and covered with microvilli, forming a brush-border membrane with a large surface area (Figure 1). This polarization of cells enables them to act as a selective barrier, where solutes, ions, drugs, metabolites and other compounds can be shuttled unidirectionally either back to the circulation or into the pre-urine. Membrane-bound carrier proteins are responsible for the selective nature of transport in PTE, by binding substrates in order to relocate them. These proteins can be divided into ion channels, solute carriers, aquaporins or efflux pumps. With regard to drug excretion, efflux pumps belonging to two families are pivotal to the process.

The Human genome organization (HUGO; http://www.genenames.org) reports a highly diverse group of membrane transport proteins that include over 300 entries, *viz.* the solute carrier (SLC) family. A number of these carriers are expressed in the PTE, providing specificity for different classes of drugs, as well as metabolites and nutrients; these transporters are involved in both excretion and reabsorption of solutes. SLC transporters facilitate the uptake of xenobiotics from the interstitium and a range of substrates including negatively charged solutes handled by organic anion transporters 1 and 3 (OAT1, *SLC22A6* and OAT3, *SLC22A8*, respectively), as well as the organic anion

transporter polypeptide 4C1 (solute carrier organic anion transporter family member 4A1; *SLCO4C1*)[16]. Alongside, the organic cation transporter 2 (OCT2; *SLC22A1*) handles positively charged solutes. The apical side of PTEC faces the lumen of the nephron and a set of transporters present at this side extrude solutes, drugs, metabolic bi-products and other compounds into the ultra-filtrate, a process which concentrates xenobiotics and metabolic waste compounds in the urine. Present at this side are the multidrug and toxin extrusion protein 1 and 2 (MATE1 (*SLC47A1*) and MATE2K (*SLC47A2*), respectively), both of which share cationic substrates. Bi-directional SLC transporters are also present at the apical side, like the urate transporter (URAT1, *SLC22A12*) that exchanges anions by urate, regulating its plasma levels. The organic anion transporter 4 (OAT4, *SLC22A11*) is responsible for the reabsorption of anionic solutes and the carnitine transporters 1 and 2 (OCTN1, *SLC22A4* and OCTN2, *SLC22A5*; respectively) reabsorb this essential amino acid.

The most prominent apical efflux transporters in the PT belong to the ABC superfamily and handle the bulk of renally excreted compounds. This transporter family is highly conserved with diverse classes of trans-membrane transporter proteins, sometimes translocating substrates against steep concentration gradients, harnessing energy from ATP hydrolysis [17]. The HUGO lists 51 human ABC transporters encoding genes that have been identified and divided into 7 families (ABC – A through G), according to their divergent evolution (http://www.genenames.org). Members of the ABC superfamily display broad substrate specificities and are involved in the transport of endogenous compounds like

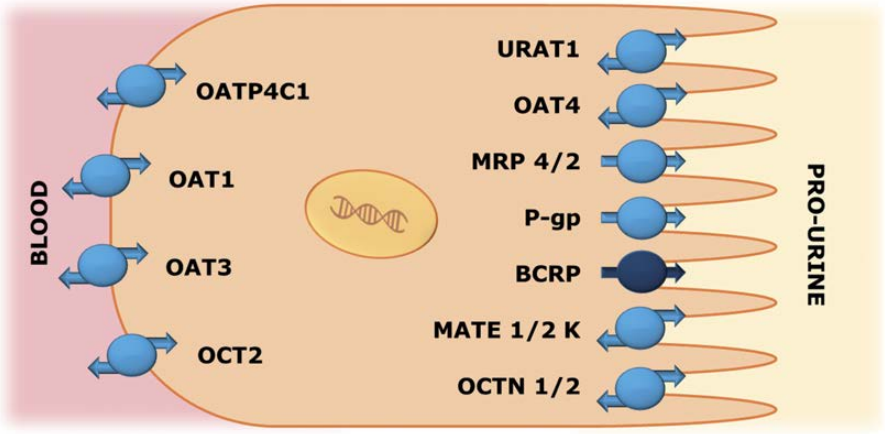


Figure 1. Drug transporters in the proximal tubule. Influx transporters facing the blood side: organic anion transporter protein 4C1 (OATP4C1), organic anion transporter 1 and 3 (OAT1 and -3) and organic cation transporter 2 (OCT2). Efflux carriers- expressed at the apical membrane – urate anion exchanger 1 (URAT1), OAT4, multidrug resistance protein 2 and 4 (MRP2 and -4), P-glycoprotein (P-gp). Breast cancer resistance protein (BCRP), multidrug and toxin extrusion protein 1 and 2K (MATE1 and -2K) and the organic carnitine transporter 1 and 2 (OCTN1 and -2). Solute carrier transporter (SLC) are depicted as , and ATP binding cassette (ABC) transporters as .

inorganic anions, metal ions, amino acids, hormones, fatty-acids, peptides, sugars and metabolic bi-products, as well as exogenous substrates such as drugs and environmental toxicants. The signature feature of this transporter superfamily is a nucleotide-binding domain (NBD), shared by all superfamily members. Traditionally, ABC transporters have been tightly associated with cancer MDR, one of the most widely studied membrane transport-related mechanisms, from which several transporters derive their namesakes [13]. ABC transporters present in the PTE include the multidrug resistance proteins 2 and 4 (MRP2, *ABCC2* and MRP4, *ABCC4*, respectively), P-gp and the breast cancer resistance protein (BCRP; *ABCG2*), the role of which in renal function has gained momentum in recent years, but has often been overlooked in comprehensive reviews [3, 18].

BCRP, FROM DRUG RESISTANCE TO RENAL EXCRETION

Since its fortuitous initial description in 1998 by Doyle *et al* in a naive MDR human breast cancer cell line [19], BCRP has been implicated in physiological processes way beyond drug-resistance and identified in several cell types and tissues. Different expression levels of BCRP have been reported in the brain and central nervous system, liver, placenta, uterus, mammary glands, prostate, testis, stomach, intestine, colon, lung and the kidney [15, 20]. This widespread presence is indicative of a mechanism of tissue-defense against xenobiotics, and the pool of substrates handled by BCRP confirms this role. Furthermore, the affinity of endogenous substrates such as steroid hormones and small metabolites for BCRP indicates that this transporter may also play a regulatory function. Moreover, a large number of chemotherapeutics and other drugs known to be substrates of both P-gp and MRP are also BCRP substrates [21].

Human BCRP is a protein roughly half the size of most members of the ABC superfamily, with 665 amino acids and a molecular mass of 72KDa, and is, encoded by a gene residing on chromosome 4, band 22 (4q22). BCRP and other members of the ABCG family are, in essence, distinct from other prominent ABC transporters such as P-gp and MRPs, which are present in their active assembled forms in the membrane. BCRP is designated a remove "half-transporter", since it requires two identical monomers to form a functional homodimer. Like all ABC transporters, BCRP is an ATP-driven extrusion pump which transports its substrates from the intracellular space as well as from lipid bilayer to the extracellular milieu [22, 23]. BCRP is highly expressed in lactating mammary epithelium where it secretes its substrates into the milk including the vitamin B_2 riboflavin [24, 25]. This transporter is also highly expressed on the Side Population (SP) of primitive stem cells [26]. Although its exact function in these cells is still unclear, BCRP likely fulfills a protective function by pumping out toxicants and harmful products of oxidative stress [27].

RENAL BCRP FUNCTION, WHY SHOULD WE CARE?

In general, the organic anion transport system constitutes the key mechanism in renal transport and, indisputably, the bulk of solutes removed via the PTEC is in fact transported into PTEC by OAT1 and OAT3. However, there is another side to renal transport, the one moving substances from PTEC into the lumen, and it is here that BCRP plays its role. Since its functional presence was reported in human PT by Huls *et al*. in 2008 [20], our understanding of BCRP in the kidney has expanded and its active excretory contribution is currently recognized. Basal BCRP expression levels in human PTEC are lower than those of other ABC transporters (*i.e.* P-gp and MRP4), nonetheless, its functional activities are comparable [28].

The list of established BCRP substrates is ever increasing. Table 1 lists a number of clinically approved drugs known to be excreted via the renal route that are BCRP substrates. As overlapping substrate specificities among the xenobiotics transporters are apparent, we depicted other renal transporters involved as well. In addition, known drug-drug interactions that result in altered plasma levels of a drug are described. This information was retrieved from the open-access database drug bank (http://www.drugbank.ca/) [29] using a comprehensive search of all approved drugs listed as interacting with BCRP/*ABCG2*, while described to be excreted by the kidney to any given extent. It is evident that majority substantial fraction are chemotherapeutics (25–50%) and also that a considerable substrate specificity overlap exists between BCRP and other members of the ABC superfamily. We identified thirty-two drugs being common transport substrates of BCRP and P-gp, seventeen drugs that share specificities for BCRP and MRP2 and eight drugs that are common substrates for MRP4 and BCRP. Given the amount of drugs and drug-interactions observed, it is evident that BCRP, together with its ABC counterparts, does play a prominent role in xenobiotic excretion in PTEC. The importance of these interactions becomes relevant when considering that the wide use of co-medication, combination therapies and concomitant use of different medicines [30]. This can give rise to an imbalance and interference in renal excretion and consequent systemic accumulation of drugs with unintended consequences.

Beyond its role in drug excretion, BCRP is also implicated in the excretion of a class of endogenous metabolites that particularly reflect kidney function, the so-called uremic solutes [4]. These solutes are often derived from metabolic processes and, under normal conditions, efficiently excreted by the kidneys. Their systemic retention can be a direct cause of a renal clearance deficit as in acute kidney injury (AKI) or chronic kidney diseases (CKD), or indirectly through competitive interactions at the transporters level as a number of uremic solutes have been identified as substrates [16]. Thus far, BCRP has been associated with the handling of several uremic solutes [31], including urate, hippuric acid, indoxyl sulfate, kynurenic acid and p-cresyl sulfate and p-cresyl-glucuronide [32-34].

Table 1. List of FDA approved drugs that are eliminated via the renal route and have been described as substrates for BCRP

Drug	Class	Renal clearance	Substrate for[1]		Known interactions
			basolateral	apical	
Apixaban	Anticoagulant	27%	n/d	P-gp	Imatinib*, Idelalisib*, Dabrafenib**
Buprenorphine	Analgesic	10-30%	n/d	P-gp	Dabrafenib**, Idelalisib*
Carboplatin	Chemotherapeutical agent	100%	n/d	MRP2	Topotecan, Paclitaxel
Cisplatin	Chemotherapeutical agent	100%	OCT2	hMATE I, hMATE II	Docetaxel, Paclitaxel, Topotecan
Clofarabine	Chemotherapeutical agent	49-60%	n/d	n/d	n/d
Cyclosporine	Immunosuppressant	6%	OAT1	P-gp, MRP2	Dabrafenib**, Doxorubicin, Etoposide, Glyburide, Imatinib*, Idelalisib*, Ezetimibe*, Methotrexate*, Mycophenolate mofetil, Rosuvastatin, Pravastatin, Pitavastatin, Topotecan, Testosterone
Daclatasvir	Antiviral	6.6%	n/d	P-gp	Dabrafenib*, Doxorubicin, Pitavastatin, Pravastatin, Topotecan, Verapamil
Dabrafenib	Chemotherapeutical agent	23%	OAT1	P-gp	Vandetanib, Teniposide, Apixaban, Buprenorphine, Cyclosporine, Doxorubicin, Estradiol, Etoposide, Imatinib, Idelalisib*, Topotecan, Sunitinib, Teniposide,

Table 1. (continued)

Drug	Class	Renal clearance	Substrate for[1]		Known interactions
			basolateral	apical	
Daunorubicin	Chemotherapeutical agent	25%	OCT2	P-gp, MRP2	Verapamil*, Paclitaxel,
Docetaxel	Chemotherapeutical agent	6%	n/d	P-gp, MRP2	Cisplatin, Dabrafenib*, Sorafenib*, Verapamil*
Doxorubicin	Chemotherapeutical agent	5-12%	n/d	P-gp, MRP2	Cyclosporine*, Daclatasvir*, Idelalisib*, Paclitaxel, Zidovudine, Sofosbuvir*, Sorafenib*, Sunitinib, Vandetanib*
Dronabinol	Psychoactive drug	n/d	n/d	P-gp	Fluorouracil*, Imatinib*
Erlotinib	Chemotherapeutical agent	8%	n/d	P-gp	Dabrafenib*, Idelalisib*, Omeprazole*, Rabeprazole*
Estradiol	Steroid hormone	n/d	OCT2, OAT3	P-gp	Dabrafenib**, Glyburide, Teriflunomide**, Verapamil*
Etoposide	Chemotherapeutical agent	56%	n/d	P-gp, MRP2	Cyclosporine, Idelalisib*, Dabrafenib**, Verapamil*
Ezetimibe	Statin (anti-cholesterol)	11%	n/d	P-gp, MRP2	Cyclosporine
Fluorouracil	Chemotherapeutical agent	20-90%	n/d	MRP4	Dronabinol
Glyburide	Anti-hyperglycemic agent	n/d	OAT1	P-gp, MRP2	Cyclosporine, Dabrafenib, Estradiol, Hydrocortisone, Sunitinib, Testosterone

Table 1. (continued)

Drug	Class	Renal clearance	Substrate for[1]		Known interactions
			basolateral	apical	
Hydrocortisone	Anti-inflammatory agent	n/d	n/d	P-gp	Glyburide, Testosterone, Verapamil*
Idelalisib	Chemotherapeutical agent	14%	n/d	P-gp	Apixaban, Buprenorphine, Cyclosporine, Dabrafenib, Doxorubicin, Docetaxel, Etoposide, Erlotinib, Imatinib, Irinotecan, Sunitinib, Vincristine, Verapamil, Teniposide, Sorafenib
Imatinib	Chemotherapeutical agent	5-13%	OCT2		Cyclosporine, Dronabinol, Idelalisib*, Apixaban, Dabrafenib*, Doxorubicin, Topotecan
Irinotecan	Chemotherapeutical agent	25-50%	n/d	P-gp, MRP4	Dabrafenib*, Erlotinib*, Idelalisib*, Lenvatinib*, Regorafenib*, Verapamil*
Lamivudine	Antiviral	5%	OCT2, OAT1	P-gp	n/d
Lansoprazole	Proton pump inhibitor	33%	n/d	P-gp	Dabrafenib**, Imatinib, Methotrexate, Mycophenolate, mofetil
Lenvatinib	Chemotherapeutical agent	25%	n/d	P-gp	Irinotecan
Methotrexate	Chemotherapeutical agent	80-90%	OAT1, OAT3	MRP2, MRP4	Cyclosporine*, Lansoprazole*, Pantaprazol*, Teriflunomide*, Verapamil*

Table 1. (continued)

Drug	Class	Renal clearance	Substrate for[1]		Known interactions
			basolateral	apical	
Mycophenolate mofetil	Immunosuppressive agent	93%	n/d	P-gp, MRP2	Cyclosporine**, Lansoprazole*, Pantaprazol*, Omeprazole*, Teriflunomide*
Omeprazole	Proton pump inhibitor	77%	n/d	P-gp	Cyclosporine, Erlotinib, Dabrafenib**, Mycophenolate mofetil
Oxaliplatin	Chemotherapeutical agent	54%	OCT2	MRP2	Paclitaxel, Topotecan
Paclitaxel	Chemotherapeutical agent	14%	n/d	P-gp, MRP2	Cisplatin, Carboplatin, Oxaliplatin, Daunorubicin, Dabrafenib**, Doxorubicin, Verapamil*
Pantaprazol	Proton pump inhibitor	71%	n/d	P-gp	Erlotinib, Methotrexate, Mycophenolate mofetil,Topotecan
Pitavastatin	Statin (anti-cholesterol)	15%	n/d	P-gp, MRP2	Cyclosporine*, Daclatasvir*, Teriflunomide*
Pravastatin	Statin (anti-cholesterol)	47%	OAT1, OAT3	P-gp, MRP2	Cyclosporine*, Daclatasvir*, Teriflunomide*
Rabeprazole	Proton pump inhibitor	90%	n/d	n/d	Dabrafenib**, Erlotinib
Regorafenib	Chemotherapeutical agent	19%	n/d	P-gp	Dabrafenib**, Irinotecan, Verapamil
Rosuvastatin	Statin (anti-cholesterol)	28%	n/d	MRP4	Cyclosporine*

Table 1. (continued)

Drug	Class	Renal clearance	Substrate for[1]		Known interactions
			basolateral	apical	
Sofosbuvir	Antiviral	80%	n/d	P-gp	Doxorubicin, Vincristine**, Verapamil*
Sorafenib	Chemotherapeutical agent	19%	n/d	P-gp, MRP2, MRP4	Carboplatin, Docetaxel, Doxorubicin, Idelalisib*, Paclitaxel
Sunitinib	Chemotherapeutical agent	16%	n/d	MRP4	Testosterone, Topotecan, Idelalisib*, Doxorubicin, Verapamil
Teniposide	Chemotherapeutical agent	4-12%	n/d	n/d	Idelalisib*, Verapamil*
Teriflunomide	Immunomodulatory agent	23%	n/d	n/d	Estradiol, Methotrexate, Mycophenolate mofetil, Pitavastatin, Pravastatin, Topotecan, Zidovudine
Testosterone	Steroid hormone	90%	OAT3	P-gp	Cyclosporine, Glyburide, Hydrocortisone, Sunitinib
Topotecan	Chemotherapeutical agent	73%	n/d	P-gp	Cisplatin, Carboplatin, Cyclosporine*, Daclatasvir*, Imatinib*, Pantaprazol*, Oxaliplatin, Teriflunomide*, Vandetanib*
Vandetanib	Chemotherapeutical agent	25%	n/d	n/d	Doxorubicin, Topotecan, Verapamil

Table 1. (continued)

Drug	Class	Renal clearance	Substrate for[1]		Known interactions
			basolateral	apical	
Verapamil	Anti-arrhythmia agent	70%	OCTN2	P-gp, MRP4	Apixaban, Cyclosporine, Idelalisib*, Daclatasvir*, Doxorubicin, Etoposide, Estradiol, Imatinib, Irinotecan, Methotrexate, Vincristine, Vandetanib*, Sunitinib*
Vincristine	Chemotherapeutical agent	10-20%	OCT3	MRP2	Cyclosporine*, Idelalisib*, Dabrafenib*, Sunitinib*, Verapamil*
Vismodegib	Chemotherapeutical agent	4%	n/d	P-gp	n/d
Zidovudine	Antiviral	29%	OCT2, OAT1, OAT3	P-gp, MRP4	Doxorubicin, Teriflunomide*

Most of these 49 compounds are also substrates for other transporters expressed in the kidney, being P-gp the most common and chemotherapeutical agents are the majority, with 25 substrates. Any known pharmacological interactions between these substrates and BCRP are also listed in the table (known interactions). The majority of the described interactions involve changes in the of plasma levels of a given substrate when co-administrated, the compounds marked with * increase in plasma levels, while compounds marked with ** decrease in plasma levels. Unknown parameters are designated not defined (n/d). Data retrieved from drugbank.ca.

[1]Substrates for this transporters in addition to BCRP.

Chemotherapeutic agents influence BCRP activity

Prior to being recognized as a renal transporter, and since its identification, BCRP has held a place as a major contributor to the chemoresistance of several cancers [35]. The presence of BCRP in multiple tumor types has been correlated to diminish therapeutic outcomes and to reduce chemotherapeutics retention and efficacy. This is consistent with its tissue protective function under physiological conditions. As highlighted in Table 1, a wealth of anticancer drugs that are BCRP substrates are cleared via the kidneys. In the current review we explore some of the most pronounced effects on BCRP activity by these chemotherapeutic agents, studied in cancer models.

In recent years, the so-called tyrosine kinase inhibitors (TKI) have gained ground as the frontline treatment for several cancers. By suppressing proliferation-related mechanisms in cancer cells, TKI can be administered in combination with other antitumor agents in order to boost their cytotoxic effects. Imatinib is an example of a TKI that is both substrate and a modulator of BCRP. It can suppress its expression in BCR-ABL$^+$ cells, a hallmark genetic alteration that enables permanent tyrosine kinase activation hence conferring upon chronic myelogenous leukemia its aggressive phenotype [36]. Head and neck carcinomas show particular upregulated kinase activity, and here too imatinib decreased BCRP efflux activity resulting in enhanced doxorubicin cytotoxicity, when combined. In this case, BCRP protein levels were intact, and determined to be inactive in the cytoplasm, a tell-tail sign of post-transcriptional regulation [37]. Consistently, in MCF7 tumor spheres exposed to both lapatinib and doxorubicin, BCRP expression was found to be reduced while intracellular accumulation of doxorubicin was enhanced [38]. Similarly, by inhibiting the epidermal growth factor receptor (EGFR) in lung cancer cells, erlotinib reduced the expression of BCRP in a time-dependent manner. Further, using MDCK-BCRP cells, Pick et al., demonstrated that imatinib and erlotinib can reduce the membrane expression and transport activity of BCRP [39]. Moreover, an overexpression of ErB2 – EGFR2 in MCF7 cells affected BCRP levels and conferred resistance to etoposide, mitoxantrone, 5-fluorouracil, paclitaxel and cisplatin [40]. Since their approval, and besides their proven clinical efficacy, TKI have been instrumental to unravel the molecular mechanisms underlying drug resistance, by shedding light on ubiquitous regulatory pathways. A better understanding of the action mechanisms of this class of drugs and how they affect BCRP activity can improve strategies to tackle BCRP-dependent drug resistance. TKI appear to harbour the potential to influence transport activity not only by direct interacting with BCRP, but also by negatively regulating its expression and function. EGFR/ErB expression is not transversal to all tumors and its expression can significantly vary within the type of cancer and within the patient population; therefore, the clinical impact of TKI-mediated BCRP regulation is still unknown and clear evidence confirming this aspect is currently absent.

BCRP is also known to play an active role in cancer resistance to several conventional anti-cancer drugs. Mimicking cytotoxic bolus drug treatment through 12- to 24-hour pulse exposure of ABCG2-silenced leukemia cells, using clinically relevant concentrations of the chemotherapeutic agents daunorubicin and mitoxantrone, resulted in a marked transcriptional up-regulation of ABCG2 [41]. Hence, antitumor drug-induced epigenetic reactivation of ABCG2 gene expression in cancer cells appears to be an early molecular event leading to MDR. These findings have important implications for the emergence, clonal selection, and expansion of malignant cells with the MDR phenotype during chemotherapy.

The well-known nephrotoxic agent, cisplatin, is widely used in the treatment of a variety of cancers, and nephrotoxicity is often a limiting factor in its use [42]. Although several transporters are involved in cisplatin handling, it has been shown *in vitro* that BCRP expression in colon cancer cells can be enhanced by cisplatin exposure [43],

demonstrating the inducible nature of BCRP in chemoresistance. In myeloid leukemia clofarabine, cytotoxicity is hampered by BCRP, an effect that is attenuated upon clofarabine phosphorylation by deoxycytidine kinase. The addition of a phosphate group to this drug reduces its affinity for BCRP [44]. This coupled force highlights the modulatory effects that enzyme activity can exert on transporter function as well as tissue specificity of metabolic processes, whereas in tissues lacking this enzyme, BCRP activity towards clofarabine will likely not be reduced. Taken together, these interactions show that the role of BCRP in chemoresistance is far more advanced than its mere presence in tumors, hence underlining the complexity of the cellular processes involved.

CELLULAR BCRP REGULATION INFLUENCES RENAL DRUG EXCRETION

So far, regulatory mechanisms that are implicated in stress responses, xenobiotic and toxin sensing and cellular homeostasis have been linked to the activation and suppression of functional BCRP activity, as summarized in Figure 2. Most of the evidence regarding BCRP regulation originates from animal models and tumor-derived cell lines. Arguably, regulatory pathways between humans and rodents may not be conserved whereas cancer cell lines are transformed and their physiology is altered, and therefore not representative of physiological conditions. Nonetheless, the mechanisms described are ubiquitous and active in several other organs, including the kidneys. Furthermore, many of the drugs that modulate/inhibit such regulatory pathways are substrates for BCRP itself [45] a characteristic that emphasizes the stress-related activation of BCRP upon xenobiotic exposure. The cellular machinery behind the activation, expression and transcription of membrane transporters has been subject of active research, and recently several studies have revealed factors responsible for their regulation.

Diverse studies implicated nuclear factor erythroid-derived 2 (Nrf2), a transcription factor that regulates the expression of stress and inflammation related genes, in BCRP regulation. In rats, Nrf2 stimulation by sulforaphane induced expression and function of BCRP in brain capillaries, an effect absent in Nrf2-null mice [46]. In line with these findings, Nrf2 knockdown with siRNA in both prostate and lung cancer cell lines reduced BCRP gene and protein expression [47]. In contrast, Hyuk-Sang Jeong *et al.,* demonstrated that suppression of Kelch-like ECH-associated protein 1 (KEAP1), down-regulates Nrf2, which led to increases in BCRP expression in human renal kidney (HK-2) cells [48]. Furthermore, this nuclear factor is also implicated in drug resistance, linked to the activation of metabolic enzymes and enhanced efflux activity in tumors [49].

Another transcription factor implicated in BCRP modulation is the ligand-activated aryl hydrocarbon receptor (Ahr), which acts as a sensor of environmental toxins, drugs and other xenobiotics, triggering the expression of metabolizing enzymes [50]. It was shown that BCRP can be affected by environmental toxins, such as polycyclic aromatic hydrocarbons (PAH), that activate Ahr and upregulate BCRP expression [51]. Furthermore, the uremic solute p-cresyl glucuronide can functionally induce BCRP through a yet

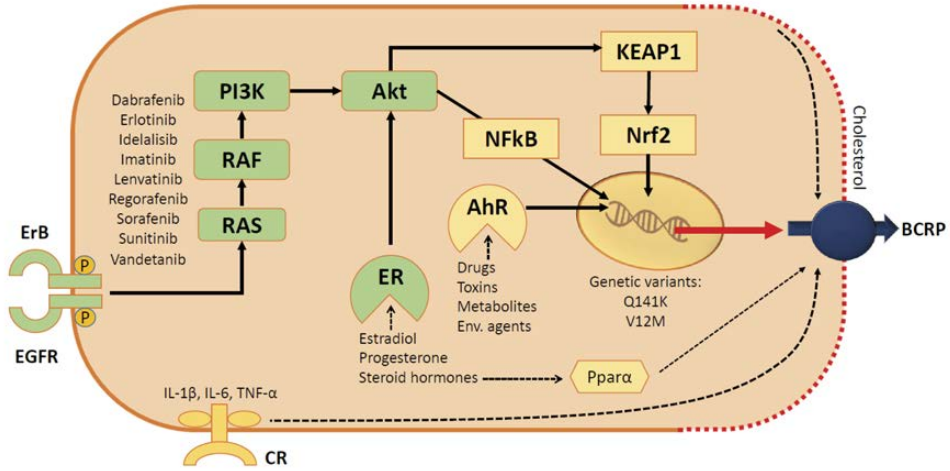


Figure 2. Proposed BCRP regulatory mechanisms. The estrogen (ER) and aryl hydrocarbon (Ahr) are intra-cellular receptor that sense exogenous stimuli that can trigger a transcriptional response, via the serine-threonine protein kinase (Akt) and further the Kelch-like ECH-associated protein 1 (KEAP1) and nuclear factor erythroid-derived 2 (Nrf2), which is known to positively regulate BCRP expression. Epidermal growth factor receptor (EGFR/HERB) dependent signaling can be inhibited up- or down- stream of the receptor, modulating the Akt cascade and resulting in BCRP suppression or activation. Gaps persist in the understanding of the full mechanism/path involved in lipid-dependent regulation, where the peroxisome proliferator-activated receptor (Pparα) receptor is involved, as well as how cytokines influence activity, acting via chemokine receptors (CR).

unknown mechanism [33]. In terms of endogenous factors, steroid hormones such as progesterone and estradiol have been described to downregulate BCRP [52]. In isolated rat and mouse brain capillaries exposure to 17-estradiol (E2) reduced the expression and efflux activity of BCRP, and furthermore E2 decreased active a-serine-threonine protein kinase (Akt) as well as phosphoinositide 3-kinase (PI3K) [53].

The role of protein kinase activity has also been associated with BCRP modulation, particularly in drug-resistance studies, where the inhibition of the PI3K/Akt signaling pathway by LY294002 reduced BCRP expression and function in both MCF7 and MDCK-BCRP cells [54]. Furthermore, PI3K/Akt inhibition blocked the targeting of BCRP to extracellular vesicles, hence overcoming MDR in breast cancer cells [55]. The use of gefitinib, a TKI used as a chemotherapeutic agent, decreased BCRP expression and activity in advanced non-small lung cancer patient cells [56]. Further studies have confirmed the role of tyrosine kinase-dependent signaling in BCRP regulation. Downstream inhibition of the constitutive EGFR pathway downregulates BCRP expression in *in vitro* [39], implicating Akt activity in BCRP gene expression. Other factors such as the peroxisome proliferator-activated receptor α (PPARα), a steroid hormone receptor that mediates lipid homeostasis, was shown to increase BCRP efflux activity in human cerebral microvascular endothelial cells - hCMEC/D3 [57]. The pro-inflammatory cytokines interleukin 1-β and 6

(IL-1β and IL-6, respectively) together with the tumor necrosis factor α (TNF-α) can also reduce the expression an activity of BCRP in these cells [58].

Plasma membrane components, such as lipids and cholesterol, have been linked to the post-transactional regulation of BCRP. This suggests that BCRP is sensitive to the lipid nature of its surroundings [59] as is its MDR efflux transporter counterpart, P-gp [60-62]. Further evidence implicating lipids in regulation of BCRP activity emerges from clinical studies, where patients with familial hypercholesterolemia undergoing statin therapy showed reduced BCRP expression and transport activity [63]. Pharmacokinetics studies have correlated the *ABCG2* 421C>A polymorphism with increased low-density lipoprotein cholesterol in response to rosuvastatin, a BCRP transport substrate, suggesting that cellular BCRP regulation can be influenced by its own substrate [63]. *In vitro* cholesterol membrane content was shown to have a significant impact on BCRP activity, where cholesterol loading has a positive impact in transport activity, and sterol-sensing motifs have been identified in the BCRP structure [64]. Furthermore, *ex vivo* cholesterol depletion was also shown to decrease BCRP expression and activity [63].

In a chronic kidney disease model, where 5/6 nephrectomy was performed in male rats, renal BCRP expression decreased and correlated with both injury severity and cytokine increase, an effect that is absent in female rats [65]. This initial evidence suggests a prominent role for BCRP in kidney disease. Adding to the complexity of BCRP regulation, a study by Martín *et al.*, showed that increased melatonin levels leads to BCRP promoter methylation and subsequent silencing of the BCRP gene and the function of its gene product. Moreover, the epigenetic alterations that occur in cancer cells may well be a hallmark in the development of transporter-mediated multidrug resistance [66]. Another aspect of BCRP regulation is related to the fact that the pathways now implicated in its activity are also responsible for the regulation of other ABC transporters that are co-expressed along with BCRP in numerous tissues [50]. These findings strongly suggest that members of the ABC superfamily that share similar substrates, and therefore similar functions, may also share the same regulatory pathways. This holds true in the blood brain barrier where BCRP and P-gp activation have common denominators, such as cytokines, Ahr and Nrf2 [50], and in tumor cell lines where TKI also inhibit both ABC transporters. Furthermore, the mechanisms that promote upregulation of BCRP may well be independent, where different stimuli led to the same result, either initiated by an exogenous source, like a toxicant triggering Ahr or by a more intrinsic mechanism, like cytokines which activate the PI3K/Akt pathway (Figure 2). The clinical implications of these cellular regulatory mechanisms, not only implicating BCRP, and how they can govern the renal clearance of drugs, is in large part yet to be resolved. EGFR signaling plays a considerable role in maintaining physiological processes, and despite the fact that current TKI seem to lack a nephrotoxic potential often observed with conventional chemotherapeutics [67], little if any clinical evidence exists concerning the effects of combination therapies in drug disposition and nephrotoxicity, where several drugs are BCRP transport substrates. This

illustrates where both renal and cancer research can overlap and highlights the need for novel and concise research that takes into account the complexity of the mechanisms underlying pharmacological drug interactions in the kidney.

THE PHARMACOGENOMICS PROFILE ALTERS BCRP-MEDIATED TRANSPORT

As many drugs compete for similar binding sites of transporters, drug-drug interactions might occur upon co-administration. Consequently, drug concentrations will rise thereby leading to a nephrotoxic event [68-70]. In addition, the administration of a compound with an intrinsic toxicity, like the antibiotic gentamicin or the immunosuppressive drug cyclosporine A, may directly result in drug-induced kidney injury [71]. These effects may be more pronounced in patients with genetic variants of BCRP, as described for uric acid. The role of pharmacogenomics has become evident over the past years and emerges as relevant for predicting the safety and efficacy of therapies [72] as well as for personalized medicine [73, 74]. To date, over 60 SNPs have been characterized in the coding sequence of BCRP, both missense and nonsense variants, two SNPs of which, namely Q141K and V12M, display a relatively high allele frequency of 12% and 16% in the global population, respectively. Interestingly, the frequency is distributed among the five known super-populations (Figure 3). Both variants are most abundantly present in the East Asian population with a frequency of about 30% for both polymorphisms. In addition to disturbed xenobiotic handling, there is a clear genome-wide association between BCRP-Q141K and gout manifestation. Gout is characterized by elevated plasma urate levels which consequently lead to the formation of crystals within joints [75-78]. Urate is derived from purine nucleotide catabolism following dietary intake and endogenous biosynthesis of purine nucleotides. In healthy subjects, urate is cleared by the gastrointestinal tract for approximately one-third, whereas two-thirds are secreted via the kidneys by glomerular filtration and active tubular secretion involving BCRP [32, 79]. The Q141K variant contributes to the development of hyperuricemia as the clearance into the pro-urine is impaired [76], leading to increased plasma urate levels and onset of gout. Next to the Q141K variant, the Q126X variant has also been associated with gout [80], although this variant is less common as it has an average allele frequency in the general population only 0.1% in the general population. Next to gout, elevated urate levels have been associated with the progression of CKD [32, 81]. Dankers *et al.,* showed that uric acid inhibited BCRP-mediated transport in a competitive manner *in vitro*. Moreover, in hyperuremic mice, levels of L-kynurenine and kynurenic acid, both of which are metabolites of tryptophan metabolism, were retained in plasma. These metabolites are known substrates for BCRP and their elimination is hampered by elevated uric acid levels through competition with the same efflux transporter [31, 32, 34]. Elevated levels of uric acid, L-kynurenine and kynurenic acid are known to exert negative effects on many biological systems within the body, e.g. on vasculature endothelial cells [81]. As BCRP is

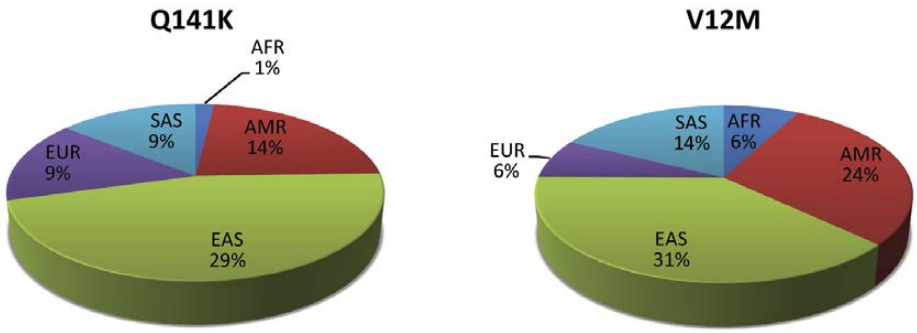


Figure 3. Distribution of the ABCG2 Q141K and V12M allele frequencies in five super populations. EUR: European, SAS: South Asian, AFR: African, AMR: Ad Mixed American, EAS: East Asian.

known to mediate transport of multiple uremic toxins, as previously described [31, 34], hyperuricemia might have a significant impact on the removal of these metabolites and therefore will contribute to the pathology of CKD.

The Q141K genotype is also linked to altered BCRP function in terms of pharmacokinetics hence consequently influencing xenobiotic disposition beyond urate [82]. For example, the plasma levels of the TKI sunitinib were found to be significantly higher in human renal cell carcinoma patients with a homo- and heterozygous genotype as compared to wild type BCRP [83, 84]. Consistently, in *abcg2*$^{-/-}$ mice the values of sunitinib C_{max} were found to be significantly higher when compared to wild type mice [84]. Furthermore, elevated plasma levels of the topoisomerase I inhibitor diflomotecan were found in heterozygous Q141K patients [85]. This may also account for numerous other anticancer agents resulting in elevated drug plasma levels and increased toxicity risk to various organs including the kidney [82]. Nevertheless, this is not seen for all drugs, as the antifolate methotrexate, which is predominantly cleared by the kidneys and associated with intrinsic renal toxicity [86], did not exhibit an altered pharmacokinetic profile in Q141K variants [87]. The BCRP-R482G and -R482T variants, on the other hand, may result in altered methotrexate pharmacokinetics as suggested by *in vitro* studies using Human Embryonic Kidney (HEK)-293 cells overexpressing these transporter variants [22, 88]. Molecular pharmacological studies demonstrated that this may be caused by reduced substrate transport and ATP turnover but most likely not due to changes in substrate binding *per se* [89]. Interestingly, recent studies showed the complex role of various BCRP residues on impaired drug export function via processes like altered posttranslational modifications, attenuated protein trafficking towards the plasma membrane and alterations in the drug binding site *in vitro* [90]. However, many of these residues, like BCRP-R482, have a very low prevalence in the population and are not yet identified in altered clinical pharmacokinetics.

The BCRP-Q141K mutant transporter is also associated with reduced the transport of the antidiabetic drug of the sulfonylureas class, glyburide, influencing diabetic treatment

[91]. Both the apparent transport K_t and V_{max} values of glyburide were significantly higher in the Q141K variant as compared to the wild type BCRP. Concurrently, the exact molecular mechanism underlying BCRP-Q141K dysfunction remains elusive as some studies showed a similar plasma membrane expression of BCRP of the wild type and mutant transporters [92, 93], whereas others detected reduced expression levels of BCRP [94, 95]. In addition, a decreased ATPase activity has been detected in Q141K BCRP mutants which might explain the less efficient BCRP-mediated glyburide export [92, 96].

To date, the knowledge on the effects of BCRP-V12M on (renal) pharmacokinetics are less pronounced and more controversial [82]. As shown by Imai *et al., in vitro*, the transport function of BCRP-V12M was not impaired as compared to wild type BCRP as determined by topotecan accumulation, a known anticancer drug substrate of BCRP[94]. In contrast, Mizuarai *et al*., showed that BCRP-V12M cells were more sensitive to topotecan treatment due to the accumulation of this cytotoxic compound [96]. These discrepancies might be related to the different species origin of cells applied in these studies. Mizuarai *et al*., used BCRP-transfected porcine renal cells (LLC-PK1) for functional testing whereas Imai *et al.,* performed functional experiments in mice fibroblasts (PA317) cells. BCRP physiology might vary fundamentally among cell models in terms of processing from gene to protein to function, and this might explain the different study outcomes [97]. A more reliable approach would be to perform CRISPR-Cas gene editing to implement polymorphisms in human renal epithelial cell models which constitute an endogenous BCRP expression system [97, 98]. This would allow to study the effect of BCRP-V12M on BCRP function in a more physiologically relevant manner.

Interestingly, the V12M SNP has been associated with alternative splicing of BCRP exon 2 in liver cells from human Hispanic origin and reduced BCRP liver expression was detected [99], potentially pointing towards alterations in drug disposition in these subjects. However, the impact on the functional level remains elusive as Chinese patients harboring acute leukemia which express the wild type BCRP genotype display a longer disease-free survival and overall less mortality as compared to BCRP hetero- or homozygous leukemia patients [26]. BCRP mRNA expression levels in these patients were solely detected in peripheral blood cells. It would have been of interest to investigate the renal BCRP mRNA expression in this population, as this could further elucidate the possible role of the V12M polymorphism in the kidney. Nonetheless, a full picture of the role of this BCRP polymorphism in drug clearance could be more complete with the protein's renal expression levels.

SEX, SPECIES AND GENDER DIFFERENCES INFLUENCE BCRP FUNCTION

BCRP expression has been shown to be sex and species-specific, which is in part related to the fact that its expression is regulated by hormones like estradiol and testosterone [100]. As such, humans often respond differently to drugs than predicted from preclinical data obtained in animal studies because the pharmacokinetic and pharmacodynamics profiles

are distinct [101]. Focusing on differences in renal BCRP expression in rodents, Dankers *et al*., showed that the BCRP gene expression in kidneys isolated from female Friend Leukemia virus B strain (FVB) mice was significantly lower as compared to male FVB mice [52]. Consistently, in a study performed by Tanaka *et al*., renal BCRP expression in female C57BL/6J mice tended to be lower as compared to male mice, though not statistically significant [100]. In the same study, Sprague-Dawley female rats showed significantly less BCRP gene expression as compared to male rats. Interestingly, renal BCRP expression in female rats was found to be significantly lower as compared to female C57BL/6J mice [100]. In general, it is well accepted that female animals display a lower renal BCRP expression than males, most likely due to the suppressive effect of estradiol as compared to males in which testosterone has an inductive effect though clear interspecies differences are observed between sexes [102, 103]. In translation to humans, Huls *et al.,* showed that kidneys of male FVB mice and rats (strain not reported, but communicated to be Wistar Hanover) exhibited a much higher renal BCRP gene and protein expression as compared to human kidneys [20]. These data emphasize that translating animal pharmacokinetics data to human requires caution, particularly when interpreting BCRP transport substrates. This observation is in line with the finding that many drugs fail in clinical studies due to adverse toxic events, simply explained by the inaccuracy of animal models to reflect the genuine pharmacokinetics profile in humans [104]. In addition, significant differences in postnatal nephron development and maturation may exist between sexes, as well as between species. The ontogeny of drugs transporters in the kidney was recently reviewed [18], however, the amount of information available is scarce, in particular for human. Allegaert *et al.,* [105] demonstrated that the renal excretion of drugs is commonly slow in neonates and dominated by glomerular filtration, which is in line with postnatal development and the harmful effects that drugs can have when used during pregnancy and in preterm-born infants, leading to renal mal-development and congenital anomalies of the kidney and urinary tract [106].

Importantly, next to sex, gender differences have been also shown to influence preclinical studies [107]. Gender is characterized by the socio-cultural environment that influences biology. For example it has been shown that housing animals in groups instead of single animals affects data variability [108]. Moreover, researcher gender is associated with handling and attitude towards male and female animals [107]. As such, animals handled by male researchers demonstrated higher stress levels as compared to animals treated by female scientists [109]. Interestingly, this researcher gender effect can be translated to BCRP function as induced stress levels can influence plasma testosterone levels [110-112]. Subsequently, this could potentially alter renal BCRP expression and function as testosterone is known to regulate BCRP [52, 100].

Thus far, these compelling findings lack thus far validation/translation at the clinical setting. It is well established that renal and malignant disease incidence and prevalence can vary greatly with age and gender. Survival rates for female patients undergoing dialysis are lower [113] and overall cancer mortality is higher among males [114]. Nonetheless,

our understanding of the role played by gender differences in transport activity in disease and drug treatments, remains insufficient.

CONCLUSIONS AND PERSPECTIVES

A substancial fraction of cancer drugs cleared by the kidneys are BCRP substrates. Despite its functional presence in the kidney, little is known about the impact of renal disease on the disposition of such drugs [115, 116]. On the other hand, renal function is a major concern in chemotherapy. For example, for cisplatin, an integral frontline treatment for several of the most common malignancies, nephrotoxicity is often a dose-limiting factor. Moreover, most cancer patients fall in the age group at risk of kidney disease. For safer therapy, a better understanding of both nephrotoxic mechanisms and kidney disease in cancer chemotherapy is warranted. In depth knowledge on the limitations of the renal function of cancer patients can contribute to better drug regimens design. Furthermore, the impact of cancer drugs on the renal contribution of BCRP is largely unknown. The same regulatory mechanisms governing drug resistance induced by drugs such as TKIs, namely imatinib, lapatinib and erlotinib, are also present in the kidneys. Despite studies reporting the relative safety of TKI's with respect to nephrotoxicity [67], virtually nothing is known on how these modulatory drugs may affect renal excretion.

AKI and CKD are a global health problem with significant morbidity and mortality, which affects 5–7% of the world population. The number of these patients continues to rise as a consequence of the increasing incidence of diabetes mellitus, the prevalence of insufficiently treated hypertension, and the aging population with its related progressive decline in renal function [117, 118]. The total medical care expenditure for CKD was nearly $58 billion in 2012 (U.S. Renal Data System, 2013). For both social and economic reasons, optimizing resources and finding novel strategies to tackle kidney disease is imperative.

Genetic factors, such as polymorphisms and differences between species, with respect to BCRP distribution and expression are recognized as important variables when it comes to drug absorption and distribution. On the other hand, the influence of sex and in particular of gender is often neglected. As such, raising awareness for the importance of sex and gender as a biological variable in biomedical research is key for future translational medicine.

The advent of personalized medicine holds the potential to both elucidating the mechanisms underlying drug resistance as well as in improving the clinical outcomes of cancer treatment [8]. The meta-data obtained by various "Omics" analyses can simultaneously provide information about genetic variants expression, cellular pathway activation, transporter expression and, together with the knowledge on drugs handled by BCRP as described in this manuscript, could significantly improve patient survival, with BCRP as a therapeutic target. However, this approach is still limited to pre-clinical setups, whereas clinical trials making a slow debut into clinical practice must be enhanced and

accelerated. On the other hand, knowledge on renal abundance of BCRP in humans is emerging, as recently reported using quantitative proteomics [118], and new approaches quantifying both apical and basolateral renal transporters distributions [119]. Hence, BCRP's role in renal drug excretion is becoming increasingly acknowledged.

Nephropharmacology is the discipline that studies the connection between clinical pharmacology and nephrology [119]. A better understanding of the role of drug transporters and their regulation in the kidney will aid in the development of safe drug therapies. The relevance of BCRP in the disposition of clinically relevant drugs in addition to its established role in chemoresistance, is now widely recognized by regulatory bodies, including the US Food and Drug Administration (FDA). Their guidelines include BCRP testing for relevant drug interactions studies [2]. Several reports, including those from the International Transporter Consortium (ITC) - http://www.ascpt.org - have identified BCRP as a prominent player in drug-interactions in the intestine, liver and blood-brain barrier, in both adult and developing human organs [18, 120]. We strongly advocate the inclusion of BCRP in studying drug interactions at the level of the kidney.

Towards this end. innovative, humanized, preclinical models that aid in drug screening during development and reliably predict drug disposition and clinical performance are needed. Recent approaches in renal cell biology focus on the generation of 3-dimensional cell culture models due to the more advanced features that these models offer by being more closely related to physiological conditions [121]. This can even be enhanced with the implementation of flow in the culture conditions. Furthermore, downscaling the size with microfluidics towards a so-called 'kidney-on-a-chip' using human or patient-derived cells has great potential for drug screenings [68]. In addition, stem cell-derived kidney organoids have been developed to understand kidney regeneration but also to present a platform that can be used for drug screening and disease modeling [122-124]. Drug development may be advanced further with humanized animal models, such as the humanized BCRP mouse in which the rodent transporter is replaced by its human orthologue [125] Finally, results of these biomedical innovations could be implemented into mechanistic models that mathematically describe underlying processes involved in renal drug handling [126]

In conclusion, we herein propose to recognize the importance of BCRP in chemoresistance and renal excretion for effective cancer treatment and, concomitantly, preserve kidney function.

ACKNOWLEDGEMENTS

This work was support by the Nephrotools consortium under the scope of EU FP7 Marie Curie actions (grant no. 289754).

REFERENCES

1. Konig J, Muller F, Fromm MF. Transporters and drug-drug interactions: important determinants of drug disposition and effects. Pharmacol Rev. 2013;65(3):944-66. doi: 10.1124/pr.113.007518. PubMed PMID: 23686349.
2. International Transporter C, Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, et al. Membrane transporters in drug development. Nat Rev Drug Discov. 2010;9(3):215-36. doi: 10.1038/nrd3028. PubMed PMID: 20190787; PubMed Central PMCID: PMCPMC3326076.
3. Nigam SK. What do drug transporters really do? Nat Rev Drug Discov. 2015;14(1):29-44. doi: 10.1038/nrd4461. PubMed PMID: 25475361; PubMed Central PMCID: PMCPMC4750486.
4. Masereeuw R, Mutsaers HA, Toyohara T, Abe T, Jhawar S, Sweet DH, et al. The kidney and uremic toxin removal: glomerulus or tubule? Semin Nephrol. 2014;34(2):191-208. doi: 10.1016/j.semnephrol.2014.02.010. PubMed PMID: 24780473.
5. Zhitomirsky B, Assaraf YG. Lysosomes as mediators of drug resistance in cancer. Drug Resist Updat. 2016;24:23-33. doi: 10.1016/j.drup.2015.11.004. PubMed PMID: 26830313.
6. Gonen N, Assaraf YG. Antifolates in cancer therapy: structure, activity and mechanisms of drug resistance. Drug Resist Updat. 2012;15(4):183-210. doi: 10.1016/j.drup.2012.07.002. PubMed PMID: 22921318.
7. Wijdeven RH, Pang B, Assaraf YG, Neefjes J. Old drugs, novel ways out: Drug resistance toward cytotoxic chemotherapeutics. Drug Resist Updat. 2016;28:65-81. doi: 10.1016/j.drup.2016.07.001. PubMed PMID: 27620955.
8. Li W, Zhang H, Assaraf YG, Zhao K, Xu X, Xie J, et al. Overcoming ABC transporter-mediated multidrug resistance: Molecular mechanisms and novel therapeutic drug strategies. Drug Resist Updat. 2016;27:14-29. doi: 10.1016/j.drup.2016.05.001. PubMed PMID: 27449595.
9. Ferreira JA, Peixoto A, Neves M, Gaiteiro C, Reis CA, Assaraf YG, et al. Mechanisms of cisplatin resistance and targeting of cancer stem cells: Adding glycosylation to the equation. Drug Resist Updat. 2016;24:34-54. doi: 10.1016/j.drup.2015.11.003. PubMed PMID: 26830314.
10. Ifergan I, Scheffer GL, Assaraf YG. Novel extracellular vesicles mediate an ABCG2-dependent anticancer drug sequestration and resistance. Cancer Res. 2005;65(23):10952-8. doi: 10.1158/0008-5472.CAN-05-2021. PubMed PMID: 16322243.
11. Jeggo PA, Pearl LH, Carr AM. DNA repair, genome stability and cancer: a historical perspective. Nat Rev Cancer. 2016;16(1):35-42. doi: 10.1038/nrc.2015.4. PubMed PMID: 26667849.
12. Ruud H. Wijdeven BP, Yehuda Assaraf, Jacques Neefjes. Old drugs, novel ways out: drug resistance towards cytotoxic chemotherapeutics. Drug Resistance Updates. 2016;28:65–81. doi: http://dx.doi.org/10.1016/j.drup.2016.07.001.
13. Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG. Cancer drug resistance: an evolving paradigm. Nat Rev Cancer. 2013;13(10):714-26. doi: 10.1038/nrc3599. PubMed PMID: 24060863.
14. Raz S, Stark M, Assaraf YG. Folylpoly-gamma-glutamate synthetase: A key determinant of folate homeostasis and antifolate resistance in cancer. Drug Resist Updat. 2016;28:43-64. doi: 10.1016/j.drup.2016.06.004. PubMed PMID: 27620954.
15. Fletcher JI, Williams RT, Henderson MJ, Norris MD, Haber M. ABC transporters as mediators of drug resistance and contributors to cancer cell biology. Drug Resist Updat. 2016;26:1-9. doi: 10.1016/j.drup.2016.03.001. PubMed PMID: 27180306.

16. Nigam SK, Wu W, Bush KT, Hoenig MP, Blantz RC, Bhatnagar V. Handling of Drugs, Metabolites, and Uremic Toxins by Kidney Proximal Tubule Drug Transporters. Clin J Am Soc Nephrol. 2015;10(11):2039-49. doi: 10.2215/CJN.02440314. PubMed PMID: 26490509; PubMed Central PMCID: PMCPMC4633783.

17. Vasiliou V, Vasiliou K, Nebert DW. Human ATP-binding cassette (ABC) transporter family. Hum Genomics. 2009;3(3):281-90. PubMed PMID: 19403462; PubMed Central PMCID: PMCPMC2752038.

18. Brouwer KL, Aleksunes LM, Brandys B, Giacoia GP, Knipp G, Lukacova V, et al. Human Ontogeny of Drug Transporters: Review and Recommendations of the Pediatric Transporter Working Group. Clin Pharmacol Ther. 2015;98(3):266-87. doi: 10.1002/cpt.176. PubMed PMID: 26088472; PubMed Central PMCID: PMCPMC4731327.

19. Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, et al. A multidrug resistance transporter from human MCF-7 breast cancer cells. Proc Natl Acad Sci U S A. 1998;95(26):15665-70. PubMed PMID: 9861027; PubMed Central PMCID: PMCPMC28101.

20. Huls M, Brown CD, Windass AS, Sayer R, van den Heuvel JJ, Heemskerk S, et al. The breast cancer resistance protein transporter ABCG2 is expressed in the human kidney proximal tubule apical membrane. Kidney Int. 2008;73(2):220-5. doi: 10.1038/sj.ki.5002645. PubMed PMID: 17978814.

21. Robey RW, To KK, Polgar O, Dohse M, Fetsch P, Dean M, et al. ABCG2: a perspective. Adv Drug Deliv Rev. 2009;61(1):3-13. doi: 10.1016/j.addr.2008.11.003. PubMed PMID: 19135109; PubMed Central PMCID: PMCPMC3105088.

22. Shafran A, Ifergan I, Bram E, Jansen G, Kathmann I, Peters GJ, et al. ABCG2 harboring the Gly482 mutation confers high-level resistance to various hydrophilic antifolates. Cancer Res. 2005;65(18):8414-22. doi: 10.1158/0008-5472.CAN-04-4547. PubMed PMID: 16166320.

23. Bram EE, Adar Y, Mesika N, Sabisz M, Skladanowski A, Assaraf YG. Structural determinants of imidazoacridinones facilitating antitumor activity are crucial for substrate recognition by ABCG2. Mol Pharmacol. 2009;75(5):1149-59. doi: 10.1124/mol.109.054791. PubMed PMID: 19251825.

24. van Herwaarden AE, Wagenaar E, Karnekamp B, Merino G, Jonker JW, Schinkel AH. Breast cancer resistance protein (Bcrp1/Abcg2) reduces systemic exposure of the dietary carcinogens aflatoxin B1, IQ and Trp-P-1 but also mediates their secretion into breast milk. Carcinogenesis. 2006;27(1):123-30. doi: 10.1093/carcin/bgi176. PubMed PMID: 16000399.

25. van Herwaarden AE, Wagenaar E, Merino G, Jonker JW, Rosing H, Beijnen JH, et al. Multidrug transporter ABCG2/breast cancer resistance protein secretes riboflavin (vitamin B2) into milk. Mol Cell Biol. 2007;27(4):1247-53. doi: 10.1128/MCB.01621-06. PubMed PMID: 17145775; PubMed Central PMCID: PMCPMC1800714.

26. Wang F, Liang YJ, Wu XP, Chen LM, To KK, Dai CL, et al. Prognostic value of the multidrug resistance transporter ABCG2 gene polymorphisms in Chinese patients with de novo acute leukaemia. Eur J Cancer. 2011;47(13):1990-9. doi: 10.1016/j.ejca.2011.03.032. PubMed PMID: 21531129.

27. Doyle MJ, Maher TJ, Li Q, Garry MG, Sorrentino BP, Martin CM. Abcg2-Labeled Cells Contribute to Different Cell Populations in the Embryonic and Adult Heart. Stem Cells Dev. 2016;25(3):277-84. doi: 10.1089/scd.2015.0272. PubMed PMID: 26573225; PubMed Central PMCID: PMCPMC4742971.

28. Caetano-Pinto P, Janssen MJ, Gijzen L, Verscheijden L, Wilmer MJ, Masereeuw R. Fluorescence-Based Transport Assays

Revisited in a Human Renal Proximal Tubule Cell Line. Mol Pharm. 2016;13(3):933-44. doi: 10.1021/acs.molpharmaceut.5b00821. PubMed PMID: 26871298.

29. Wishart DS, Knox C, Guo AC, Shrivastava S, Hassanali M, Stothard P, et al. DrugBank: a comprehensive resource for in silico drug discovery and exploration. Nucleic Acids Res. 2006;34(Database issue):D668-72. doi: 10.1093/nar/gkj067. PubMed PMID: 16381955; PubMed Central PMCID: PMCPMC1347430.

30. Conde-Estevez D. Targeted cancer therapy: interactions with other medicines. Clin Transl Oncol. 2016. doi: 10.1007/s12094-016-1509-x. PubMed PMID: 27112938.

31. Mutsaers HA, van den Heuvel LP, Ringens LH, Dankers AC, Russel FG, Wetzels JF, et al. Uremic toxins inhibit transport by breast cancer resistance protein and multidrug resistance protein 4 at clinically relevant concentrations. PLoS One. 2011;6(4):e18438. doi: 10.1371/journal.pone.0018438. PubMed PMID: 21483698; PubMed Central PMCID: PMCPMC3070735.

32. Dankers AC, Mutsaers HA, Dijkman HB, van den Heuvel LP, Hoenderop JG, Sweep FC, et al. Hyperuricemia influences tryptophan metabolism via inhibition of multidrug resistance protein 4 (MRP4) and breast cancer resistance protein (BCRP). Biochim Biophys Acta. 2013;1832(10):1715-22. doi: 10.1016/j.bbadis.2013.05.002. PubMed PMID: 23665398.

33. Mutsaers HA, Caetano-Pinto P, Seegers AE, Dankers AC, van den Broek PH, Wetzels JF, et al. Proximal tubular efflux transporters involved in renal excretion of p-cresyl sulfate and p-cresyl glucuronide: Implications for chronic kidney disease pathophysiology. Toxicol In Vitro. 2015;29(7):1868-77. doi: 10.1016/j.tiv.2015.07.020. PubMed PMID: 26216510.

34. Jansen JF, M.; Wilmer, M.J.; Peters, J.G.; Kreuser, U.M.; Bilos, A.; Mensink, R.A.; Boltje, T.J.; Stamatialis, D.; Wetzels, J.F.; van den Heuvel, L.P.; Hoenderop, J.G.; Masereeuw, R. Bioengineered kidney tubules efficiently excrete uremic toxins. Sci Rep. 2016;provisionally accepted.

35. Natarajan K, Xie Y, Baer MR, Ross DD. Role of breast cancer resistance protein (BCRP/ABCG2) in cancer drug resistance. Biochem Pharmacol. 2012;83(8):1084-103. doi: 10.1016/j.bcp.2012.01.002. PubMed PMID: 22248732; PubMed Central PMCID: PMCPMC3307098.

36. Nakanishi T, Shiozawa K, Hassel BA, Ross DD. Complex interaction of BCRP/ABCG2 and imatinib in BCR-ABL-expressing cells: BCRP-mediated resistance to imatinib is attenuated by imatinib-induced reduction of BCRP expression. Blood. 2006;108(2):678-84. doi: 10.1182/blood-2005-10-4020. PubMed PMID: 16543472.

37. Chu TS, Chen JS, Lopez JP, Pardo FS, Aguilera J, Ongkeko WM. Imatinib-mediated inactivation of Akt regulates ABCG2 function in head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg. 2008;134(9):979-84. doi: 10.1001/archotol.134.9.979. PubMed PMID: 18794444.

38. Chun SY, Kwon YS, Nam KS, Kim S. Lapatinib enhances the cytotoxic effects of doxorubicin in MCF-7 tumorspheres by inhibiting the drug efflux function of ABC transporters. Biomed Pharmacother. 2015;72:37-43. doi: 10.1016/j.biopha.2015.03.009. PubMed PMID: 26054673.

39. Pick A, Wiese M. Tyrosine kinase inhibitors influence ABCG2 expression in EGFR-positive MDCK BCRP cells via the PI3K/Akt signaling pathway. ChemMedChem. 2012;7(4):650-62. doi: 10.1002/cmdc.201100543. PubMed PMID: 22354538.

40. Zhang W, Ding W, Chen Y, Feng M, Ouyang Y, Yu Y, et al. Up-regulation of breast cancer resistance protein plays a role in HER2-mediated chemoresistance through PI3K/Akt and nuclear factor-kappa B signaling pathways in MCF7 breast cancer

cells. Acta Biochim Biophys Sin (Shanghai). 2011;43(8):647-53. doi: 10.1093/abbs/gmr050. PubMed PMID: 21712253.

41. Bram EE, Stark M, Raz S, Assaraf YG. Chemotherapeutic drug-induced ABCG2 promoter demethylation as a novel mechanism of acquired multidrug resistance. Neoplasia. 2009;11(12):1359-70. PubMed PMID: 20019844; PubMed Central PMCID: PMCPMC2794517.

42. Oh GS, Kim HJ, Shen A, Lee SB, Khadka D, Pandit A, et al. Cisplatin-induced Kidney Dysfunction and Perspectives on Improving Treatment Strategies. Electrolyte Blood Press. 2014;12(2):55-65. doi: 10.5049/EBP.2014.12.2.55. PubMed PMID: 25606044; PubMed Central PMCID: PMCPMC4297704.

43. Herraez E, Gonzalez-Sanchez E, Vaquero J, Romero MR, Serrano MA, Marin JJ, et al. Cisplatin-induced chemoresistance in colon cancer cells involves FXR-dependent and FXR-independent up-regulation of ABC proteins. Mol Pharm. 2012;9(9):2565-76. doi: 10.1021/mp300178a. PubMed PMID: 22800197.

44. Nagai S, Takenaka K, Nachagari D, Rose C, Domoney K, Sun D, et al. Deoxycytidine kinase modulates the impact of the ABC transporter ABCG2 on clofarabine cytotoxicity. Cancer Res. 2011;71(5):1781-91. doi: 10.1158/0008-5472.CAN-10-1919. PubMed PMID: 21245102; PubMed Central PMCID: PMCPMC3531552.

45. Nakanishi T, Ross DD. Breast cancer resistance protein (BCRP/ABCG2): its role in multidrug resistance and regulation of its gene expression. Chin J Cancer. 2012;31(2):73-99. doi: 10.5732/cjc.011.10320. PubMed PMID: 22098950; PubMed Central PMCID: PMCPMC3777471.

46. Wang X, Campos CR, Peart JC, Smith LK, Boni JL, Cannon RE, et al. Nrf2 upregulates ATP binding cassette transporter expression and activity at the blood-brain and blood-spinal cord barriers. J Neurosci. 2014;34(25):8585-93. doi: 10.1523/JNEUROSCI.2935-13.2014. PubMed PMID: 24948812; PubMed Central PMCID: PMCPMC4061395.

47. Singh A, Wu H, Zhang P, Happel C, Ma J, Biswal S. Expression of ABCG2 (BCRP) is regulated by Nrf2 in cancer cells that confers side population and chemoresistance phenotype. Mol Cancer Ther. 2010;9(8):2365-76. doi: 10.1158/1535-7163.MCT-10-0108. PubMed PMID: 20682644; PubMed Central PMCID: PMCPMC2955865.

48. Jeong HS, Ryoo IG, Kwak MK. Regulation of the expression of renal drug transporters in KEAP1-knockdown human tubular cells. Toxicol In Vitro. 2015;29(5):884-92. doi: 10.1016/j.tiv.2015.03.013. PubMed PMID: 25841332.

49. Bai X, Chen Y, Hou X, Huang M, Jin J. Emerging role of NRF2 in chemoresistance by regulating drug-metabolizing enzymes and efflux transporters. Drug Metab Rev. 2016:1-27. doi: 10.1080/03602532.2016.1197239. PubMed PMID: 27320238.

50. Miller DS. Regulation of ABC transporters blood-brain barrier: the good, the bad, and the ugly. Adv Cancer Res. 2015;125:43-70. doi: 10.1016/bs.acr.2014.10.002. PubMed PMID: 25640266.

51. Hessel S, Lampen A, Seidel A. Polycyclic aromatic hydrocarbons in food--efflux of the conjugated biomarker 1-hydroxypyrene is mediated by Breast Cancer Resistance Protein (ABCG2) in human intestinal Caco-2 cells. Food Chem Toxicol. 2013;62:797-804. doi: 10.1016/j.fct.2013.10.007. PubMed PMID: 24140465.

52. Dankers AC, Sweep FC, Pertijs JC, Verweij V, van den Heuvel JJ, Koenderink JB, et al. Localization of breast cancer resistance protein (Bcrp) in endocrine organs and inhibition of its transport activity by steroid hormones. Cell Tissue Res. 2012;349(2):551-

63. doi: 10.1007/s00441-012-1417-5. PubMed PMID: 22581381; PubMed Central PMCID: PMCPMC3414710.

53. Hartz AM, Madole EK, Miller DS, Bauer B. Estrogen receptor beta signaling through phosphatase and tensin homolog/phosphoinositide 3-kinase/Akt/glycogen synthase kinase 3 down-regulates blood-brain barrier breast cancer resistance protein. J Pharmacol Exp Ther. 2010;334(2):467-76. doi: 10.1124/jpet.110.168930. PubMed PMID: 20460386; PubMed Central PMCID: PMCPMC2913769.

54. Komeili-Movahhed T, Fouladdel S, Barzegar E, Atashpour S, Hossein Ghahremani M, Nasser Ostad S, et al. PI3K/Akt inhibition and down-regulation of BCRP re-sensitize MCF7 breast cancer cell line to mitoxantrone chemotherapy. Iran J Basic Med Sci. 2015;18(5):472-7. PubMed PMID: 26124933; PubMed Central PMCID: PMCPMC4475655.

55. Goler-Baron V, Sladkevich I, Assaraf YG. Inhibition of the PI3K-Akt signaling pathway disrupts ABCG2-rich extracellular vesicles and overcomes multidrug resistance in breast cancer cells. Biochem Pharmacol. 2012;83(10):1340-8. doi: 10.1016/j.bcp.2012.01.033. PubMed PMID: 22342288.

56. Galetti M, Petronini PG, Fumarola C, Cretella D, La Monica S, Bonelli M, et al. Effect of ABCG2/BCRP Expression on Efflux and Uptake of Gefitinib in NSCLC Cell Lines. PLoS One. 2015;10(11):e0141795. doi: 10.1371/journal.pone.0141795. PubMed PMID: 26536031; PubMed Central PMCID: PMCPMC4633241.

57. Hoque MT, Shah A, More V, Miller DS, Bendayan R. In vivo and ex vivo regulation of breast cancer resistant protein (Bcrp) by peroxisome proliferator-activated receptor alpha (Pparalpha) at the blood-brain barrier. J Neurochem. 2015;135(6):1113-22. doi: 10.1111/jnc.13389. PubMed PMID: 26465636; PubMed Central PMCID: PMCPMC4729455.

58. Poller B, Drewe J, Krahenbuhl S, Huwyler J, Gutmann H. Regulation of BCRP (ABCG2) and P-glycoprotein (ABCB1) by cytokines in a model of the human blood-brain barrier. Cell Mol Neurobiol. 2010;30(1):63-70. doi: 10.1007/s10571-009-9431-1. PubMed PMID: 19629677.

59. Hegedus C, Telbisz A, Hegedus T, Sarkadi B, Ozvegy-Laczka C. Lipid regulation of the ABCB1 and ABCG2 multidrug transporters. Adv Cancer Res. 2015;125:97-137. doi: 10.1016/bs.acr.2014.10.004. PubMed PMID: 25640268.

60. Drori S, Eytan GD, Assaraf YG. Potentiation of anticancer-drug cytotoxicity by multidrug-resistance chemosensitizers involves alterations in membrane fluidity leading to increased membrane permeability. Eur J Biochem. 1995;228(3):1020-9. PubMed PMID: 7737146.

61. Assaraf YG, Borgnia MJ. Probing the interaction of the multidrug-resistance phenotype with the polypeptide ionophore gramicidin D via functional channel formation. Eur J Biochem. 1994;222(3):813-24. PubMed PMID: 7517866.

62. Borgnia MJ, Eytan GD, Assaraf YG. Competition of hydrophobic peptides, cytotoxic drugs, and chemosensitizers on a common P-glycoprotein pharmacophore as revealed by its ATPase activity. J Biol Chem. 1996;271(6):3163-71. PubMed PMID: 8621716.

63. Hu M, To KK, Mak VW, Tomlinson B. The ABCG2 transporter and its relations with the pharmacokinetics, drug interaction and lipid-lowering effects of statins. Expert Opin Drug Metab Toxicol. 2011;7(1):49-62. doi: 10.1517/17425255.2011.538383. PubMed PMID: 21091277.

64. Pal A, Mehn D, Molnar E, Gedey S, Meszaros P, Nagy T, et al. Cholesterol potentiates ABCG2 activity in a heterologous expression system: improved in vitro model to study function of human ABCG2. J

Pharmacol Exp Ther. 2007;321(3):1085-94. doi: 10.1124/jpet.106.119289. PubMed PMID: 17347325.

65. Lu H, Klaassen C. Gender differences in mRNA expression of ATP-binding cassette efflux and bile acid transporters in kidney, liver, and intestine of 5/6 nephrectomized rats. Drug Metab Dispos. 2008;36(1):16-23. doi: 10.1124/dmd.107.014845. PubMed PMID: 17855625.

66. Arrigoni E, Galimberti S, Petrini M, Danesi R, Di Paolo A. ATP-binding cassette transmembrane transporters and their epigenetic control in cancer: an overview. Expert Opin Drug Metab Toxicol. 2016:1-14. doi: 10.1080/17425255.2016.1215423. PubMed PMID: 27459275.

67. Cosmai L, Gallieni M, Porta C. Renal toxicity of anticancer agents targeting HER2 and EGFR. J Nephrol. 2015;28(6):647-57. doi: 10.1007/s40620-015-0226-9. PubMed PMID: 26341657.

68. Wilmer MJ, Ng CP, Lanz HL, Vulto P, Suter-Dick L, Masereeuw R. Kidney-on-a-Chip Technology for Drug-Induced Nephrotoxicity Screening. Trends Biotechnol. 2016;34(2):156-70. doi: 10.1016/j.tibtech.2015.11.001. PubMed PMID: 26708346.

69. Emami Riedmaier A, Nies AT, Schaeffeler E, Schwab M. Organic anion transporters and their implications in pharmacotherapy. Pharmacol Rev. 2012;64(3):421-49. doi: 10.1124/pr.111.004614. PubMed PMID: 22457399.

70. Crean D, Bellwon P, Aschauer L, Limonciel A, Moenks K, Hewitt P, et al. Development of an in vitro renal epithelial disease state model for xenobiotic toxicity testing. Toxicol In Vitro. 2015;30(1 Pt A):128-37. doi: 10.1016/j.tiv.2014.11.015. PubMed PMID: 25536518.

71. de Arriba G, Calvino M, Benito S, Parra T. Cyclosporine A-induced apoptosis in renal tubular cells is related to oxidative damage and mitochondrial fission. Toxicol Lett. 2013;218(1):30-8. doi: 10.1016/j.toxlet.2013.01.007. PubMed PMID: 23347876.

72. Awdishu L, Joy MS. Role of Pharmacogenomics in Kidney Disease and Injury. Adv Chronic Kidney Dis. 2016;23(2):106-19. doi: 10.1053/j.ackd.2016.01.018. PubMed PMID: 26979149.

73. De Mattia E, Cecchin E, Toffoli G. Pharmacogenomics of intrinsic and acquired pharmacoresistance in colorectal cancer: Toward targeted personalized therapy. Drug Resist Updat. 2015;20:39-70. doi: 10.1016/j.drup.2015.05.003. PubMed PMID: 26027741.

74. Gillis NK, McLeod HL. The pharmacogenomics of drug resistance to protein kinase inhibitors. Drug Resist Updat. 2016;28:28-42. doi: 10.1016/j.drup.2016.06.008. PubMed PMID: 27620953; PubMed Central PMCID: PMCPMC5022787.

75. Dehghan A, Kottgen A, Yang Q, Hwang SJ, Kao WL, Rivadeneira F, et al. Association of three genetic loci with uric acid concentration and risk of gout: a genome-wide association study. Lancet. 2008;372(9654):1953-61. doi: 10.1016/S0140-6736(08)61343-4. PubMed PMID: 18834626; PubMed Central PMCID: PMCPMC2803340.

76. Ichida K, Matsuo H, Takada T, Nakayama A, Murakami K, Shimizu T, et al. Decreased extra-renal urate excretion is a common cause of hyperuricemia. Nat Commun. 2012;3:764. doi: 10.1038/ncomms1756. PubMed PMID: 22473008; PubMed Central PMCID: PMCPMC3337984.

77. Woodward OM, Kottgen A, Coresh J, Boerwinkle E, Guggino WB, Kottgen M. Identification of a urate transporter, ABCG2, with a common functional polymorphism causing gout. Proc Natl Acad Sci U S A. 2009;106(25):10338-42. doi: 10.1073/pnas.0901249106. PubMed PMID: 19506252; PubMed Central PMCID: PMCPMC2700910.

78. Yang Q, Kottgen A, Dehghan A, Smith AV, Glazer NL, Chen MH, et al. Multiple genetic loci influence serum urate levels and their relationship with gout and cardiovascular disease risk factors. Circ Cardiovasc Genet. 2010;3(6):523-30. doi: 10.1161/CIRCGENETICS.109.934455. PubMed PMID: 20884846; PubMed Central PMCID: PMCPMC3371395.

79. Choi HK, Mount DB, Reginato AM, American College of P, American Physiological S. Pathogenesis of gout. Ann Intern Med. 2005;143(7):499-516. PubMed PMID: 16204163.

80. Matsuo H, Ichida K, Takada T, Nakayama A, Nakashima H, Nakamura T, et al. Common dysfunctional variants in ABCG2 are a major cause of early-onset gout. Sci Rep. 2013;3:2014. doi: 10.1038/srep02014. PubMed PMID: 23774753; PubMed Central PMCID: PMCPMC3684804.

81. Duranton F, Cohen G, De Smet R, Rodriguez M, Jankowski J, Vanholder R, et al. Normal and pathologic concentrations of uremic toxins. J Am Soc Nephrol. 2012;23(7):1258-70. doi: 10.1681/ASN.2011121175. PubMed PMID: 22626821; PubMed Central PMCID: PMCPMC3380651.

82. Noguchi K, Katayama K, Sugimoto Y. Human ABC transporter ABCG2/BCRP expression in chemoresistance: basic and clinical perspectives for molecular cancer therapeutics. Pharmgenomics Pers Med. 2014;7:53-64. doi: 10.2147/PGPM.S38295. PubMed PMID: 24523596; PubMed Central PMCID: PMCPMC3921828.

83. Miura Y, Imamura CK, Fukunaga K, Katsuyama Y, Suyama K, Okaneya T, et al. Sunitinib-induced severe toxicities in a Japanese patient with the ABCG2 421 AA genotype. BMC Cancer. 2014;14:964. doi: 10.1186/1471-2407-14-964. PubMed PMID: 25515134; PubMed Central PMCID: PMCPMC4301945.

84. Mizuno T, Fukudo M, Terada T, Kamba T, Nakamura E, Ogawa O, et al. Impact of genetic variation in breast cancer resistance protein (BCRP/ABCG2) on sunitinib pharmacokinetics. Drug Metab Pharmacokinet. 2012;27(6):631-9. PubMed PMID: 22673043.

85. Sparreboom A, Gelderblom H, Marsh S, Ahluwalia R, Obach R, Principe P, et al. Diflomotecan pharmacokinetics in relation to ABCG2 421C>A genotype. Clin Pharmacol Ther. 2004;76(1):38-44. doi: 10.1016/j.clpt.2004.03.003. PubMed PMID: 15229462.

86. Kumar N, Shirali AC. What is the best therapy for toxicity in the setting of methotrexate-associated acute kidney injury: high-flux hemodialysis or carboxypeptidase G2? Semin Dial. 2014;27(3):226-8. doi: 10.1111/sdi.12220. PubMed PMID: 24620824.

87. Yanagimachi M, Goto H, Kaneko T, Naruto T, Sasaki K, Takeuchi M, et al. Influence of pre-hydration and pharmacogenetics on plasma methotrexate concentration and renal dysfunction following high-dose methotrexate therapy. Int J Hematol. 2013;98(6):702-7. doi: 10.1007/s12185-013-1464-z. PubMed PMID: 24241962.

88. Chen ZS, Robey RW, Belinsky MG, Shchaveleva I, Ren XQ, Sugimoto Y, et al. Transport of methotrexate, methotrexate polyglutamates, and 17beta-estradiol 17-(beta-D-glucuronide) by ABCG2: effects of acquired mutations at R482 on methotrexate transport. Cancer Res. 2003;63(14):4048-54. PubMed PMID: 12874005.

89. Ejendal KF, Diop NK, Schweiger LC, Hrycyna CA. The nature of amino acid 482 of human ABCG2 affects substrate transport and ATP hydrolysis but not substrate binding. Protein Sci. 2006;15(7):1597-607. doi: 10.1110/ps.051998406. PubMed PMID: 16815914; PubMed Central PMCID: PMCPMC2242565.

90. Haider AJ, Cox MH, Jones N, Goode AJ, Bridge KS, Wong K, et al. Identification of residues in ABCG2 affecting protein trafficking and drug transport, using co-evolutionary analysis of ABCG sequences.

Biosci Rep. 2015;35(4). doi: 10.1042/BSR20150150. PubMed PMID: 26294421; PubMed Central PMCID: PMCPMC4613716.

91. Pollex EK, Anger G, Hutson J, Koren G, Piquette-Miller M. Breast cancer resistance protein (BCRP)-mediated glyburide transport: effect of the C421A/Q141K BCRP single-nucleotide polymorphism. Drug Metab Dispos. 2010;38(5):740-4. doi: 10.1124/dmd.109.030791. PubMed PMID: 20159988.

92. Morisaki K, Robey RW, Ozvegy-Laczka C, Honjo Y, Polgar O, Steadman K, et al. Single nucleotide polymorphisms modify the transporter activity of ABCG2. Cancer Chemother Pharmacol. 2005;56(2):161-72. doi: 10.1007/s00280-004-0931-x. PubMed PMID: 15838659.

93. Zamber CP, Lamba JK, Yasuda K, Farnum J, Thummel K, Schuetz JD, et al. Natural allelic variants of breast cancer resistance protein (BCRP) and their relationship to BCRP expression in human intestine. Pharmacogenetics. 2003;13(1):19-28. PubMed PMID: 12544509.

94. Imai Y, Nakane M, Kage K, Tsukahara S, Ishikawa E, Tsuruo T, et al. C421A polymorphism in the human breast cancer resistance protein gene is associated with low expression of Q141K protein and low-level drug resistance. Mol Cancer Ther. 2002;1(8):611-6. PubMed PMID: 12479221.

95. Kondo C, Suzuki H, Itoda M, Ozawa S, Sawada J, Kobayashi D, et al. Functional analysis of SNPs variants of BCRP/ABCG2. Pharm Res. 2004;21(10):1895-903. PubMed PMID: 15553238.

96. Mizuarai S, Aozasa N, Kotani H. Single nucleotide polymorphisms result in impaired membrane localization and reduced atpase activity in multidrug transporter ABCG2. Int J Cancer. 2004;109(2):238-46. doi: 10.1002/ijc.11669. PubMed PMID: 14750175.

97. Jansen J, Schophuizen CM, Wilmer MJ, Lahham SH, Mutsaers HA, Wetzels JF, et al. A morphological and functional comparison of proximal tubule cell lines established from human urine and kidney tissue. Exp Cell Res. 2014;323(1):87-99. doi: 10.1016/j.yexcr.2014.02.011. PubMed PMID: 24560744.

98. Freedman BS, Brooks CR, Lam AQ, Fu H, Morizane R, Agrawal V, et al. Modelling kidney disease with CRISPR-mutant kidney organoids derived from human pluripotent epiblast spheroids. Nat Commun. 2015;6:8715. doi: 10.1038/ncomms9715. PubMed PMID: 26493500; PubMed Central PMCID: PMCPMC4620584.

99. Poonkuzhali B, Lamba J, Strom S, Sparreboom A, Thummel K, Watkins P, et al. Association of breast cancer resistance protein/ABCG2 phenotypes and novel promoter and intron 1 single nucleotide polymorphisms. Drug Metab Dispos. 2008;36(4):780-95. doi: 10.1124/dmd.107.018366. PubMed PMID: 18180275.

100. Tanaka Y, Slitt AL, Leazer TM, Maher JM, Klaassen CD. Tissue distribution and hormonal regulation of the breast cancer resistance protein (Bcrp/Abcg2) in rats and mice. Biochem Biophys Res Commun. 2005;326(1):181-7. doi: 10.1016/j.bbrc.2004.11.012. PubMed PMID: 15567169.

101. Soldin OP, Mattison DR. Sex differences in pharmacokinetics and pharmacodynamics. Clin Pharmacokinet. 2009;48(3):143-57. doi: 10.2165/00003088-200948030-00001. PubMed PMID: 19385708; PubMed Central PMCID: PMCPMC3644551.

102. Gal Z, Hegedus C, Szakacs G, Varadi A, Sarkadi B, Ozvegy-Laczka C. Mutations of the central tyrosines of putative cholesterol recognition amino acid consensus (CRAC) sequences modify folding, activity, and sterol-sensing of the human ABCG2 multidrug transporter. Biochim Biophys Acta. 2015;1848(2):477-87. doi: 10.1016/j.bbamem.2014.11.006. PubMed PMID: 25445676.

103. Martin V, Sanchez-Sanchez AM, Herrera F, Gomez-Manzano C, Fueyo J, Alvarez-Vega MA, et al. Melatonin-induced methylation

of the ABCG2/BCRP promoter as a novel mechanism to overcome multidrug resistance in brain tumour stem cells. Br J Cancer. 2013;108(10):2005-12. doi: 10.1038/bjc.2013.188. PubMed PMID: 23632480; PubMed Central PMCID: PMCPMC3670480.

104. Guengerich FP. Mechanisms of drug toxicity and relevance to pharmaceutical development. Drug Metab Pharmacokinet. 2011;26(1):3-14. PubMed PMID: 20978361; PubMed Central PMCID: PMCPMC4707670.

105. Allegaert K, Peeters MY, Verbesselt R, Tibboel D, Naulaers G, de Hoon JN, et al. Inter-individual variability in propofol pharmacokinetics in preterm and term neonates. Br J Anaesth. 2007;99(6):864-70. doi: 10.1093/bja/aem294. PubMed PMID: 17965417.

106. Schreuder MF, Bueters RR, Huigen MC, Russel FG, Masereeuw R, van den Heuvel LP. Effect of drugs on renal development. Clin J Am Soc Nephrol. 2011;6(1):212-7. doi: 10.2215/CJN.04740510. PubMed PMID: 21071516.

107. Klein SL, Schiebinger L, Stefanick ML, Cahill L, Danska J, de Vries GJ, et al. Opinion: Sex inclusion in basic research drives discovery. Proc Natl Acad Sci U S A. 2015;112(17):5257-8. doi: 10.1073/pnas.1502843112. PubMed PMID: 25902532; PubMed Central PMCID: PMCPMC4418862.

108. Prendergast BJ, Onishi KG, Zucker I. Female mice liberated for inclusion in neuroscience and biomedical research. Neurosci Biobehav Rev. 2014;40:1-5. doi: 10.1016/j.neubiorev.2014.01.001. PubMed PMID: 24456941.

109. Sorge RE, Martin LJ, Isbester KA, Sotocinal SG, Rosen S, Tuttle AH, et al. Olfactory exposure to males, including men, causes stress and related analgesia in rodents. Nat Methods. 2014;11(6):629-32. doi: 10.1038/nmeth.2935. PubMed PMID: 24776635.

110. Mahmoud R, Wainwright SR, Galea LA. Sex hormones and adult hippocampal neurogenesis: Regulation, implications, and potential mechanisms. Front Neuroendocrinol. 2016. doi: 10.1016/j.yfrne.2016.03.002. PubMed PMID: 26988999.

111. Sabolic I, Asif AR, Budach WE, Wanke C, Bahn A, Burckhardt G. Gender differences in kidney function. Pflugers Arch. 2007;455(3):397-429. doi: 10.1007/s00424-007-0308-1. PubMed PMID: 17638010.

112. Ullah H, Jahan S, Ain QU, Shaheen G, Ahsan N. Effect of bisphenol S exposure on male reproductive system of rats: A histological and biochemical study. Chemosphere. 2016;152:383-91. doi: 10.1016/j.chemosphere.2016.02.125. PubMed PMID: 26994432.

113. Hecking M, Bieber BA, Ethier J, Kautzky-Willer A, Sunder-Plassmann G, Saemann MD, et al. Sex-specific differences in hemodialysis prevalence and practices and the male-to-female mortality rate: the Dialysis Outcomes and Practice Patterns Study (DOPPS). PLoS Med. 2014;11(10):e1001750. doi: 10.1371/journal.pmed.1001750. PubMed PMID: 25350533; PubMed Central PMCID: PMCPMC4211675.

114. Cook MB, McGlynn KA, Devesa SS, Freedman ND, Anderson WF. Sex disparities in cancer mortality and survival. Cancer Epidemiol Biomarkers Prev. 2011;20(8):1629-37. doi: 10.1158/1055-9965.EPI-11-0246. PubMed PMID: 21750167; PubMed Central PMCID: PMCPMC3153584.

115. Lameire N, Vanholder R, Van Biesen W, Benoit D. Acute kidney injury in critically ill cancer patients: an update. Crit Care. 2016;20(1):209. doi: 10.1186/s13054-016-1382-6. PubMed PMID: 27480256; PubMed Central PMCID: PMCPMC4969681.

116. Wong G, Staplin N, Emberson J, Baigent C, Turner R, Chalmers J, et al. Chronic kidney disease and the risk of cancer: an individual patient data meta-analysis of 32,057 participants from six prospective studies. BMC Cancer. 2016;16:488. doi: 10.1186/s12885-016-2532-6. PubMed PMID: 27421889; PubMed Central PMCID: PMCPMC4947287.

117. Jha V, Garcia-Garcia G, Iseki K, Li Z, Naicker S, Plattner B, et al. Chronic kidney disease: global dimension and perspectives. Lancet. 2013;382(9888):260-72. doi: 10.1016/S0140-6736(13)60687-X. PubMed PMID: 23727169.

118. Fallon JK, Smith PC, Xia CQ, Kim MS. Quantification of Four Efflux Drug Transporters in Liver and Kidney Across Species Using Targeted Quantitative Proteomics by Isotope Dilution NanoLC-MS/MS. Pharm Res. 2016;33(9):2280-8. doi: 10.1007/s11095-016-1966-5. PubMed PMID: 27356525.

119. Uchida Y, Toyohara T, Ohtsuki S, Moriyama Y, Abe T, Terasaki T. Quantitative Targeted Absolute Proteomics for 28 Transporters in Brush-Border and Basolateral Membrane Fractions of Rat Kidney. J Pharm Sci. 2016;105(2):1011-6. doi: 10.1002/jps.24645. PubMed PMID: 26367854.

120. Leung KC, Tonelli M, James MT. Chronic kidney disease following acute kidney injury-risk and outcomes. Nat Rev Nephrol. 2013;9(2):77-85. doi: 10.1038/nrneph.2012.280. PubMed PMID: 23247572.

121. Atkinson AJ, Jr., Huang SM. Nephropharmacology: drugs and the kidney. Clin Pharmacol Ther. 2009;86(5):453-6. doi: 10.1038/clpt.2009.191. PubMed PMID: 19844216.

122. Mao Q, Unadkat JD. Role of the breast cancer resistance protein (BCRP/ABCG2) in drug transport--an update. AAPS J. 2015;17(1):65-82. doi: 10.1208/s12248-014-9668-6. PubMed PMID: 25236865; PubMed Central PMCID: PMCPMC4287283.

123. Sanchez-Romero N, Schophuizen CM, Gimenez I, Masereeuw R. In vitro systems to study nephropharmacology: 2D versus 3D models. Eur J Pharmacol. 2016;790:36-45. doi: 10.1016/j.ejphar.2016.07.010. PubMed PMID: 27395797.

124. Chuah JK, Zink D. Stem cell-derived kidney cells and organoids: Recent breakthroughs and emerging applications. Biotechnol Adv. 2016. doi: 10.1016/j.biotechadv.2016.12.001. PubMed PMID: 28017905.

125. Schutgens F, Verhaar MC, Rookmaaker MB. Pluripotent stem cell-derived kidney organoids: An in vivo-like in vitro technology. Eur J Pharmacol. 2016;790:12-20. doi: 10.1016/j.ejphar.2016.06.059. PubMed PMID: 27375081.

126. Sachs N, Clevers H. Organoid cultures for the analysis of cancer phenotypes. Curr Opin Genet Dev. 2014;24:68-73. doi: 10.1016/j.gde.2013.11.012. PubMed PMID: 24657539.

127. Dallas S, Salphati L, Gomez-Zepeda D, Wanek T, Chen L, Chu X, et al. Generation and Characterization of a Breast Cancer Resistance Protein Humanized Mouse Model. Mol Pharmacol. 2016;89(5):492-504. doi: 10.1124/mol.115.102079. PubMed PMID: 26893303.

128. Scotcher D, Jones C, Posada M, Rostami-Hodjegan A, Galetin A. Key to Opening Kidney for In Vitro-In Vivo Extrapolation Entrance in Health and Disease: Part I: In Vitro Systems and Physiological Data. AAPS J. 2016;18(5):1067-81. doi: 10.1208/s12248-016-9942-x. PubMed PMID: 27365096.

CHAPTER 3

Fluorescence-based transport assays revisited in a human renal proximal tubule cell line

Pedro Caetano-Pinto[1,2], Manoe J. Janssen[1,2]
Linda Gijzen[1], Laurens Verscheijden[1],
Martijn J.G. Wilmer[1], Rosalinde Masereeuw[1,2]

[1]Department of Pharmacology and Toxicology, Radboud university medical center, Radboud Institute for Molecular Life Sciences, Nijmegen, The Netherlands
[2]Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, The Netherlands

Molecular Pharmaceutics. 2016Mar7;13(3):93344.
doi:10.1021/acs.molpharmaceut.5b00821.

ABSTRACT

Apical transport is key in renal function, and the activity of efflux transporters and receptor-mediated endocytosis is pivotal in this process. The conditionally immortalized proximal tubule epithelial cell line (ciPTEC) endogenously expresses these systems. Here, we used ciPTEC to investigate the activity of three major efflux transporters, *viz.* breast cancer resistance protein (BCRP), multidrug resistance protein 4 (MRP4) and P-glycoprotein (P-gp), as well as protein uptake through receptor-mediated endocytosis, using a fluorescence-based setup for transport assays. To this end, cells were exposed to Hoechst33342, chloromethylfluorescein-diacetate (CMFDA) and calcein-AM in presence or absence of model inhibitors for BCRP (KO143), P-gp (PSC833) or MRP's (MK571). Overexpression cell lines MDCKII-BCRP and MDCKII-P-gp were used as positive controls and membrane vesicles over-expressing one transporter were used to determine substrate and inhibitor specificities. Receptor-mediated endocytosis was investigated by determining the intracellular accumulation of fluorescently labeled receptor associated protein (RAP-GST).

In ciPTEC, BCRP and P-gp showed similar expressions and activities while MRP4 was more abundantly expressed. Hoechst33342, GS-MF and calcein are retained in the presence of KO143, MK571 and PSC833, showing clearly redundancy between the transporters. Noteworthy is the fact that both KO143 and MK571 can block BCRP, P-gp and MRP's, while PSC833 appears a potent inhibitor for BCRP and P-gp, but not the MRP's. Furthermore, ciPTEC accumulate RAP-GST in intracellular vesicles in a dose and time dependent manner, which was reduced in megalin-deficient cells.

In conclusion, fluorescent probe-based assays are fast and reproducible in determining apical transport mechanisms, *in vitro*. We demonstrate that typical substrates and inhibitors are not specific for the designated transporters, reflecting the complex interactions that can take place *in vivo.* The set of tools we describe are also compatible with innovative kidney culture models, and allows studying transport mechanisms that are central to drug absorption, disposition and detoxification.

Key words

ABC membrane transporters, receptor-mediated endocytosis, human proximal tubule cells, advanced *in vitro* models, fluorescence functional assays

INTRODUCTION

Kidney proximal tubular epithelial cells (PTEC) play an active role in transporting xenobiotics, including drugs and their metabolic waste products, from the blood into the urine. In addition, PTEC are essential in reabsorbing hormones, proteins, nutrients, electrolytes and drugs from the glomerular filtrate. These processes result in exposure of the PTEC to many potentially harmful compounds, making them an easy target for drug related toxicity. Energy dependent efflux across kidney tubular apical membrane, mediated by members of the ABC (ATP-binding cassette) transporter family, is a key mechanism in the renal clearance and extensively contributes to this tissue's secretory function [1, 2]. The four major types of ABC efflux transporters responsible for urinary excretion are P-glycoprotein (P-gp or *ABCB1*), multidrug resistance proteins 2 and 4 (MRP2 and MRP4; *ABCC2/4*) and breast cancer resistance protein (BCRP or *ABCG2*) [2]. Together, these polyspecific transporters eliminate a great variety of xenobiotics [3, 4]. The substrates can be neutral, anionic or cationic by nature, or anionic after being conjugated to glutathione, glucuronic acid or sulphate. Although there is a significant substrate overlap between the transporters, their binding properties allow for different substrate specificities. BCRP and MRP's have a high affinity for conjugated substrates, while P-gp is extensively described to handle unconjugated as well as cationic xenobiotics [5, 6]. During renal excretion, xenobiotics can also accumulate in proximal tubules and, over time, contribute to nephrotoxicity [7]. Furthermore, interferences with renal excretion can also lead to accumulation in the systemic circulation, subsequently giving rise to other adverse effects [8].

In addition to active secretion, PTEC efficiently reabsorb compounds from the glomerular filtrate, recovering low molecular weight proteins and important nutrients, vitamins and hormones bound to carrier proteins [9, 10]. These compounds bind with high affinity to megalin complexes on the apical membrane of the PTEC and are internalized through receptor-mediated endocytosis. Interference with megalin-mediated endocytosis consequently leads to systemic loss of nutrients and vitamins as well as increased levels of protein in the urine. Urinary proteins directly contribute to chronic tubulointerstitial damage by inducing proinflammatory and profibrotic processes, ultimately resulting in progressive renal damage [11, 12]. It is therefore important to determine which compounds affect PTEC function and protein reabsorption.

Information on ABC transporter function was largely retrieved from overexpression cell models or vesicles derived from such cell lines [13-15]. However, in these models only one or a few individual transporter(s) can be expressed, which is far from the physiological situation [16-18]. Moreover, regulatory pathways towards the transporters cannot or only partially be investigated. In addition, the megalin transport mechanism has been studied mainly in non-human derived cells, such as opossum kidney (OK) and the rat yolk sac cells (BN16)[19, 20]. To overcome these limitations, the conditionally immortalized human proximal tubule cell line (ciPTEC) has proven to be a valuable *in vitro* tool to study

several pharmacological aspects of renal clearance [21-24]. Endogenous expression of the ABC efflux pumps and the endocytosis machinery, together with relevant metabolic enzymes, allows for an integrated approach on studying the effect of compounds on PTEC function. A popular strategy to evaluate xenobiotic handling in *in vitro* systems relies on fluorescent probes that are substrates for certain transporters or precursors that acquire fluorescence by metabolic activity [25, 26]. Previous studies have shown that Hoechst33342 can be used to study the activity of BCRP and P-gp, whereas calcein-AM is widely used to assess P-gp function. Furthermore, we and others previously demonstrated 5-chloromethylfluorescein-diacetate (CMFDA) to become an MRP's substrate after hydrolysis and glutathione conjugation [27, 28]. These compounds have in common that they can easily pass the plasma membrane by diffusion. When combining these substrates with efflux modulators, either competitors or non-competitive inhibitors, the intracellular fluorescence increases due to substrate retention [29]. This accumulation can be measured and correlated to transport activity. In the case of endocytosis, receptor associated protein (RAP) is efficiently bound to megalin and internalized as a complex, which is then quantified and used to evaluate defects in protein reabsorption *in vitro* [30, 31].

The aim of the present study was to investigate the activity of multiple transport systems that act in concert at the proximal tubule apical membrane, reflecting the complex interactions that can take place *in vivo, by* using fluorescent probes that accumulate intracellularly upon inhibition.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma (Zwijndrecht, The Netherlands) unless stated otherwise. [6, 7'-^{3}H (*n*)]-estradiol 17 β-D-glucuronide ([^{3}H]- $H_2$17βD-G) and [6', 7'-^{3}H (*n*)]-estrone-sulfate ammonium salt ([^{3}H]-E1S) were obtained from Perkin Elmer (Groningen, The Netherlands). Stock solutions of all compounds used for transport assays were prepared according to specification in either dimethyl sulfoxide (DMSO) or dH_2O. RAP-GST was kindly provided by De Matteis (TIGEM, Napels, Italy) and produced and purified as described previously [32].

Cell lines and cell culture

The ciPTEC line was derived from a healthy donor, as described previously [23, 33], and the megalin-deficient ciPTEC from a Dent's disease patient (with CLC-5 mutation c.del132-241 resulting in low-molecular-weight proteinuria, hypercalciuria, nephrolithiasis, and renal failure) was kindly provided by Prof. R. Thakker, University of Oxford, UK [24]. Cells were cultured in phenol red free DMEM/F12 (Invitrogen, Breda, The Netherlands) supplemented with 10% (v/v) FCS (MP Biomedicals, Uden, The Netherlands), containing insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml),

epithelial growth factor (10 ng/ml), and tri-iodothyronine (40 pg/ml), without penicillin / streptomycin (pen/strep). ciPTEC assays were performed with cells seeded at a density of 48,000 cells per cm^2 in 96 well plates (Costar 3614, Corning, NY, USA) cultured for 24 h at 33ºC and matured for 7 days at 37ºC. Cells were sub-cultured at a dilution of 1:3 to 1:6 at 33°C and experiments performed between passages 30 and 40. The Madin-Darby canine kidney (MDCKII) cells stably overexpressing BCRP or P-gp [34, 35], were kindly provided by Dr. A. Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands) and Dr. M. Gottesman (Laboratory of Cell Biology, National Cancer Institute, USA), respectively. MDCKII cells were cultured in DMEM (Invitrogen, Breda, The Netherlands) supplemented with 10% (v/v) FCS (MP Biomedicals, Uden, The Netherlands) and 1% pen/strep, 5 units/ml (v/v). MDCK II assays were performed with cells seeded at a density of 30,000 cells per cm^2 and cultured until confluence at 37°C. All MDCK cells lines were sub-cultured at a dilution of 1:3 at 37°C in a 5% CO_2 atmosphere.

Fluorescent based functional assays in a 96 well plate set-up

The functional activity of BCRP, MRP4 and P-gp in ciPTEC, MDCKII-BCRP or MDCKII-P-gp cells was evaluated using fluorescent substrates that accumulate intracellularly upon inhibition of the efflux transporters. Hoechst33342, calcein-AM (Life Technologies, Carlsbad, CA, USA) and CMFDA (Life Technologies, Carlsbad, CA, USA) were used to evaluate transporter activity. The inhibitors KO143, MK571 and PSC833 (purchased from Tocris, Bristol, UK) were used as typical inhibitors for BCRP, MRP4 and P-gp, respectively. A two fold dilution range was made for the different inhibitors starting at concentrations of 15 µM for KO143, 50 µM for MK571 and 6 µM for PSC833. To test for Hoechst retention, the cells were incubated with 1.25 µM Hoechst in combination with a gradient of each of the inhibitors for 30 min at 37°C. Subsequently, cells were washed with ice-cold Krebs-Henseleit buffer and fluorescence was measured immediately after (appropriate filter settings; wavelengths: excitation-350nm, emission-460nm). To determine GS-MF retention, the cells were incubated with 1.25 µM CMFDA in combination with a gradient of each of the inhibitors for 30 min, washed and kept for an additional 30 min at 37°C before fluorescence was measured (appropriate filter settings; wavelengths: excitation-492nm, emission-517nm). Calcein retention was assessed by incubating the cells with 1 µM calcein-AM for 60 min in combination with a gradient of each of the inhibitors. Afterwards, cells were washed with cold buffer and lysed with a solution of 1% Triton X100, kept at 37°C for an additional 60 min before fluorescence was measured (appropriate filter settings wavelengths: excitation-485nm, emission-530nm). Each solution was prepared in buffer at pH of 7.4, and fluorescence readings were performed using a Victor X3 Multi-label plate reader (Perkin Elmer, Waltham, USA). All experiments were performed in a 96 well plate set-up in triplo with a minimum of three separate experiments.

Fluorescence based, receptor mediated endocytosis assay

Endocytosis was evaluated by RAP-GST uptake over time and at different concentrations in ciPTEC cultured in 96 well black chimney plates (Costar 3690, Corning, NY, USA). Cells were incubated with RAP-GST at a 3-fold dilution range (starting at 7.5 µg/ml) for 2 h, or with one concentration of RAP-GST (2.5 µg/ml) for different times (1 or 6 h) and on ice. To compare the uptake between normal ciPTEC and megalin-deficient ciPTEC were incubated for 1 h with RAP-GST (2.5 µg/ml). After incubation, cells were fixed in 4% PFA/PBS for 15 min, washed with PBS and permeabilized for 10 min with 0.1% Triton/PBS. Next, samples were incubated overnight at 4°C with anti-GST antibody (GE Healthcare 27457701, Goat polyclonal, 1:1600 in 1% BSA/PBS), washed 3 times with PBS and incubated for 2 h at room temperature with fluorescently labeled secondary antibody (Molecular probes A11055, Donkey anti goat, Alexa Fluor 488, 1:500 in 1%BSA/PBS). Finally, samples were washed 3 times with PBS and fluorescence was recorded with the Victor X3 Multi-label plate reader.

Cytoplasmic enzyme activity

Enzymatic activity required to form fluorescent products from calcein-AM and CMFDA was evaluated by exposing cell lysates of the cell lines to the same concentration of fluorescent substrates used in the functional assays (*viz.* Hoechst33342 at 1.25 µM, CMFDA at 1.25 µM and calcein-AM at 1 µM). Cell lysates were obtained from matured ciPTEC and MDCKII cells by exposing cell suspensions (80,000 cells per 100 µL) to heat shock, first immersing the cells in liquid nitrogen until completely frozen and subsequent immersion in heated tap water. This procedure was repeated three times. Afterwards, the suspensions were centrifuged for 15 min at 10.000 rpm and the supernatant collected and stored at -80°C. The assay was performed in 96-well black chimney plates after addition of 50 µL of cell lysates followed by 50 µL of each of the fluorescent substrates. Fluorescence was monitored over time for 90 min at 37°C, with measurements performed every 2 min using a Victor X3 Multi-label plate reader.

Confocal microscopy

To visualize the uptake of fluorescent substrates and their accumulation, matured ciPTEC, cultured in a Lab-TeK8 chambers slide (Thermo Scientific, Waltham, MA, USA), were incubated with the substrates Hoechst33342 (1.25 µM) combined with KO143 (10 µM), CMFDA (1.25 µM) combined with MK571 (10 µM) and calcein-AM (1 µM) combined with PSC833 (5 µM) for 30 min. Images were acquired via an CV7000S high-content imager (Yokogawa, Tokio, Japan). For microscopic evaluation of receptor-mediated endocytosis, cells were cultured on a 8-well chambered coverslip (Ibidi 80826, Planegg-Martinsried, Germany), incubated for 1 h with RAP-GST (2.5 µg/ml) before fixation, permeabilization and fluorescent labeling with an Alexa 488 labeled antibody (see also receptor mediated endocytosis assay). After nuclear stain (DAPI; 300 ng/ml) images were acquired via an Olympus FV1000 confocal microscope (Zoeterwoude, The Netherlands

MRP4 and BCRP membrane vesicle uptake transport inhibition assay

Substrate and inhibitor specificity was investigated further using the membrane vesicle uptake assays, as established in our laboratory [36-38]. The uptake of radiolabeled substrates [^{3}H]-$H_2$17βG and [^{3}H]-E1S by MRP4 and BCRP vesicles was analysed as described previously [15, 36, 39]. Briefly, human embryonic kidney (HEK) 293 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (Invitrogen life sciences, Breda, the Netherlands) containing 10% (v/v) fetal calf serum (MP Biomedicals, Uden, the Netherlands) at 37 °C in a 5% (v/v) CO_2 atmosphere. To functionally overexpress MRP4 and BCRP, cells were transduced with baculoviruses of human MRP4, BCRP or enhanced yellow fluorescent protein (eYFP; as a negative control), generated via the Bac-to-Bac system (Invitrogen). Cell membranes were isolated and resuspended in isotonic buffer (10 mM Tris-HEPES and 250 mM sucrose, pH 7.4, adjusted with HEPES). Membrane vesicles were prepared via ultrafiltration. Afterwards, vesicles were frozen in liquid nitrogen and stored at –80 °C. For the uptake assays, 25 µl of Tris-Sucrose (TS) buffer containing 4 mM ATP, 10mM $MgCl_2$ and radiolabeled substrate was added to 5 µl of membrane vesicles (1.5 mg/ml). The transport assay was performed in the presence of the highest concentrations of inhibitors and substrates used in the 96-well plate assays described: KO143 (15 µM), MK571 (50 µM), PSC833 (6 µM), Hoechst33342 (1.25 µM), CMFDA (1.25 µM), calcein-AM (1 µM) and DMSO (2%, vehicle). Transport was achieved by incubating the mixture at 37 °C for 1 min (BCRP vesicles) or 5 min (MRP4 vesicles), the transport was stopped by placing the samples on ice and adding 150 µl ice cold TS buffer. Afterwards, the samples were transferred to a 96 well filter plate (Millipore, Etten-Leur, The Netherlands), pre-incubated with TS buffer and filtered using a Multiscreen HTS-Vacuum Manifold filtration device (Millipore). Subsequently, 2 ml of scintillation liquid was added to each filter and radioactivity was measured by liquid scintillation counting. EYFP-membrane vesicles and AMP were used as negative controls, and all experiments were performed in triplicate.

Gene expression

Transporters gene expression in matured ciPTEC and Human Kidney (acquired from healthy donors under consent) samples was performed by isolating total using an RNeasy Mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturers recommendations. Subsequently, cDNA was generated using the Omniscript RT-kit (Qiagen). Following cDNA-synthesis, quantitative PCR was performed using a CFX96 Real-Time PCR detection system (Bio-rad, Veenendaal, The Netherlands). GAPDH was used as housekeeping gene and relative expression levels were calculated as fold change using the $2^{-\Delta\Delta CT}$ method. The primer-probe sets were obtained from Applied Biosystems: GAPDH - hs99999905_m1; BCRP - hs00184979_m1; MRP4 - hs00195260_m1 and Pgp - hs00184500_m1.

Pharmacokinetic and statistical data analysis

Efflux activity was calculated by normalizing fluorescence intensity (upon inhibition), expressed in arbitrary units (a.u.), to baseline values (no inhibitor) after subtraction of background, prior to all calculations. As inhibition of efflux activity led to an increase in total fluorescence, the efflux activity is depicted as the inverse of the fold increase in fluorescence.

Non-linear regression with variable slope constraining the top to 100% was used to fit the normalized data, using GraphPad Prism 5.02 (GraphPad software, San Diego, CA, USA). Differences between groups were considered to be statistically significant when $p < 0.05$ when performing a one-tailed Student t-test. All data is presented as mean±SEM of at least three separate experiments performed in triplicate, unless stated otherwise. Statistical analysis to determine differences between IC_{50} values was performed within every substrate via two-way ANOVA. Statistical significant differences were set to $p<0.05$.

RESULTS

To study the transport activity in absence and presence of inhibitors, we compared three different cell models. The ciPTEC show many characteristics of human PTEC, including cell polarization, monolayer organization, expression of tight junction proteins, as well as transporter and metabolic enzyme activity [18, 19, 23, 32]. We investigated the relative expression levels of the ABC transporters BCRP, P-gp, MRP2 and MRP4 in comparison to the human kidney. Table 1 shows that both in ciPTEC and in human kidney MRP4 is the most abundantly expressed transporter relatively to BCRP and Pgp, while MRP2 is only moderately expressed in ciPTEC. BCRP and Pgp show comparable expression levels.

Table 1. Efflux transporters relative expression in ciPTEC and Human Kidney (HuKid)a.

	ΔCt		$2^{-\Delta Ct}$		Relative expression ciPTEC vs HuKid
	ciPTEC	HuKid	ciPTEC	HuKid	
BCRP	10.8±0.7	6.7±1.0	$6.5x10^{-4}$±$3.2x10^{-4}$	0.01±0.008	6.5%
Pgp	8.8±0.4	4.5±0.7	$2.3x10^{-3}$±$9.2x10^{-4}$	0.05±0.02	4.6%
MRP4	6.6±0.6	3.9±0.3	$1.1x10^{-2}$±$5.4x10^{-3}$	0.07±0.02	15.7%
MRP2	15.6±0.2	b	$2.0x10^{-5}$±$2.8x10^{-6}$	b	b

[a] Values are shown in mean ± standard deviation. [b] MRP2 could not be determined.

The MDCK overexpression lines were used as positive controls for BCRP and P-gp activity. Hoechst33342 was used to evaluate BCRP activity directly, whereas calcein-AM and CMFDA gain fluorescent properties upon hydrolysis (methyl group cleavage) in the cytoplasm, and, in case of CMFDA, further conjugation to carboxyfluorescein-glutathione (GS-MF) with the replacement of the chlorine group. The fluorescent end

products were used to determine P-gp (calcein) and MRP's (GS-MF) activities, respectively. The inhibitors KO143, MK571 and PSC833 were used to block BCRP, MRP4 and P-gp activity, respectively [29, 40, 41]. To evaluate their effect on the different cell lines, we tested the three inhibitors in combination with all three substrates.

ciPTEC and MDCKII overexpression cells have differential fluorescent substrate retention

Substrate retention levels varied between the different cell lines used as an indication of the transporters activity in each cell type. MDCKII-BCRP cells showed the lowest Hoechst33342 accumulation while ciPTEC presented the highest (Figure 1A), consistent with the high expression of BCRP in the MDCKII overexpression cell line. For GS-MF, ciPTEC and MDCKII-Pgp presented similar intensities and MDCKII-BCRP the highest (Figure 1B). When incubated with calcein-AM, the highest retention was observed in ciPTEC while MDCKII-P-gp showed minor accumulation (Figure 1C), in agreement with the overexpression of the transporter in the latter cell line.

ciPTEC and MDCKII cells have similar enzyme activities

Since enzyme activity is required to convert CMFDA and calcein-AM into their fluorescent counterparts (GS-MF and calcein, respectively), the efficiency of this conversion was compared in cell lysates of ciPTEC and MDCKII cells. As expected, Hoechst33342 fluorescence was not influenced when incubated with cell lysates nor showed a time dependent effect (Figure 1D). When cell lysates were incubated with either CMFDA (Figure 1E) or calcein-AM (Figure 1F) at 37°C, the fluorescence behaviour over time was similar in ciPTEC and MDCKII, although ciPTEC showed a more rapid initial increase in signal. Both substrates also showed to be slowly transformed over time in buffer (without cells). The inhibitors used in the functional assays (KO143, MK571 and PSC833) did not interfere with the enzyme activity in converting the substrates (data no shown).

Fluorescent substrates accumulate in ciPTEC upon efflux transport inhibition

Model efflux inhibitors increased the intracellular accumulation of fluorescent substrates in ciPTEC, as demonstrated in figure 2. In the presence of KO143, Hoechst33342 fluorescence increased 1.8-fold (Figure 2A), GS-MF retention increased 3.0-fold in presence of MK571 (Fig. 2-D) and PSC833 increased calcein fluorescence 2.1-fold (Figure 2G). These results indicate that efflux can effectively be blocked in ciPTEC. The substrates' intracellular accumulations upon efflux inhibition could be confirmed with confocal microscopy. A clear nuclear accumulation was observed for Hoechst33342 (Figure 2B-C), whereas GS-MF (Figure 2E-F) and calcein (Figure 2H-I) accumulated predominantly in the cytoplasm.

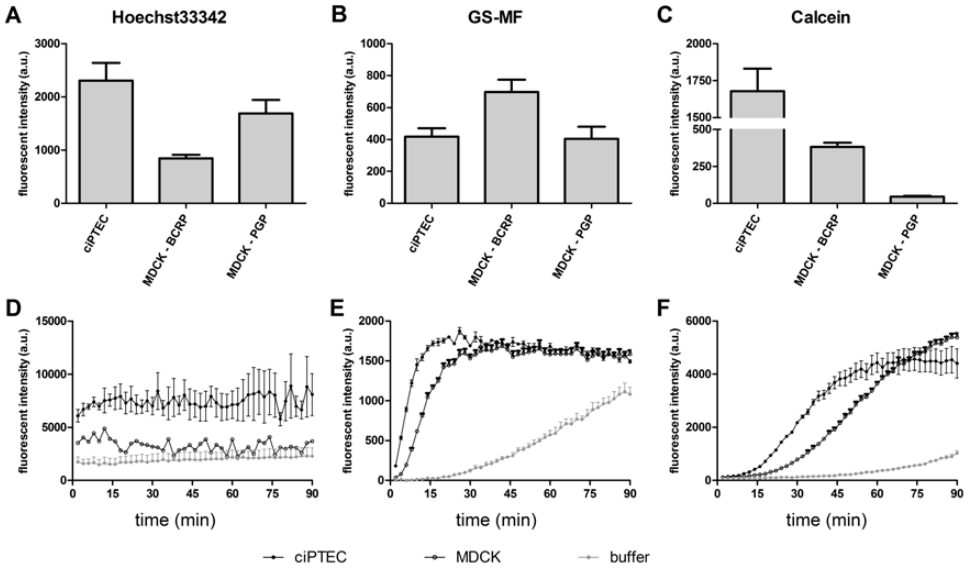


Figure 1. Differential fluorescent substrates retention and enzyme activity in ciPTEC and MDCKII cell lines. Hoechst33342 (1.25 µM) retention (A) was lower in MDCKII-BCRP and MDCKII-Pgp. CMFDA/GS-MF (1.25 µM; B) was similarly retained in ciPTEC and MDCKII-Pgp, but in a lower fashion than in MDCKII-BCRP. Calcein (1 µM; C) was efficiently effluxed of MDCKII-Pgp cells while it accumulated greatly in ciPTEC. Differential substrates retention between the cell lines is indicative of the disparities in endogenous and over-expressed transport systems. By using cell lysates of both ciPTEC and MDCKII parent cell line, it was possible to determine the intracellular enzyme activity over time (90 min), in the presence of the substrates Hoechst33342 (1.25 µM; D), CMFDA (1.25 µM; E) and calcein-AM (1 µM; F).

Inhibitors and substrates used for efflux pumps lack specificity

The efflux of the model substrates Hoechst33342, GS-MF and calcein was investigated further in a concentration dependent manner with all combinations of inhibitors, to determine efflux activity and substrate and inhibitor specificities (Figures 3-5). Inhibition curves were analysed by non-linear curve fitting and a summary of the IC_{50} values obtained for all interactions is presented in Table 2.

Hoechst33342 efflux.

The prototype inhibitor for BCRP activity and Hoechst33342 retention is KO143 (Figure 3A), which reduced the efflux in MDCKII-BCRP and MDCKII-P-gp by 85 and 80% (Figure 1), respectively. In ciPTEC, efflux was reduced by 46.9±5.0%, suggesting efflux is only partially BCRP mediated. On the other hand, Hoechst33342 efflux in ciPTEC could be inhibited by MK571 (Figure 3B), suggesting Hoechst33342 efflux is mediated by MRP transport as well. In presence of MK571 (Figure 3B), ciPTEC and MDCKII-BCRP presented comparable efflux inhibitions (7.1±0.3% and 9±2%, respectively). When exposed to the Pgp blocker PSC833, Hoechst33342 efflux was reduced to 46±8% in ciPTEC,

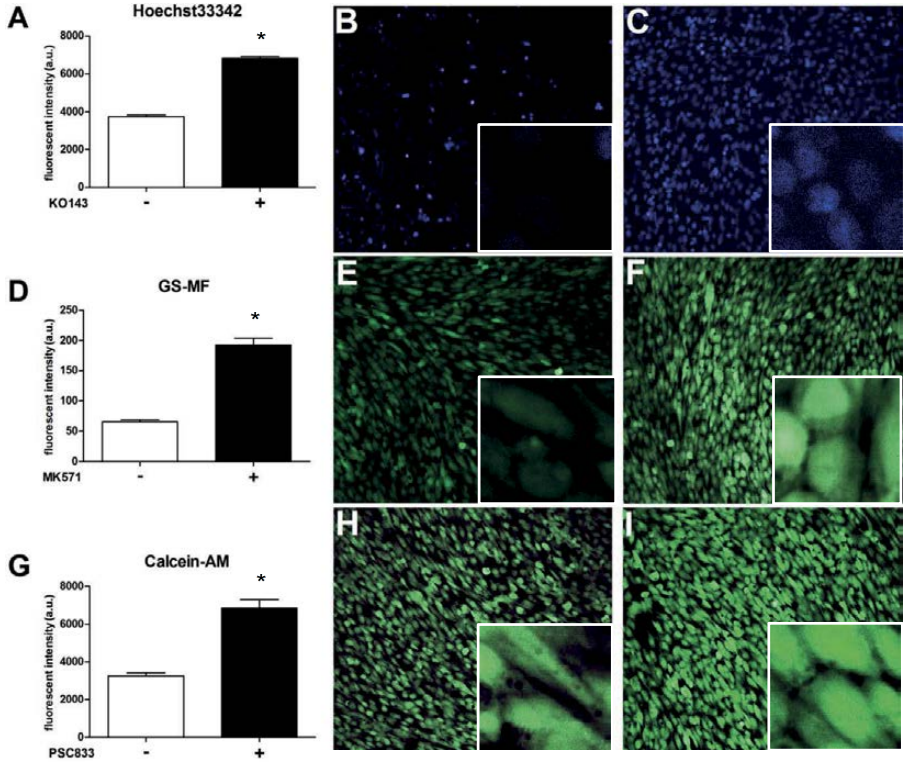


Figure 2. Fluorescent substrates accumulate in ciPTEC in the presence of efflux transport blockers. An increase in fluorescence was observed when cells were incubated with the fluorescent substrates Hoechst (1.25 µM; A), CMFDA (1.25 µM; D), Calcein-AM (1 µM; G) without inhibitors (B, E, H) or in presence of inhibitors (C, F, I). Inhibitors promoted the intracellular accumulation of the fluorescent substrates, as depicted in the representative images and quantified in the bar graphics (black bars; n=3, $*p < 0.05$).

to 24±11% in MDCKII-BCRP and to 27±14% in MDCKII-P-gp (Figure 3C), supporting the role of P-gp in Hoechst3342 efflux as well.

CMFDA/GS-MF efflux

For CMFDA/GS-MF efflux inhibition KO143 showed a comparable potency (Table 2) in ciPTEC and MDCKII-BCRP, while the highest inhibition, up to 28±11%, was found in MDCKII-P-gp cells (Figure 4A). MK571 (Figure 4B) promoted a considerable inhibition in all cell lines tested, with the highest decrease of efflux in ciPTEC (up to 7.0±1.8%). PSC833 (Figure 4C) promoted similar maximal inhibitions in all cell lines, except for MDCKII-Pgp, which was most sensitive (11.0±1.8%). The highest reduction in overall transport was found in ciPTEC. Taken together, these results underline the cross-over in inhibitor specificities, pointing to a role for BCRP, P-gp and MRP transporters in handling CMFDA and/or its derivatives.

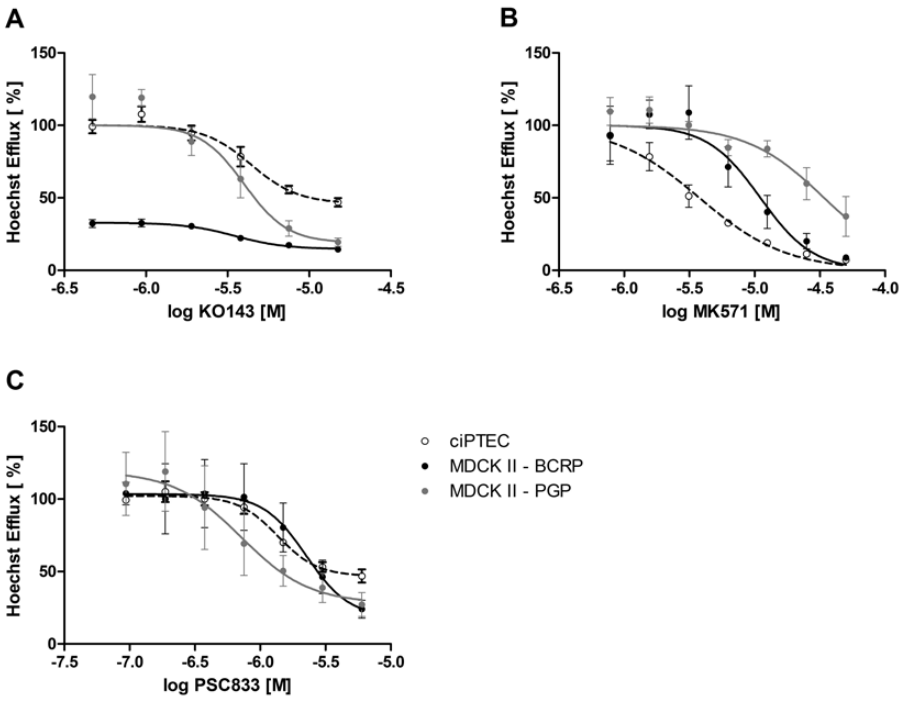


Figure 3. Dose response effects of the inhibitors KO143 (A), MK571 (B) and PSC833 (C) on Hoechst33342 retention in ciPTEC (dotted line), MDCK II-BCRP (black line) and MDCK II-Pgp (grey line). To determine IC_{50} values, non-linear regression with variable slope constraining the top to 100% was performed using at least six concentrations of each inhibitors. Activity was considered 100% in the respective cell lines, in absence of any inhibitor.

Calcein efflux

In MDCKII-P-gp, KO143 could inhibit calcein efflux up to 12±6% (Figure 5A), whereas in ciPTEC the maximum inhibition was only 63±27%. When exposed to MK571 (Figure 5B) MDCKII-BCRP and ciPTEC showed similar inhibitions in efflux; up to 25±7% and 25±8%, respectively. PSC833 (Figure 5C), affected calcein efflux mostly in MDCKII-P-gp cells (5.3±1.6%) with the highest potency (Table 2) ciPTEC and MDCKII-BCRP presented similar IC_{50} values, where ciPTEC showed the lowest efflux reduction (32.7±10.9%). These findings are consistent with KO143 exhibiting inhibitory properties towards P-gp, and further underscores a lack of substrate and inhibitor specificity.

Fluorescent substrates and inhibitors affect BCRP and MRP4 activity in vesicles

Membrane vesicles were used as single expression transporter systems for P-gp, BCRP and MRP4 in order to determine the specific effects of the substrates and inhibitors used. Using NMQ as a substrate [42], it was not possible to determine the interaction

Table 2. Inhibitory potencies of KO143, MK571 and PSC833 on Hoechst33342, GS-MF and calcein efflux by ciPTEC, values are presented as mean ± SD.

		IC_{50} (M)		
Substrate	Cell type	KO143	MK571	PSC833
Hoechst33342	ciPTEC	$4.4x10^{-6}$±$0.9x10^{-6}$	$5.1x10^{-6}$±$0.9x10^{-6}$	$1.3x10^{-6}$±$0.3x10^{-6e}$
	MDCKII-BCRP	$3.5x10^{-6}$±$0.1x10^{-6}$	$10.1x10^{-6}$±$3.7x10^{-6d}$	$2.0x10^{-6}$±$1.1x10^{-6e}$
	MDCKII-Pgp	$3.6x10^{-6}$±$1.5x10^{-6}$	$24.2x10^{-6}$±$2.9x10^{-6a, b, d}$	$0.9x10^{-6}$±$0.5x10^{-6e}$
CMFDA/GS-MF	ciPTEC	$2.7x10^{-6}$±$0.2x10^{-6}$	$6.4x10^{-6}$±$1.3x10^{-6}$	$1.0x10^{-6}$±$0.2x10^{-6}$
	MDCKII- BCRP	$2.3x10^{-6}$±$0.9x10^{-6}$	$8.5x10^{-6}$±$2.4x10^{-6}$	$1.2x10^{-6}$±$0.4x10^{-6}$
	MDCKII-Pgp	$3.6x10^{-6}$±$1.7x10^{-6}$	$22.2x10^{-6}$±$10.3x10^{-6a, b, d}$	$0.7x10^{-6}$±$0.3x10^{-6e}$
Calcein	ciPTEC	$2.5x10^{-6}$±0.5 $x10^{-6}$	$10.2x10^{-6}$±$3.1x10^{-6d}$	$0.7x10^{-6}$±$0.5x10^{-6e}$
	MDCKII- BCRP	$2.4x10^{-6}$±$0.4x10^{-6}$	$9.9x10^{-6}$±$5.8x10^{-6d}$	$1.2x10^{-6}$±$0.7x10^{-6e}$
	MDCKII-Pgp	$1.7x10^{-6}$±$0.1x10^{-6}$	$5.3x10^{-6}$±$2.3x10^{-6}$	$0.8x10^{-6}$±$0.1x10^{-6}$

Statistical significant differences ($p<0.05$) between cell types (same inhibitor), are represented by: [a](ciPTEC), [b](MDCK-BCRP), [c](MDCK-Pgp). Statistical significant differences ($p<0.05$) between inhibitors (same cell type) are represented by: [d](KO143), [e](MK571), [f](PSC833).

between the compounds used and P-gp (data not shown). To evaluate BCRP and MRP4 activities, we used [^{3}H]-E1S and [^{3}H]-$H_2$17βG as substrates, respectively. The radioactive substrate uptake inhibition in BCRP or MRP4 vesicles is a direct indication of the potency of the compound tested. In BCRP vesicles (Figure 6A), [^{3}H]-E1S uptake was significantly reduced by KO143, MK571, PSC833 and CMFDA, compared to the control condition. On the other hand, MRP4 mediated uptake of [^{3}H]-$H_2$17βG (Figure 6B) was only significantly reduced by KO143 and MK571 compared to control.

Endocytosis mediated reabsorption in ciPTEC

Protein reabsorption is another important function of proximal tubular cells and essential for renal physiology and drug safety assessments. The megalin ligand RAP-GST is efficiently taken up by ciPTEC and has been used to study the receptor mediated endocytosis machinery in patient cells in comparison to ciPTEC from healthy controls [30, 31]. The quantification is traditionally done on fluorescent microscopy images, which is a very laborious and time-consuming process. Here, we optimized the staining and quantification for a 96-well plate format with detection in a fluorescent plate reader. We could show that RAP-GST was efficiently taken up by the cells in a time and concentration dependent manner (Figure 7A-B). To evaluate the initial binding of ligand to the megalin receptors on the cell (prior to internalization), we incubated the cells for 30 min on ice in the presence of ligand (Figure 7B). The binding on ice was 24% of the uptake after 1 h, suggesting that within this time frame the initial amount of receptor on the surface can be internalized and replenished on the cell surface several times. Dent's disease ciPTEC

showed a RAP-GST uptake of 57% of the control cells. Furthermore, the fluorescent labelling was consistent with endocytotic vesicles and showed minor background fluorescence.

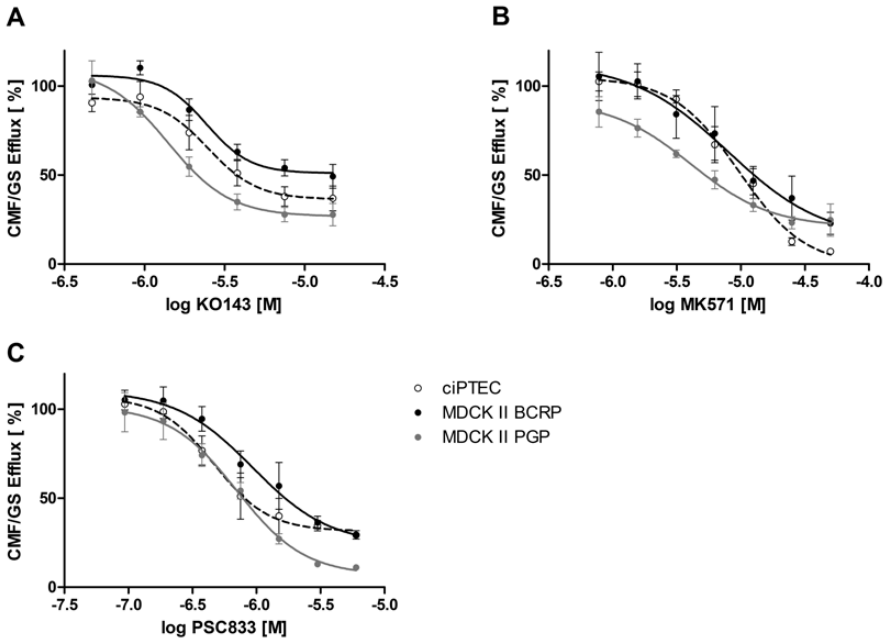


Figure 4. Dose response effects of the inhibitors KO143 (A), MK571 (B) and PSC833 (C) on CMFDA/GS-MF retention in ciPTEC (dotted line), MDCKII-BCRP (black line) and MDCKII-Pgp (grey line). To determine IC50 values, non-linear regression with variable slope constraining the top to 100% was performed using at least six concentrations of each inhibitor. Activity was considered 100% in the respective cell lines in absence of any inhibitor.

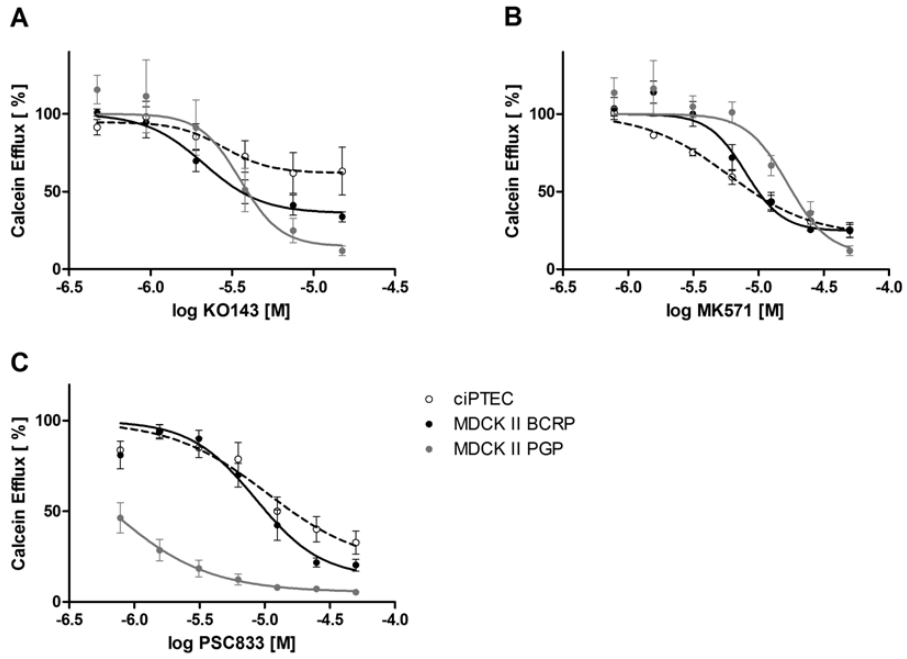


Figure 5. Dose response effects of the inhibitors KO143 (A), MK571 (B) and PSC833 (C) on calcein retention in ciPTEC (dotted line), MDCKII-BCRP (black line) and MDCKII-Pgp (grey line). To determine IC50 values, non-linear regression with variable slope constraining the top to 100% was performed using at least six concentrations of each inhibitor. Activity was considered 100% in the respective cell lines in absence of any inhibitor.

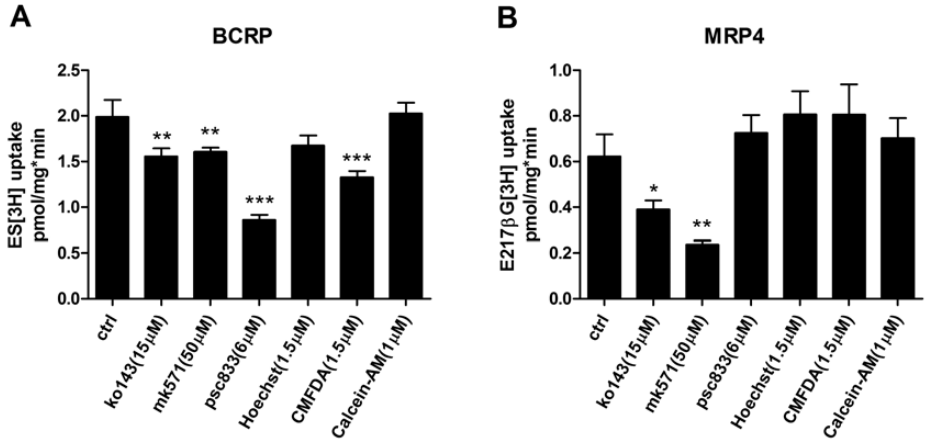


Figure 6. ATP dependent uptake of E1S-^{3}H mediated by BCRP (A) or E217βB-^{3}H uptake mediated by MRP4 (B) was studied in HEK293 cell derived vesicles. DMSO was used as vehicle (ctrl), along with inhibitors KO143, MK571, PSC833 and substrates Hoechst33342 and CMFDA. BCRP mediated uptake is significantly decreased by KO143, MK571, PSC833 and CMFDA. MRP4 is affected only by KO143 and MK571. Statistical analysis was performed via unpaired Student's *t*-test. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control.

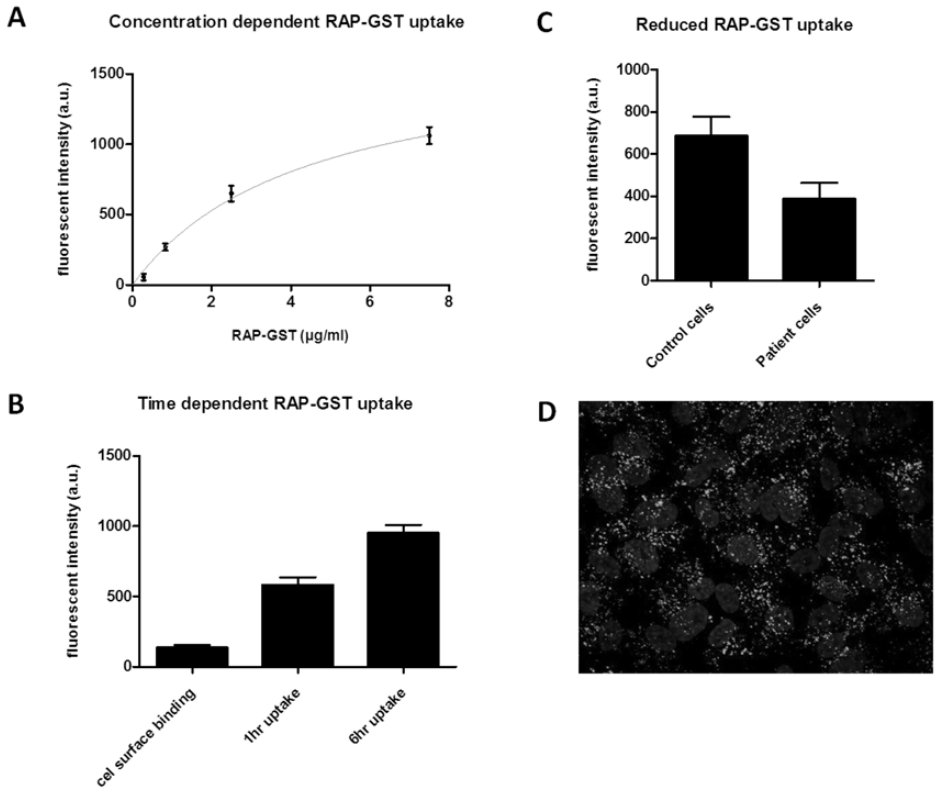


Figure 7. Endocytosis mediated protein reabsorption in ciPTEC. Cells were incubated at 37˚C with different concentration RAP-GST for 2 h (A) or with 2.5 µg/ml RAP-GST for different periods of time (B). Protein surface binding was determined by incubating cells with RAP-GST on ice for 30 min (B). Protein uptake was compared between control ciPTEC cells and cells from patients with Dent's disease (C). Immunofluorescent image of fluorescently labelled RAP-GST after uptake in control ciPTEC (D).

DISCUSSION

The renal proximal tubule is a versatile part of the nephron, which plays a crucial role in the active secretion and reuptake of numerous exogenous (*eg.* drugs), as well as endogenous compounds. These processes are mediated by a wide variety of transport proteins, present in the basolateral and apical membranes of PTEC. In this study, we describe the application of fluorescent-based assays in a 96-well plate format to provide reproducible data on transporter activity and drug interactions on transport activities present in the proximal tubule apical membrane. We tested the efflux of several classical substrates (Hoechst33342, CMFDA/GS-MF and calcein(-AM)) in cell models expressing different transporters simultaneously or in parallel and demonstrated that the substrates are not transported by one particular transporter, as was previously described [43]. Furthermore, we used several known inhibitors (KO143, MK571 and

PSC833) for the relevant transporters and showed that these inhibitors (partially) inhibit the function of other transporters as well. We believe that our data provides insight into the complex interactions that take place *in vivo* and which should be taken into account when screening for drug interactions using advanced human cell model systems, such as ciPTEC, where the membrane transporters central to this study, BCRP, P-gp and MRP4, are expressed at the protein levels [23, 33]. The expression and activity of organic cation transporter 2 (OCT2) was been reported [22],as well as broad panel of phase I metabolic enzymes, including Cytochromes P450 (CYP) YP3A4, CYP4A11, CYP2D6, UDP-glucuronosyltransferases (UGT) 1A1, UGT1A9, UGT2B7 and UGT2B28 show similar expression to primary human proximal tubule cells [21, 44]. Moreover, UGT protein expression and functionality, which is pivotal for the metabolic activation of the substrates CMFDA and Calcein-AM, was also demonstrated in ciPTEC[21].

A graphical summary of our findings is provided in figure 8, in which the substrates enzymatic transformations are depicted as well as the transporters involved in their handling and the blockers responsible for inhibitions. Overall, Hoechst33342, GS-MF and calcein are retained in the presence of KO143, MK571 and PSC833, showing clearly the redundancy between the transporters. Noteworthy is the fact that both KO143 and MK571 can block BCRP, P-gp and MRP's, while PSC833 appears a potent inhibitor for BCRP and P-gp, but not the MRPs. The inhibitor concentrations ranges used in this study were chosen based on the concentration where maximum substrate retention was achieved by the model inhibitors in ciPTEC, with no toxic effects (data not shown). Arguably, the concentrations used were high enough to promote lack of specificity. These concentrations were necessary to achieve inhibition of particular transporters, since at lower concentrations, in ciPTEC, all inhibitors either yield none or little inhibition, this can be accounted by the multiple transporters expressed simultaneously in this cell type and given the cross-over between inhibitors, higher concentrations are needed to discriminate between the particular transporters. However, inhibitory potencies revealed that, despite cross-over, the different transporters do maintain different specificities towards the inhibitors. Previous reports showed that the presence and activity of transport systems *in vitro* can be assessed with a similar approach, and that immortalized cells with poorly retained proximal tubule phenotype, such as HK-2 cells, yield low fluorescent functional properties [25, 29].

After comparing the results obtained in our cell assay with the membrane vesicles assay, the redundancy in substrate and inhibitor specificities could be confirmed. Vesicular transport assays are widely employed for drug screening because they lack complex physiological processes, such as enzyme activity and post-translational regulation, hence, providing an accurate indication of substrate and inhibitor interaction. Our results demonstrated that KO143 and MK571 inhibited BCRP and MRP4 mediated transport, and PSC833 only inhibited BCRP. On the other hand, the interaction of Hoechst33342 with BCRP and MRP4 could not be identified using the vesicular system. This may be due to the low affinity of Hoechst33342 for BCRP in comparison with E1S, which has a high

affinity for the transporter [38]. Note that the concentration used for Hoechst33342 was low, and the same as used for the cellular assays. Furthermore, under these experimental conditions it was not possible to determine the interaction between the compounds and P-gp in membrane vesicles. This is most likely due to the lipophilicity of the substrates, because we used cell-permeable probes. It was recently reported that P-gp activity measurements in vesicular transport assays is jeopardized by the lipophilicity of substrates [45]. Therefore, these substrates require cellular assays, for which overexpression models are well suited to study a particular transporter in its' subcellular environment. Additionally, MDCKII–BCRP/-P-gp cell lines express the enzymes that allowed us to use CMFDA and calcein-AM as substrate. Still, the overexpression systems lack a proper clinical translation [46] as a result of their non-human origin and supra-physiological condition. Although the expression of the transporters studied are low in ciPTEC relative to human kidney (Table 1), the order of expression is comparable, where MRP4 is the highest expressed, followed by P-gp and then BCRP.

Receptor mediated endocytosis is important for the function of proximal tubular cells and a deficiency in protein reabsorption will lead to loss of nutrients, proteinuria, inflammation and renal damage. Drugs, including aminoglycosides, cisplatin, statins and many others can directly interfere with the different steps of the receptor mediated

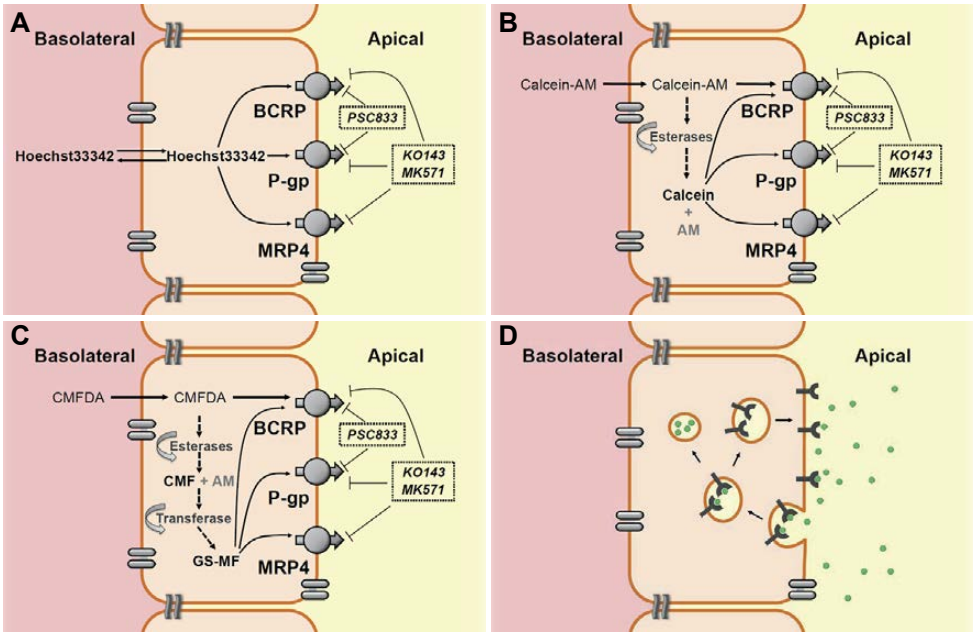


Figure 8. Graphic presentation of substrate handling, transport inhibition and reabsorption in ciPTEC. Hoechst (A), Calcein (B) and CMFDA (C) intracellular fate and the transporters responsible for efflux are depicted in combination with the inhibitors used. RAP-GST uptake (D) through receptor mediated endocytosis represents the protein reabsorption capacity of these cells.

endocytosis pathway, for instance by affecting membrane composition, clathrin availability, dynamin activity, actin skeleton function, vesicle stabilisation or membrane potential [47]. It has been described that cisplatin can affect receptor-mediated endocytosis by inhibiting vacuolar H-ATPase, and statins have been found to inhibit fluorescent protein uptake in opossum kidney cells but the mechanism remains unclear [48, 49]. Also in inherited diseases such as Dent's disease or nephropathic cystinosis defects in protein reabsorption can lead to proteinurea and subsequent kidney damage [50]. Furthermore, some compounds are taken up in high concentrations by the proximal tubular cells through receptor- mediated endocytosis and result in nephrotoxicity. Aminoglycosides such as gentamicin and amikacin, bind the megalin receptor with high affinity and accumulate in the proximal tubule cells [51]. To evaluate protein reabsorption by the proximal tubular cells we visualized the uptake of proteins in vesicles and compared the cell surface binding on ice with active RAP-GST uptake over time. This 96-well system provides a fast and reproducible approach to measure receptor mediated endocytosis and can be used to show reduced uptake in patient cells.

Renal models are widely used to study renal clearance and physiology. While animal studies are often indispensable for providing insight into the clinical relevance of preclinical observations, they are not as easily controlled and hampered by ethical concerns. Interspecies variability and extrarenal influences, such as body temperature, hormone levels, nervous regulation, hemodynamics, and GFR, can complicate data interpretation even further. By making use of human cell models, many of these factors can be overruled, enabling the study of very specific processes in a fast or high throughput fashion. As a proximal tubule cell model, ciPTEC has proven to be a robust model with its' epithelial characteristics, such as basolateral-to-apical polarization, tight-junction formation and the presence of physiologically relevant enzymatic and transporter activities. Previous findings from our group demonstrated interactions of transporters with endogenous metabolites, sometimes leading to toxicity[22]. This underscores the ability to use ciPTEC in unravelling renal physiology and clearance processes, and studying mechanisms leading to renal toxicity. A limitation of our system is that it will only be representative for processes associated with the renal proximal tubules. Drug interactions and drug induced kidney injury may also be a result of damage to other segments and cellular components of the kidney. However, no representative human cell models of these segments are currently available. Nevertheless, recent advances in the generation of complex human *in vitro* models from renal cell lineages [52] and nephron organoids from induced pluripotent stem cells (hiPS) [53-55] are promising. The importance of validating such models and exploring their potential as predictive tools are key issues that need to be addressed. In addition, recent developments in tissue culture systems acknowledge the importance of the tissue microenvironment in optimal functioning and responsiveness. This can be addressed by culturing cells in a dynamic, 3D, environment, allowing optimal communication between cells. Nephron organoids present such 3D environment, as well as PTEC cultured in microfluidic systems [56] (viz. kidney-on-a-chip) or bioartificial tubules

[57]. These models are also compatible with high-resolution and real-time molecular imaging, advancing drug interaction studies and their related intracellular effects when applied with suitable fluorescent probes. Our present work describes a set of tools that could be implemented in such platform and allows studying transport mechanisms that are central to drug absorption, disposition and detoxification.

In conclusion, we describe an approach that overcomes different limitations of widely used *in vitro* models, namely vesicles and over-expression cell lines, in order to obtain a more physiological picture of transport systems at the proximal tubule apical membrane. Fluorescent probes are central to this work, not only because they are easy to handle and data acquisition is straightforward allowing generating, validating and reproducing functional transport data, but also because we prove that, when chosen wisely, a combination of probes and inhibitors can by itself shed light on complex transport mechanisms.

ACKNOWLEDGMENTS

This work was support by the Nephrotools consortium under the scope of EU FP7 Marie Curie actions (grant nr. 289754), a grant from the Dutch Kidney Foundation (grant nr. KJPB 11.023), as well as the Netherlands Organization for Scientific Research (Zon-MW, grant nr. 40-42900-98-2002). The membrane vesicles were kindly provided by Dr. Jan Koenderink and Jeroen van den Heuvel (www.pharmtox.nl).

REFERENCES

1. Varma MV, Feng B, Obach RS, Troutman MD, Chupka J, Miller HR, et al. Physicochemical determinants of human renal clearance. J Med Chem. 2009;52(15):4844-52. Epub 2009/05/19. doi: 10.1021/jm900403j. PubMed PMID: 19445515.

2. Masereeuw R, Russel FG. Regulatory pathways for ATP-binding cassette transport proteins in kidney proximal tubules. AAPS J. 2012;14(4):883-94. Epub 2012/09/11. doi: 10.1208/s12248-012-9404-z. PubMed PMID: 22961390; PubMed Central PMCID: PMC3475864.

3. Mutsaers HA, van den Heuvel LP, Ringens LH, Dankers AC, Russel FG, Wetzels JF, et al. Uremic toxins inhibit transport by breast cancer resistance protein and multidrug resistance protein 4 at clinically relevant concentrations. PLoS One. 2011;6(4):e18438. Epub 2011/04/13. doi: 10.1371/journal.pone.0018438. PubMed PMID: 21483698; PubMed Central PMCID: PMC3070735.

4. Pavek P, Merino G, Wagenaar E, Bolscher E, Novotna M, Jonker JW, et al. Human breast cancer resistance protein: interactions with steroid drugs, hormones, the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, and transport of cimetidine. J Pharmacol Exp Ther. 2005;312(1):144-52. Epub 2004/09/15. doi: 10.1124/jpet.104.073916. PubMed PMID: 15365089.

5. Leslie EM, Deeley RG, Cole SP. Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense. Toxicol Appl Pharmacol. 2005;204(3):216-37. Epub 2005/04/23. doi: 10.1016/j.taap.2004.10.012. PubMed PMID: 15845415.

6. International Transporter C, Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, et al. Membrane transporters in drug development. Nat Rev Drug Discov. 2010;9(3):215-36. Epub 2010/03/02. doi: 10.1038/nrd3028. PubMed PMID: 20190787; PubMed Central PMCID: PMC3326076.

7. Perazella MA. Drug-induced renal failure: update on new medications and unique mechanisms of nephrotoxicity. Am J Med Sci. 2003;325(6):349-62. Epub 2003/06/18. PubMed PMID: 12811231.

8. Kitterer D, Schwab M, Alscher MD, Braun N, Latus J. Drug-induced acid-base disorders. Pediatr Nephrol. 2014. Epub 2014/11/06. doi: 10.1007/s00467-014-2958-5. PubMed PMID: 25370778.

9. Christensen EI, Birn H, Storm T, Weyer K, Nielsen R. Endocytic receptors in the renal proximal tubule. Physiology (Bethesda). 2012;27(4):223-36. doi: 10.1152/physiol.00022.2012. PubMed PMID: 22875453.

10. Wagner MC, Campos-Bilderback SB, Chowdhury M, Flores B, Lai X, Myslinski J, et al. Proximal Tubules Have the Capacity to Regulate Uptake of Albumin. J Am Soc Nephrol. 2015. doi: 10.1681/ASN.2014111107. PubMed PMID: 26054544.

11. Abbate M, Zoja C, Remuzzi G. How does proteinuria cause progressive renal damage? J Am Soc Nephrol. 2006;17(11):2974-84. doi: 10.1681/Asn.2006040377. PubMed PMID: WOS:000241912100008.

12. Gorriz JL, Martinez-Castelao A, Nephrology SS. Proteinuria: detection and role in native renal disease progression. Transplant Rev-Orlan. 2012;26(1):3-13. doi: 10.1016/j.trre.2011.10.002. PubMed PMID: WOS:000311531000002.

13. Karlsson JE, Heddle C, Rozkov A, Rotticci-Mulder J, Tuvesson O, Hilgendorf C, et al. High-activity p-glycoprotein, multidrug resistance protein 2, and breast cancer resistance protein membrane vesicles prepared from transiently transfected human embryonic kidney 293-epstein-barr virus nuclear antigen cells. Drug Metab Dispos. 2010;38(4):705-14. Epub 2010/01/15. doi: 10.1124/dmd.109.028886. PubMed PMID: 20071452.

14. El-Sheikh AA, Greupink R, Wortelboer HM, van den Heuvel JJ, Schreurs M, Koenderink JB, et al. Interaction of immunosuppressive drugs with human organic anion transporter (OAT) 1 and OAT3, and multidrug resistance-associated protein (MRP) 2 and MRP4. Transl Res. 2013;162(6):398-409. doi: 10.1016/j.trsl.2013.08.003. PubMed PMID: 24036158.

15. Wittgen HG, van den Heuvel JJ, van den Broek PH, nter-Heidorn H, Koenderink JB, Russel FG. Cannabinoid type 1 receptor antagonists modulate transport activity of multidrug resistance-associated proteins MRP1, MRP2, MRP3, and MRP4. Drug Metab Dispos. 2011;39(7):1294-302.

16. Gartzke D, Fricker G. Establishment of optimized MDCK cell lines for reliable efflux transport studies. J Pharm Sci. 2014;103(4):1298-304. Epub 2014/02/18. doi: 10.1002/jps.23901. PubMed PMID: 24532159.

17. Konig J, Muller F, Fromm MF. Transporters and drug-drug interactions: important determinants of drug disposition and effects. Pharmacological reviews. 2013;65(3):944-66. Epub 2013/05/21. doi: 10.1124/pr.113.007518. PubMed PMID: 23686349.

18. Fahrmayr C, Konig J, Auge D, Mieth M, Munch K, Segrestaa J, et al. Phase I and II metabolism and MRP2-mediated export of bosentan in a MDCKII-OATP1B1-CYP3A4-UGT1A1-MRP2 quadruple-transfected cell line. Br J Pharmacol. 2013;169(1):21-33. Epub 2013/02/08. doi: 10.1111/bph.12126. PubMed PMID: 23387445; PubMed Central PMCID: PMC3632236.

19. Nagai J, Sato K, Yumoto R, Takano M. Megalin/cubilin-mediated uptake of FITC-labeled IgG by OK kidney epithelial cells. Drug Metab Pharmacokinet. 2011;26(5):474-85. PubMed PMID: 21712611.

20. Vegt E, van Eerd JE, Eek A, Oyen WJ, Wetzels JF, de Jong M, et al. Reducing renal uptake of radiolabeled peptides using albumin fragments. J Nucl Med. 2008;49(9):1506-11. doi: 10.2967/jnumed.108.053249. PubMed PMID: 18703613.

21. Mutsaers HA, Wilmer MJ, Reijnders D, Jansen J, van den Broek PH, Forkink M, et al. Uremic toxins inhibit renal metabolic capacity through interference with glucuronidation and mitochondrial respiration. Biochim Biophys Acta. 2013;1832(1):142-50. Epub 2012/09/29. doi: 10.1016/j.bbadis.2012.09.006. PubMed PMID: 23017367.

22. Schophuizen CM, Wilmer MJ, Jansen J, Gustavsson L, Hilgendorf C, Hoenderop JG, et al. Cationic uremic toxins affect human renal proximal tubule cell functioning through interaction with the organic cation transporter. Pflugers Arch. 2013;465(12):1701-14. Epub 2013/07/03. doi: 10.1007/s00424-013-1307-z. PubMed PMID: 23812163.

23. Wilmer MJ, Saleem MA, Masereeuw R, Ni L, van der Velden TJ, Russel FG, et al. Novel conditionally immortalized human proximal tubule cell line expressing functional influx and efflux transporters. Cell Tissue Res. 2010;339(2):449-57. Epub 2009/11/11. doi: 10.1007/s00441-009-0882-y. PubMed PMID: 19902259; PubMed Central PMCID: PMC2817082.

24. Gorvin CM, Wilmer MJ, Piret SE, Harding B, van den Heuvel LP, Wrong O, et al. Receptor-mediated endocytosis and endosomal acidification is impaired in proximal tubule epithelial cells of Dent disease patients. P Natl Acad Sci USA. 2013;110(17):7014-9. doi: 10.1073/pnas.1302063110. PubMed PMID: WOS:000318677300086.

25. Bircsak KM, Gibson CJ, Robey RW, Aleksunes LM. Assessment of drug transporter function using fluorescent cell imaging. Curr Protoc Toxicol. 2013;57:Unit 23 6. Epub 2014/02/11. doi: 10.1002/0471140856.tx2306s57. PubMed PMID: 24510579; PubMed Central PMCID: PMC3920305.

26. Mahringer A, Delzer J, Fricker G. A fluorescence-based in vitro assay for drug interactions with breast cancer resistance protein (BCRP, ABCG2). Eur J Pharm Biopharm. 2009;72(3):605-13. Epub 2009/07/03. doi: 10.1016/j.ejpb.2009.01.010. PubMed PMID: 19572416.

27. Weiss J, Theile D, Ketabi-Kiyanvash N, Lindenmaier H, Haefeli WE. Inhibition of MRP1/ABCC1, MRP2/ABCC2, and MRP3/ABCC3 by nucleoside, nucleotide, and non-nucleoside reverse transcriptase inhibitors. Drug Metab Dispos. 2007;35(3):340-4. Epub 2006/12/19. doi: 10.1124/dmd.106.012765. PubMed PMID: 17172311.

28. Luna-Tortos C, Fedrowitz M, Loscher W. Evaluation of transport of common antiepileptic drugs by human multidrug resistance-associated proteins (MRP1, 2 and 5) that are overexpressed in pharmacoresistant epilepsy. Neuropharmacology. 2010;58(7):1019-32. Epub 2010/01/19. doi: 10.1016/j.neuropharm.2010.01.007. PubMed PMID: 20080116.

29. Jenkinson SE, Chung GW, van Loon E, Bakar NS, Dalzell AM, Brown CD. The limitations of renal epithelial cell line HK-2 as a model of drug transporter expression and function in the proximal tubule. Pflugers Arch. 2012;464(6):601-11. Epub 2012/09/28. doi: 10.1007/s00424-012-1163-2. PubMed PMID: 23014881.

30. Ivanova EA, De Leo MG, Van Den Heuvel L, Pastore A, Dijkman H, De Matteis MA, et al. Endo-lysosomal dysfunction in human proximal tubular epithelial cells deficient for lysosomal cystine transporter cystinosin. PLoS One. 2015;10(3):e0120998. doi: 10.1371/journal.pone.0120998. PubMed PMID: 25811383; PubMed Central PMCID: PMCPMC4374958.

31. Vicinanza M, Di Campli A, Polishchuk E, Santoro M, Di Tullio G, Godi A, et al. OCRL controls trafficking through early endosomes via PtdIns4,5P(2)-dependent regulation of endosomal actin. Embo J. 2011;30(24):4970-85. doi: 10.1038/emboj.2011.354. PubMed PMID: 21971085; PubMed Central PMCID: PMCPMC3242071.

32. Bu GJ, Geuze HJ, Strous GJ, Schwartz AL. 39-Kda Receptor-Associated Protein Is an Er Resident Protein and Molecular Chaperone for Ldl Receptor-Related Protein. Embo J. 1995;14(10):2269-80. PubMed PMID: WOS:A1995QZ86200015.

33. Jansen J, Schophuizen CM, Wilmer MJ, Lahham SH, Mutsaers HA, Wetzels JF, et al. A morphological and functional comparison of proximal tubule cell lines established from human urine and kidney tissue. Exp Cell Res. 2014;323(1):87-99. Epub 2014/02/25. doi: 10.1016/j.yexcr.2014.02.011. PubMed PMID: 24560744.

34. Jonker JW, Smit JW, Brinkhuis RF, Maliepaard M, Beijnen JH, Schellens JHM, et al. Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan. J Natl Cancer I. 2000;92(20):1651-6. doi: DOI 10.1093/jnci/92.20.1651. PubMed PMID: WOS:000089830600009.

35. Pastan I, Gottesman MM, Ueda K, Lovelace E, Rutherford AV, Willingham MC. A Retrovirus Carrying an Mdr1 Cdna Confers Multidrug Resistance and Polarized Expression of P-Glycoprotein in Mdck Cells. P Natl Acad Sci USA. 1988;85(12):4486-90. doi: DOI 10.1073/pnas.85.12.4486. PubMed PMID: WOS:A1988N986900080.

36. El-Sheikh AA, van den Heuvel JJ, Koenderink JB, Russel FG. Interaction of nonsteroidal anti-inflammatory drugs with multidrug resistance protein (MRP) 2/ABCC2- and MRP4/ABCC4-mediated methotrexate transport. JPharmacolExpTher. 2007;320(1):229-35.

37. Wittgen HG, van den Heuvel JJ, van den Broek PH, Siissalo S, Groothuis GM, de Graaf IA, et al. Transport of the coumarin metabolite 7-hydroxycoumarin glucuronide is mediated via multidrug resistance-associated proteins 3 and 4.

Drug metabolism and disposition: the biological fate of chemicals. 2012;40(6):1076-9. Epub 2012/03/15. doi: 10.1124/dmd.111.044438. PubMed PMID: 22415933.

38. Dankers AC, Sweep FC, Pertijs JC, Verweij V, van den Heuvel JJ, Koenderink JB, et al. Localization of breast cancer resistance protein (Bcrp) in endocrine organs and inhibition of its transport activity by steroid hormones. Cell and tissue research. 2012;349(2):551-63. Epub 2012/05/15. doi: 10.1007/s00441-012-1417-5. PubMed PMID: 22581381; PubMed Central PMCID: PMC3414710.

39. Van Aubel RA, Koenderink JB, Peters JG, Van Os CH, Russel FG. Mechanisms and interaction of vinblastine and reduced glutathione transport in membrane vesicles by the rabbit multidrug resistance protein Mrp2 expressed in insect cells. Mol Pharmacol. 1999;56(4):714-9. Epub 1999/09/25. PubMed PMID: 10496953.

40. Muenster U, Grieshop B, Ickenroth K, Gnoth MJ. Characterization of substrates and inhibitors for the in vitro assessment of Bcrp mediated drug-drug interactions. Pharm Res. 2008;25(10):2320-6. Epub 2008/06/05. doi: 10.1007/s11095-008-9632-1. PubMed PMID: 18523872.

41. Videmann B, Tep J, Cavret S, Lecoeur S. Epithelial transport of deoxynivalenol: involvement of human P-glycoprotein (ABCB1) and multidrug resistance-associated protein 2 (ABCC2). Food Chem Toxicol. 2007;45(10):1938-47. Epub 2007/06/05. doi: 10.1016/j.fct.2007.04.011. PubMed PMID: 17543436.

42. Gozalpour E, Greupink R, Bilos A, Verweij V, van den Heuvel JJ, Masereeuw R, et al. Convallatoxin: a new P-glycoprotein substrate. Eur J Pharmacol. 2014;744:18-27. doi: 10.1016/j.ejphar.2014.09.031. PubMed PMID: 25264938.

43. Fardel O, Le Vee M, Jouan E, Denizot C, Parmentier Y. Nature and uses of fluorescent dyes for drug transporter studies. Expert Opin Drug Met. 2015;11(8):1233-51. doi: 10.1517/17425255.2015.1053462. PubMed PMID: WOS:000358976900005.

44. Huls M, Brown CD, Windass AS, Sayer R, van den Heuvel JJ, Heemskerk S, et al. The breast cancer resistance protein transporter ABCG2 is expressed in the human kidney proximal tubule apical membrane. Kidney Int. 2008;73(2):220-5. doi: 10.1038/sj.ki.5002645. PubMed PMID: 17978814.

45. Gozalpour E, Wittgen HG, van den Heuvel JJ, Greupink R, Russel FG, Koenderink JB. Interaction of digitalis-like compounds with p-glycoprotein. Toxicol Sci. 2013;131(2):502-11. doi: 10.1093/toxsci/kfs307. PubMed PMID: 23104431.

46. Pan C, Kumar C, Bohl S, Klingmueller U, Mann M. Comparative proteomic phenotyping of cell lines and primary cells to assess preservation of cell type-specific functions. Mol Cell Proteomics. 2009;8(3):443-50. doi: 10.1074/mcp.M800258-MCP200. PubMed PMID: 18952599; PubMed Central PMCID: PMCPMC2649808.

47. Dutta D, Donaldson JG. Search for inhibitors of endocytosis: Intended specificity and unintended consequences. Cell Logist. 2012;2(4):203-8. doi: 10.4161/cl.23967. PubMed PMID: 23538558; PubMed Central PMCID: PMCPMC3607622.

48. Takano M, Nakanishi N, Kitahara Y, Sasaki Y, Murakami T, Nagai J. Cisplatin-induced inhibition of receptor-mediated endocytosis of protein in the kidney. Kidney Int. 2002;62(5):1707-17. doi: 10.1046/j.1523-1755.2002.00623.x. PubMed PMID: 12371971.

49. Sidaway JE, Davidson RG, McTaggart F, Orton TC, Scott RC, Smith GJ, et al. Inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase reduce receptor-mediated endocytosis in opossum kidney cells. J Am Soc Nephrol. 2004;15(9):2258-65. doi:

10.1097/01. ASN.0000138236. 82706.EE. PubMed PMID: 15339975.

50. Wilmer MJ, Emma F, Levtchenko EN. The pathogenesis of cystinosis: mechanisms beyond cystine accumulation. Am J Physiol Renal Physiol. 2010;299(5):F905-16. doi: 10.1152/ajprenal.00318.2010. PubMed PMID: 20826575.

51. Nagai J, Takano M. Molecular aspects of renal handling of aminoglycosides and strategies for preventing the nephrotoxicity. Drug Metab Pharmacokinet. 2004;19(3):159-70. PubMed PMID: 15499183.

52. Lam AQ, Freedman BS, Bonventre JV. Directed differentiation of pluripotent stem cells to kidney cells. Semin Nephrol. 2014;34(4):445-61. doi: 10.1016/j.semnephrol.2014.06.011. PubMed PMID: 25217273.

53. Xia Y, Sancho-Martinez I, Nivet E, Rodriguez Esteban C, Campistol JM, Izpisua Belmonte JC. The generation of kidney organoids by differentiation of human pluripotent cells to ureteric bud progenitor-like cells. Nat Protoc. 2014;9(11):2693-704. doi: 10.1038/nprot.2014.182. PubMed PMID: 25340442.

54. Takasato M, Er PX, Chiu HS, Maier B, Baillie GJ, Ferguson C, et al. Kidney organoids from human iPS cells contain multiple lineages and model human nephrogenesis. Nature. 2015;526(7574):564-8. doi: 10.1038/nature15695. PubMed PMID: 26444236.

55. Morizane R, Lam AQ, Freedman BS, Kishi S, Valerius MT, Bonventre JV. Nephron organoids derived from human pluripotent stem cells model kidney development and injury. Nat Biotechnol. 2015. doi: 10.1038/nbt.3392. PubMed PMID: 26458176.

56. Jang KJ, Mehr AP, Hamilton GA, McPartlin LA, Chung S, Suh KY, et al. Human kidney proximal tubule-on-a-chip for drug transport and nephrotoxicity assessment. Integr Biol (Camb). 2013;5(9):1119-29. doi: 10.1039/c3ib40049b. PubMed PMID: 23644926.

57. Jansen J, De Napoli IE, Fedecostante M, Schophuizen CM, Chevtchik NV, Wilmer MJ, et al. Human proximal tubule epithelial cells cultured on hollow fibers: living membranes that actively transport organic cations. Sci Rep. 2015;5:16702. doi: 10.1038/srep16702. PubMed PMID: 26567716; PubMed Central PMCID: PMCPMC4644946.

CHAPTER 4

Proximal tubular efflux transporters involved in renal excretion of p-cresyl sulfate and p-cresyl glucuronide: Implications for chronic kidney disease pathophysiology

Henricus A.M. Mutsaers[1,2,3,4*], Pedro Caetano-Pinto[1,7*], Andries E.M. Seegers[1], Anita C.A. Dankers[1], Petra H.H. van den Broek[1], Jack F.M. Wetzels[5], Jan A.J.G. van den Brand[5], Lambertus P. van den Heuvel[3,6], Joost G. Hoenderop[2], Martijn J.G. Wilmer[1], Rosalinde Masereeuw[1,7]

[1]Departments of Pharmacology and Toxicology, [2]Physiology, [3]Pediatrics and [5]Nephrology, Radboud University Medical Center, Radboud Institute for Molecular Life Sciences, Nijmegen, The Netherlands, [4]Department of Pharmaceutical Technology and Biopharmacy, University of Groningen, Groningen, The Netherlands, [6]Department of Pediatrics, Catholic University Leuven, Leuven, Belgium [7]Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, The Netherlands,

*These authors contributed equally to the manuscript

Toxicology In Vitro. 2015 Oct;29(7):1868-77.
doi: 10.1016/j.tiv.2015.07.020

ABSTRACT

The uremic solutes *p*-cresyl sulfate (pCS) and *p*-cresyl glucuronide (pCG) accumulate in patients with chronic kidney disease (CKD), and might contribute to disease progression. Moreover, retention of these solutes may directly be related to renal tubular function. Here, we investigated the role of the efflux transporters Multidrug Resistance Protein 4 (MRP4) and Breast Cancer Resistance Protein (BCRP) in pCS and pCG excretion, and studied the impact of both solutes on the phenotype of human conditionally immortalized renal proximal tubule epithelial cells (ciPTEC).

Our results show that p-cresol metabolites accumulate during CKD, with a shift from sulfation to glucuronidation upon progression. Moreover, pCS inhibited the activity of MRP4 by 40% and BCRP by 25%, whereas pCG only reduced MRP4 activity by 75%. Moreover, BCRP-mediated transport of both solutes was demonstrated. Exposure of ciPTEC to pCG caused epithelial-to-mesenchymal transition, indicated by increased expression of vimentin and Bcl-2, and diminished E-cadherin. This was associated with altered expression of key tubular transporters.

In conclusion, BCRP is likely involved in the renal excretion of both solutes, and pCG promotes phenotypical changes in ciPTEC, supporting the notion that uremic toxins may be involved in CKD progression by negatively affecting renal tubule cell phenotype and functionality.

Key words: Efflux Transporters, nephrotoxicity, proximal tubule cells, uremic retention solutes, chronic kidney disease

INTRODUCTION

A hallmark of chronic kidney disease (CKD) is the retention and accumulation of a wide variety of potential toxic metabolites [1, 2] associated with the plethora of pathologies observed in uremic patients, including renal fibrosis and cardio-vascular disease [3, 4]. In the healthy population, these so-called uremic retentions solutes are cleared by the kidney via glomerular filtration and transporter-mediated tubular excretion. In early uremic toxin research, *p*-cresol, a phenol derived from tyrosine metabolism, was widely studied and a broad array of pathophysiological effects observed in CKD patients were contributed to this compound, including endothelial and immunological dysfunction [5, 6]. However, years ago, it was demonstrated by several groups that it is not *p*-cresol itself but the metabolites, *p*-cresyl sulfate (pCS) and *p*-cresyl glucuronide (pCG), that are retained during CKD [7-9]. *P*-cresol is formed in the gut during protein fermentation and is subsequently conjugated to either sulfate or glucuronic acid in the intestinal wall resulting in the formation of pCS or pCG, respectively [10]. In a study by Aronov *et al*., it was elegantly demonstrated that the colon indeed plays a key role in the production of pCS [11]. In addition, our group recently showed renal *p*-cresol metabolism and subsequent pCG formation in human proximal tubular cells [12]. Thus, the origin of pCS and pCG is reasonably well understood; however the molecular transport mechanisms involved in physiological urinary excretion of both *p*-cresol metabolites are not fully elucidated.

Whereas recent evidence shows that the human organic anion transporters 1 and 3 (hOAT1 and hOAT3) are involved in the uptake of pCS [13], both solutes have not yet been described as substrates for any particular efflux transporter. Two pumps that likely contribute to the renal excretion of the *p*-cresol metabolites are multidrug resistance protein 4 (MRP4) and breast cancer resistance protein (BCRP). Both pumps are transmembrane proteins from the ATP-binding cassette (ABC) transporters superfamily and are expressed in several tissues with a barrier function, including liver, intestine, brain and kidney. Both transporters are known to extrude a wide variety of drugs and endogenous compounds against steep concentration gradients [14, 15]. Previously, we reported that several uremic toxins, such as kynurenic acid, hippuric acid and indoxyl sulfate, inhibited substrate-specific transport by MRP4 and BCRP at clinically relevant concentrations [16]. Since pCS and pCG share structural characteristics with the previously studied uremic solutes, we hypothesized that both pumps are also involved in the transport of pCS and pCG into the proximal tubule lumen.

Since it was reported that pCS accumulates in CKD patients, it has been widely used as a model solute to elucidate the capacity of different dialysis modalities to remove protein-bound uremic toxins from circulation in CKD patients [17]. In addition, several studies have shown that pCS can contribute to the pathophysiology of CKD. For instance, pCS can increase endothelial micro-particles shedding by endothelial cells *in vitro*, indicating pCS involvement in endothelial dysfunction [17], it can contribute to inflammation [18],

it has pro-apoptotic effects [19], suppresses the expression of the renoprotective antiaging gene Klotho both *in vitro* and *in vivo* [20], and causes insulin resistance and metabolic disturbances associated with CKD in mice by activating the ERK1/2 pathway [21]. Furthermore, pCS is often studied in conjunction with indoxyl sulfate, a protein-bound uremic toxin derived from tryptophan, and it was recently reported by Kim *et al*. that indoxyl sulfate induces epithelial-to-mesenchymal transition (EMT) in proximal tubular cells [22]. Conversely, little is known about the biological activity of pCG and the nephrotoxic potential of both *p*-cresol conjugates.

This study aimed to elucidate the renal handling of pCS and pCG by investigating the interaction between both solutes and the renal efflux transporters MRP4 and BCRP. Moreover, the human renal proximal tubule cell line (ciPTEC) was used to investigate the potential contribution of both *p*-cresol metabolites to CKD progression related to tubular changes.

MATERIALS AND METHODS

Ethics statement and patient sample selection

The Radboud University Medical Centre ethical committee on research involving human subjects approved this study, and written informed consent was obtained from each patient and each healthy volunteer. Stored serum samples from chronic renal failure (CRF) patients were obtained from a random subset of the MASTERPLAN study population [23]. Patient characteristics were obtained from the case report forms. Samples for the hemodialysis patients were collected at the start of the dialysis session as a part of routine care and other lab parameters were obtained from electronic patient records.

Chemicals

All chemicals were obtained from Sigma (Zwijndrecht, The Netherlands) unless stated otherwise. Both pCS and pCG were kindly provided by Profs. R. Vanholder and G. Glorieux (University Hospital Ghent, Belgium). pCS was synthesized as a potassium salt as described previously [24]. pCG was produced from glucuronyl-trichloroacetimidate and *p*-cresol as previously described [25]. Since pCS and pCG were obtained as potassium and an ammonium salt, respectively, KCl and NH_4Cl solutions were used as control. [3′,5′,7′-^{3}H(*n*)]-methotrexate disodium salt ([^{3}H]-MTX) was purchased from Moravek (Brea, USA) and [6′,7′-^{3}H(*n*)]-estrone-sulfate ammonium salt ([^{3}H]-E1S) was obtained from Perkin Elmer (Groningen, The Netherlands).

High-performance liquid chromatography (HPLC)

Blood samples were obtained from 79 CRF patients during regular check-up, 12 patients with end-stage renal disease (ESRD) before hemodialysis and 4 healthy controls. Clinical characteristics of study subjects are listed in Table 1. None of the subjects had been

fasting at the time of blood sampling. Blood was collected in an EDTA Vacutainer and was immediately centrifuged at 3,000 x g for 10 min. Subsequently, plasma was collected and stored at -20°C. Before chromatography an aliquot of plasma was diluted in H_2O (1:1) and deproteinized with perchloric acid (final concentration 3.3% (v/v)). Next, samples were centrifuged at 12,000 x g for 3 min and 50 µl of the supernatant was injected into the HPLC-system (Spectra-Physics Analytical, Spectrasystem SCM400). For the detection of pCS and pCG, chromatography was performed on a C18 HPLC column (GraceSmart RP 18 5u 150 x 4.6 mm) with eluent A (95% (v/v) 50 mM KH_2PO_4 (pH 3.0) and 5% (v/v) acetonitrile) and eluent B (50 mM KH_2PO_4 (pH 3.0), methanol and acetonitrile in a 1:1:1 ratio) using the following gradient: 0-15 min, 100-20% eluent A; 15-16 min, 20-100% eluent A; 16-21 min, 100% eluent A. The flow rate was 1 ml/min and the *p*-cresol conjugates were detected at a wavelength of 220 nm. Standards of the compounds were also run in order to quantify the amount of metabolites found in the samples. Acquired data were processed with PC1000 software (Spectrasystem).

Transduction of Human Embryonic Kidney cells and membrane vesicle preparation

Human embryonic kidney (HEK293; purchased at American Type Culture Collection, Manassas, VA) cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen life sciences, Breda, The Netherlands) containing 10% (v/v) fetal calf serum (MP Biomedicals, Uden, The Netherlands) at 37°C in a 5% (v/v) CO_2 atmosphere. To functionally overexpress MRP4 and BCRP, cells were transduced with baculoviruses of human MRP4, BCRP or enhanced yellow fluorescent protein (eYFP; as a negative control), generated via the Bac-to-Bac system (Invitrogen) as previously described [26]. Subsequently, cell membranes were isolated and resuspended in isotonic buffer (10 mM Tris-HEPES and 250 mM sucrose, pH 7.4, adjusted with HEPES). Membrane vesicle were prepared via ultrafiltration as described previously [26]. Afterwards, vesicles were frozen in liquid nitrogen and stored at -80 °C.

Membrane vesicle transport and cellular uptake assays

A well-established rapid filtration technique [16, 26-28], was used to study the uptake of [^{3}H]-MTX and [^{3}H]-E1S into MRP4 or BCRP membrane vesicles, and the impact of both solutes on this process. In short, 25 µl of TS buffer containing 4 mM ATP, 10 mM $MgCl_2$ and radiolabeled substrate was added to 5 µl of the membrane vesicles (1.5 mg/ml). The transport assay was performed in the absence or presence of various concentrations of pCS or pCG. Transport was started by incubating the mixture at 37 °C for 1 min (BCRP) or 10 min (MRP4), as established [26, 29]. Uptake was stopped by placing the samples on ice and the addition of 150 µl ice cold TS buffer. Subsequently, the samples were transferred to a 96 well filter plate (Millipore, Etten-leur, The Netherlands) pre-incubated with TS buffer and

filtered using a Multiscreen HTS-Vacuum Manifold filtration device (Millipore). Afterwards, 2 ml of scintillation liquid was added to each filter and radioactivity was determined using liquid scintillation counting. As negative controls ATP was substituted for AMP and eYFP-membrane vesicles were used. Each experiment was performed in triplicate. The direct uptake of pCS and pCG by MRP4 and BCRP expressing vesicles was studied by incubating vesicles with pCS or pCG for 10 min at 37°C. After filtration, perchloric acid (PCA) 3.3% was used as solvent to extract the vesicles content. The concentration of pCS and pCG was subsequently determined by liquid chromatography/tandem mass spectrometry (LC/MS-MS). To this end, 10 µl of extraction was injected into the LC/MS-MS system (Thermo Fischer Scientific, Breda, The Netherlands). Separation was performed at a flow rate of 150 µl/min with eluent A (5 mM ammonium formate+0.01% (v/v) trifluoroacetic acid) and eluent B (50% acetonitrile), the eluted solutes were directly passed through a TSQ Vantage tandem mass spectrometer (Thermo Fischer Scientific, Breda, The Netherlands), with ion spray voltage of 4 kV, source temperature of 350°C and collision gas pressure of 1.5 bar. pCS and pCG and the internal standard 1-methyl-tryptophan were quantified. A calibration curve of pCS and pCG was made to quantify the amount in the samples and the results were corrected using the internal standard. The same method was used to determine the intracellular accumulation of pCS and pCG in ciPTEC following treatment with either 1 or 2 mM of the solutes for 48 h, in the absence or presence of the BCRP inhibitor KO143 (10 µM). Acquired data were processed with Thermo Xcaliber software (Thermo Fischer Scientific, Breda, the Netherlands). Net ATP-dependent uptake was also determined, as described above.

Proximal tubule cell culture

The ciPTEC line was generated as previously described by Wilmer *et al*. [30]. The cells were cultured in phenol red free DMEM/F12 (Gibco/Invitrogen, Breda, the Netherlands) supplemented with 10% (v/v) fetal calf serum (FCS; MP Biomedicals, Uden, The Netherlands), insulin (5 µg/ml), transferrin (5 µg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), epithelial growth factor (10 ng/ml), and tri-iodothyronine (40 pg/ml) at 33ºC in a 5% (v/v) CO_2 atmosphere. Propagation of cells was maintained by subculturing the cells at a dilution of 1:3 to 1:6 at 33°C. For all experiments, cells were cultured at 33ºC to 40% confluency, followed by maturation for 7 days at 37ºC. Experiments were performed on the cells between passages 30 and 40.

Flow cytometry

Protein expression of vimentin, E-cadherin and snail1 was analysed by flow cytometry. Briefly, ciPTEC were treated for 48 h with either pCS or pCG (0-2 mM). Cells were harvested using trypsin-EDTA and fixated with 4% paraformaldehyde/0.1% saponin. All staining were performed using a 1:100 antibody dilution and incubated for 30 min at room temperature. Primary antibodies used were: mouse-α-human Vimentin-PE; rat-α-human E-cadherin; mouse-α-human snail1 (Abcam, Cambridge, UK). And as secondary

antibodies we used: Alexa488 goat-α-rat for E-cadherin, and Alexa488 goat-α-mouse for snail1 (Life Technologies, Carlsbad, USA). Samples were acquired with a BD FACSCalibur (Becton Dickinson, Breda, The Netherlands). Analysis was performed using Flow Jo software (TreeStar, Ashland, USA), gating on viable cells.

Quantitative PCR

For gene expression, total RNA was isolated from ciPTEC exposed to pCS or pCG (0-2mM) for 48 h using an RNeasy Mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturers recommendations. Subsequently, cDNA was generated using the Omniscript RT-kit (Qiagen). Following cDNA-synthesis, quantitative PCR was performed using a CFX96 Real-Time PCR detection system (Bio-rad, Veenendaal, The Netherlands). GAPDH was used as housekeeping gene and relative expression levels were calculated as fold change using the $2^{-\Delta\Delta CT}$ method. The primer-probe sets were obtained from Applied Biosystems: GAPDH, hs99999905_m1; OATP4C1, hs00698884_m1; Bcl-2, hs00608023_m1; snail1, hs00195591_m1; BCRP, hs00184979_m1; MRP4, hs00195260_m1; KIM-1, hs03054855_g1; Vanin-1, hs01546812_m1.

Fluorescent microscopy

Morphological changes after exposure to solutes in ciPTEC were assessed using both standard bright field and fluorescent microscopy, using the fluorescent cytoplasm marker carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Life Technologies). Samples were incubated with CFDA-SE (10 μM) for 10 min prior to fixation with 4% paraformaldehyde. Images were acquired via a Leica DMI6000B-automated high-content microscope (Leica, Wetzlar, Germany).

Functional assays for BCRP and MRP activity

The function of BCRP and MRP4 was evaluated using fluorescent substrates that accumulate intracellular upon inhibition of the pump activity. Hoechst33342 was used to evaluate BCRP activity and glutathione methylfluorescein (GSMF), a bi-product of 5-Chloromethylfluorescein Diacetate (CMFDA; Life Technologies), to assess MRP4 activity. To investigate ciPTEC efflux activity, cells were seeded in 96 well plates and, after maturation, incubated for 30 min with 1.25 μM of the fluorescent substrates in Krebs buffer in absence or presence of a concentration range of a specific inhibitor for either BCRP (KO143) or MRP's (MK571). Hoechst33342 fluorescence was measured directly after incubation, whereas GSMF fluorescence was monitored after an additional 30 min at 37°C. To investigate pCS and pCG effect on transport activity, cells were seeded as described and treated for 48 h with either pCS or pCG (0-2 mM) prior to incubation with either Hoechst33342 or CMFDA (1.25 μM) for 30 min at 37°C. All fluorescent assays were performed in triplicate and measures performed in a Victor 3 Multilabel plate reader (Perkin Elmer, Waltham, USA).

Statistics

Statistics were performed using GraphPad Prism 5.02 via one-way analysis of variance (ANOVA) followed by Bonferroni's or Dunnett's Multiple Comparison Test, or by an unpaired Student's *t*-test. Differences between groups were considered to be statistically significant when $p<0.05$.

RESULTS

Accumulation of pCS and pCG and changes in p-cresol metabolism in CKD patients

Figure 1 illustrates that mean pCS levels markedly increased from 20 µM in healthy controls to 62 µM in non-dialysis CRF patients and to 116 µM in dialysis patients. The concentration of pCG increased from 0.3 µM (control) to 1 µM (CRF) and 30 µM (ESRD). In addition, HPLC revealed that the *p*-cresol metabolite fraction shifted from sulfation to glucuronidation with a pCG percentage of 1.3% in CRF patients and 21% in ESRD patients (Figure 1C). Note that with the PCA extraction method used, the compounds are stable in the extract for up to 16 h, where after degradation into p-cresol was observed (data not shown).

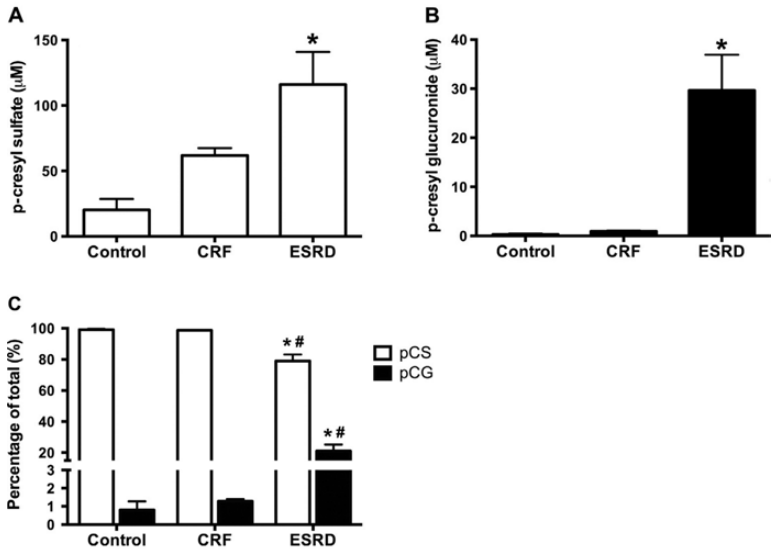


Figure 1. Accumulation of p-cresol metabolites during CKD. HPLC was used to measure the total plasma concentrations of (A) pCS and (B) pCG. Plasma samples were obtained from healthy volunteers (n=4) and patients with CRF (n=79) or ESRD (n=12). Standards of the compounds were also analyzed in order to quantify the amount of toxins found in the samples. Acquired HPLC data were processed with PC1000 software (Spectrasystem) and LC/MS data were processed with Xcaliber software (Thermo scientific). (C) Percentage pCS and pCS of total metabolites. Statistical analysis was performed via one-way ANOVA followed by the Dunnett's Multiple Comparison Test for each toxin. Results are presented as mean ± SEM. * = $p < 0.05$ compared to Control. # = $p < 0.05$ compared to CRF.

Apical transporters involved in pCS and pCG clearance

Next, we aimed to elucidate the transporters involved in the renal clearance of both *p*-cresol metabolites. Using membrane vesicles, the highest concentration tested (1 mM) of pCS inhibited MRP4-mediated [^{3}H]-MTX uptake with 40% and BCRP-mediated [^{3}H]-E1S uptake with 25% (Figure 2A-B). In contrast, pCG reduced [^{3}H]-MTX uptake by MRP4, in a concentration-dependent manner by 18% at 1 µM and 75% at 1 mM (Figure 2A). To identify whether pCG and pCS are substrates for MRP4 and BCRP, accumulation of the solutes in transporter-expressing vesicles was investigated. As shown in Figure 2C, we did not observe uptake of pCS in MRP4 expressing vesicles, whereas pCG appeared to be transported, although not significantly ($p = 0.07$). Conversely, direct uptake of both pCS and pCG in BCRP vesicles was clearly demonstrated (Figure 2D). Lastly, we set out to confirm these results in a more physiological model. Both pCS and pCG are taken up by ciPTEC as demonstrated by their accumulation in ciPTEC after exposure to either 1 or 2 mM of the solutes for 48 h (Figure 2E). Intracellular levels of pCS were determined to be 204 ± 65 and 344 ±1 11 ng/ml, respectively, while pCG levels were 239 ± 50 and 440 ± 174 ng/ml, respectively, indicating a dose-dependent uptake of both metabolites. Moreover, inhibition of BCRP activity by KO143 increased the intracellular levels of pCS (Figure 2E), further supporting the notion that this transporter is involved in renal pCS handling. In contrast, suppressing BCRP functionality did not alter intracellular pCG concentrations (Figure 2E), possibly due to MRP4-mediated transport of pCG. Nor did the MRP4 inhibitor, MK571, have any effect (data not shown). Taken together, these findings suggest that both *p*-cresol metabolites are actively transported by BCRP, and pCG appears to be both a substrate and a potent inhibitor of MRP4.

Table 1. Characteristics of study subjects.

	CRF	ESRD[a]	Control
Number	79	12	4
Age (years)	59 ± 14	55 ± 15	40 ± 12
Women (%)	29	25	50
Ureum (mmol/l)	12 ± 4	21 ± 7	ND
Creatinine (µmol/l)	171 ± 58	774 ± 242	ND
eGFR (ml/min/1.73 m^2) [b]	37 ± 14	NA	ND
Dialysis strategy	NA	11 HD, 1 CAPD	NA

Values are shown as mean ± SD. CRF, chronic renal failure; ESRD, end-stage renal disease; ND, not determined; NA, not applicable; HD, hemodialysis; CAPD, continuous ambulatory peritoneal dialysis. [a]Clinical data of 3 subjects was undisclosed, 8 samples were whole blood. [b]eGFR was calculated using the Modification of Diet in Renal Disease (MDRD) equation (www.nkdep.nih.gov).

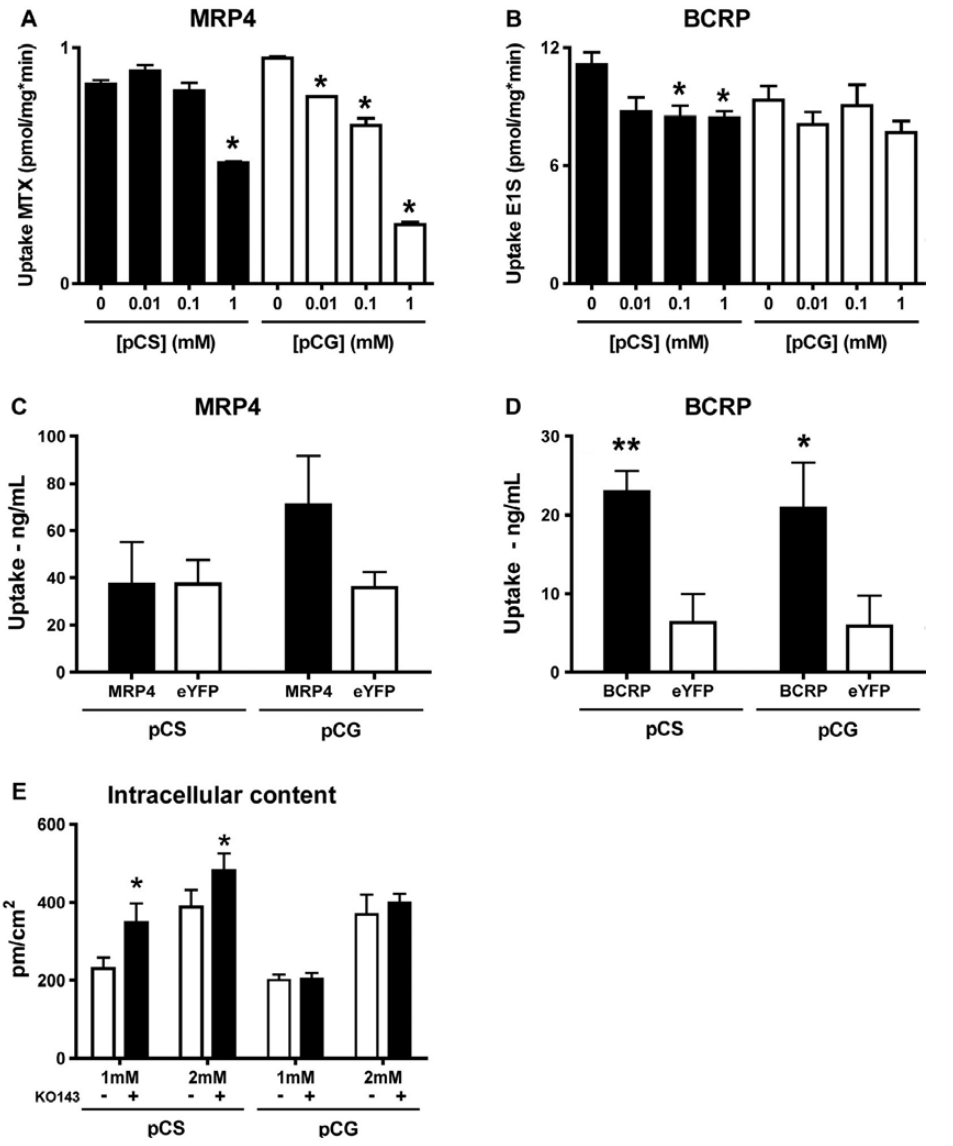


Figure 2. Uremic toxins are substrates and/or inhibit MRP4- and BCRP-mediated transport. A rapid filtration technique was used to study (A) MRP4-mediated [3H]-MTX uptake or (B) BCRP-mediated [3H]-E1S uptake into membrane vesicles in the presence of various concentrations of p-cresol metabolites. Radioactivity was determined using liquid scintillation counting. Results are presented as mean ± SEM of one representative experiment performed in triplicate. Statistical analysis was performed via one-way ANOVA followed by the Dunnett's Multiple Comparison Test for each toxin. Uptake of pCS and pCG into (C) MRP4- or (D) BCRP-overexpressing vesicles as determined by LC/MS-MS. (E) Intracellular content of pCS and pCG in ciPTEC after 48 h exposure, in the absence or presence of the BCRP inhibitor KO143, as determined by LC/MS-MS. Results are presented as mean ± SEM of three independent experiments performed at least in duplicate Statistical analysis was performed by an unpaired Student's t-test for each toxin. * =$p<0.05$ and ** =$p<0.01$ compared to eYFP transduced cells.

Induction of EMT by pCG

The toxicity of pCS has been widely investigated [20], however, little is known about the biological activity of pCG. Previously, our group demonstrated that pCS and pCG (1 and 2 mM) mitigated metabolic activity in ciPTEC, without affecting cell viability [12]. Here, we studied the impact of both solutes on ciPTEC phenotype emphasizing on EMT, a known consequence of pCS exposure [31]. Figure 3 shows that pCG concentration-dependently increased the expression of the mesenchymal marker vimentin (Figure 3A-D), while decreasing the expression of the epithelial marker E-cadherin, although this effect was not significant (Figure 3B-E). Snail is an important transcription factor involved in EMT and snail protein levels were unaltered following pCG exposure (Figure 3C-F), whereas, gene expression was significantly down-regulated (Figure 3G). Moreover, expression of the anti-apoptotic gene Bcl-2 was induced by pCG (Figure 3H), in concordance with transitioning epithelial cells being resistant to apoptosis [32]. Next, it was investigated whether pCS and pCG induced cell injury by studying the expression of two early markers of tubular damage: kidney injury molecule-1 (KIM-1) and vanin-1 [33, 34]. Figure 3I shows that exposure to pCG resulted in a slight reduction in KIM-1 expression while pCS had no effect. Furthermore, vanin-1 levels remained unchanged by either pCS or pCG (data not shown). These findings suggest that both solutes did not induce tubular cell damage, in line with the observed induction of EMT. In confirmation, treatment with pCG resulted in transformation of the typical epithelial cobblestone morphology into a more prolonged, fibroblast-like, morphology (Figure 3K-N).

Since pCG exposure led to loss of proximal tubular characteristics, gene expression of key transporters was investigated. Following treatment with pCG, the influx

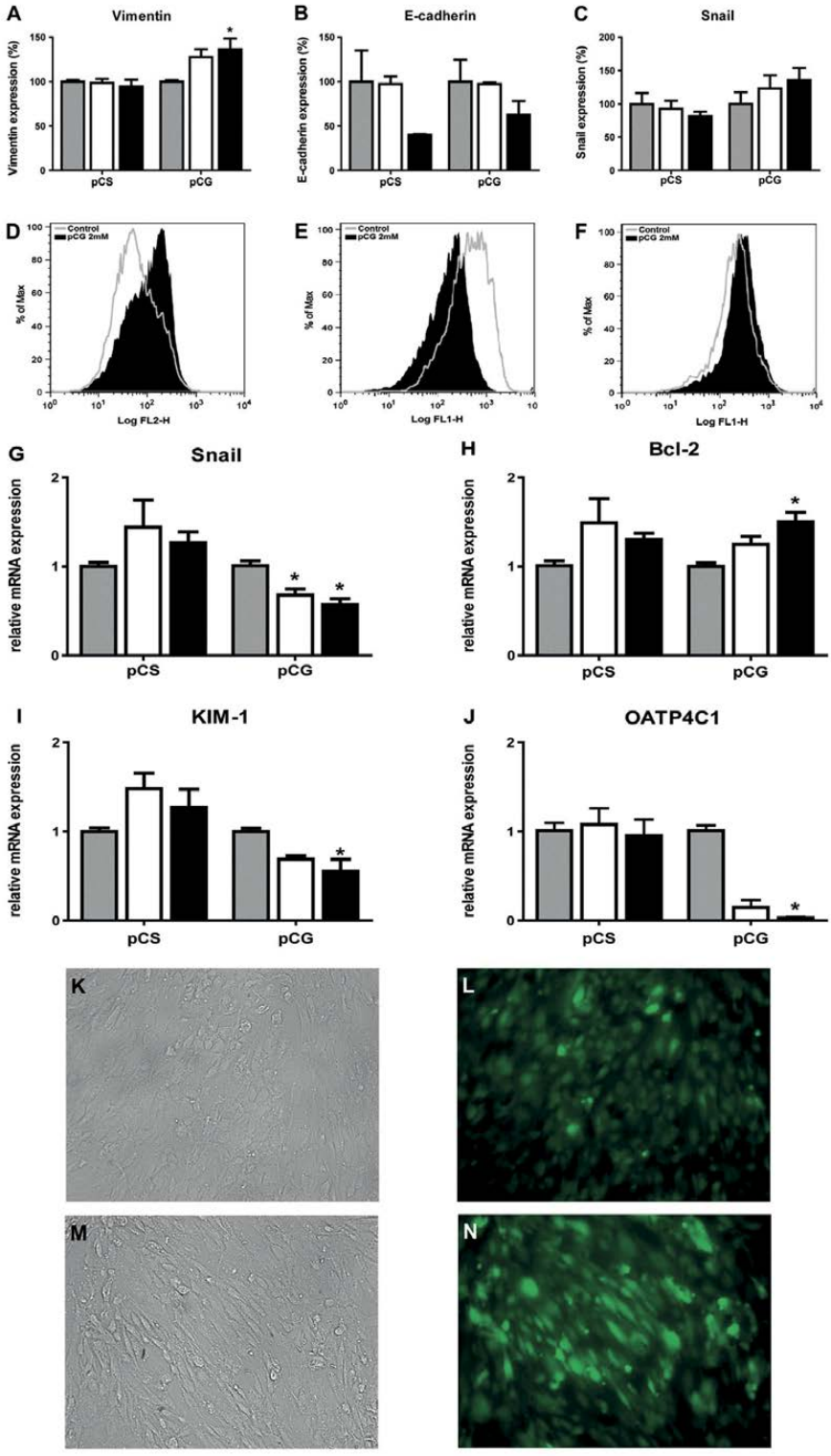
transporter potentially involved in p-cresol metabolite handling [35], organic anion transporting polypeptide (OATP4C1), was significantly down-regulated (Figure 3J), whereas expression of BCRP increased more than 2-fold (Figure 4A), consistent with effects previously reported upon renal stress or injury [36]. In addition, no changes in MRP4 mRNA levels were observed, as demonstrated in Figure 4B. Together with the observed induction of EMT, these results suggest that pCG exposure causes cell stress whereas pCS does not exert such biological activity in ciPTEC.

Exposure to pCG results in a functional up-regulation of BCRP

To investigate further the functional consequences of cell stress induced by pCG, the transport activity of BCRP and MRP4 was studied. Figure 4C shows an increase in fluorescence when cells were exposed to Hoechst33342 in presence of the BCRP inhibitor KO143, indicating inhibition of efflux. Similarly, exposing cells to CMFDA (precursor of GSMF) in presence of the MRP blocker MK571 also resulted in augmented fluorescence (Fig. 4D). Thus, fluorescent substrates are viable tools to monitor BCRP and MRP4 efflux activity in ciPTEC. Following exposure to pCG (2 mM, 48h) Hoechst33342 retention was approximately reduced by 50%, which corresponded to a 2-fold increase in BCRP

activity (Figure 4E). This effect is consistent with the observed increase in BCRP gene expression (Figure 4A). Moreover, no significant differences were observed in GSMF fluorescence (Figure 4F), also correlating with the lack of effect of pCG and pCS on MRP4 expression (Figure 4B). These findings further support the hypothesis that pCG promotes stress in cells.

Figure 3. Induction of EMT by pCS and pCG in ciPTEC. Cells were exposed for 48 h to salt control solution (grey bar), 1 mM (white bars) or 2 mM (black bars) of pCS or pCG. Following treatment, cells were harvested and stained with either (A,D) mouse-α-human Vimentin-PE, (B,E) rat-α-human E-cadherin or (C,F) mouse-α-human Snail. Representative histograms show the salt control (grey line) and the treatment with pCG 2mM (black) for (D) Vimentin, (E) E-cadherin and (F) Snail. Quantification of staining was done with a BD FACSCalibur flow cytometer using channel FL-2 or FL1 and analyzed with FlowJo software gating on viable cells. Furthermore, gene expression of (G) Snail, (H) Bcl-2, (I) KIM-1 and (J) OATP4C1 was studied using qPCR. GAPDH was used as reference gene and relative expression levels were calculated using the 2-ΔΔCT method. Representative images of cells incubated with either control salt solution (K,L) and 2 mM pCG (M,N), Images were acquired under bright field and fluorescent microscopy, cytoplasm was stained with CFDA-SE. Statistical analysis was performed via one-way ANOVA followed by the Bonferonni's Multiple Comparison Test for each toxin. Results are presented as mean ± SEM of at least three independent experiments performed in duplicate. * indicates $p<0.05$ compared with control.


A
Vimentin
Vimentin expression (%)
pCS
pCG
B
E-cadherin
E-cadherin expression (%)
C
Snail
Snail expression (%)
D
E
F
Control
pCG 2mM
% of Max
Log FL2-H
Log FL1-H
G
Snail
relative mRNA expression
H
Bcl-2
I
KIM-1
J
OATP4C1
K
L
M
N

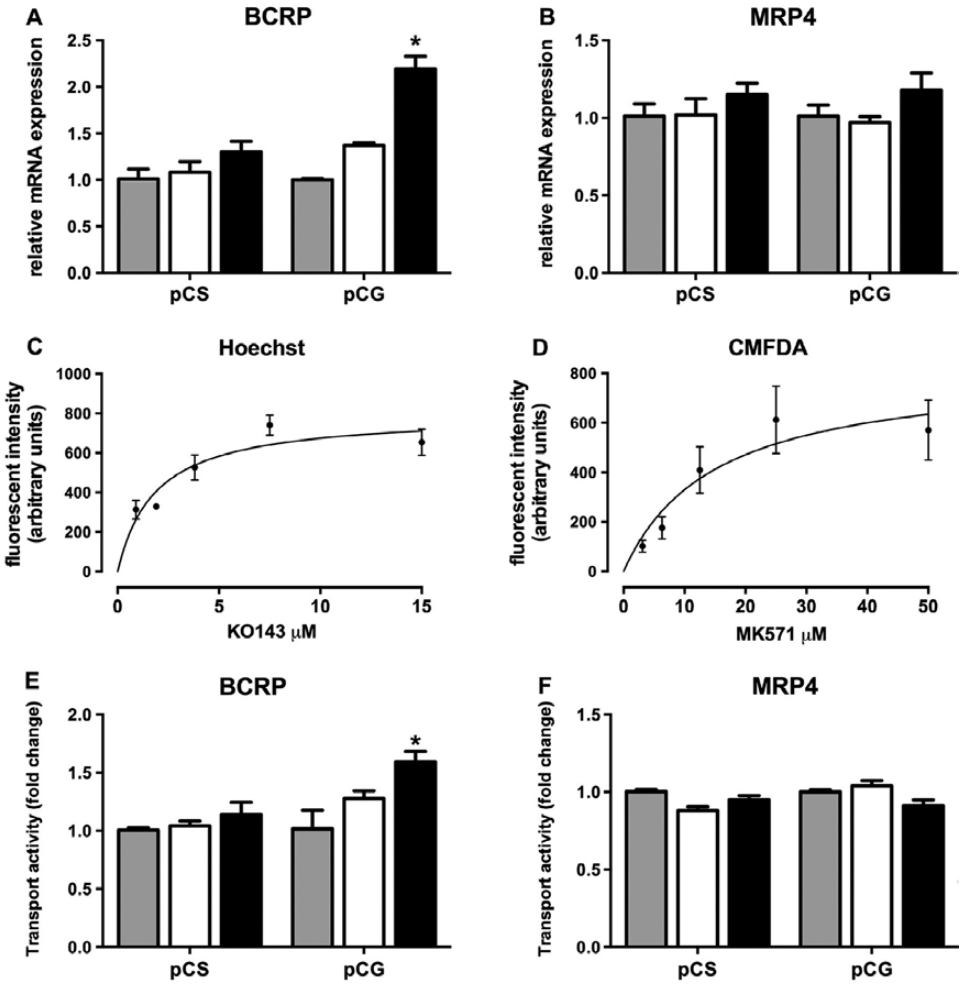


Figure 4. Impact of pCS and pCG on expression and function of apical transporters. To determine the impact of pCS and pCG, cells were exposed for 48 h to salt control solution (grey bar), 1 mM (white bars) or 2 mM (black bars) of pCS or pCG. Following treatment, ciPTEC were harvested and total mRNA was isolated. Afterwards, cDNA was synthesized and (A) BCRP and (B) MRP4 expression was studied using qPCR. GAPDH was used as housekeeping gene and relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. (C) To determine BCRP functional activity in ciPTEC, cells were incubated with Hoechst33342 (1.25 μM) in combination with a gradient of the inhibitor KO143, (D) for MRP activity cells were incubated with CMFDA (1.25 μM) and GSMF accumulation was measured in combination with a MK571 inhibitor gradient. Results are expressed in fluorescent intensity, compensated for background (arbitrary fluorescent units). Changes in transport activity of BCRP and MRP following pCS and pCG treatment were determined by incubating the cells, after the treatment, with (E) 1.25 μM Hoechst33342 and (F) 1.25 μM CMFDA. Results are expressed in activity fold compared to control (i.e. no toxin exposure). Statistical analysis was performed via one-way ANOVA followed by the Bonferonni's Multiple Comparison Test for each toxin. Values are shown as mean ± SEM of minimally two independent experiments performed in triplicate.

DISCUSSION

CKD is characterized by the accumulation of a diverse range of metabolites, which are normally cleared by the healthy kidney. Yet, it remains unclear which solutes are the true culprits in CKD progression and contribute to the myriad of comorbidities associated with the disease. The results of the present study demonstrate that the uremic retention solutes, pCS and pCG, accumulate in chronic kidney disease patients and pCG exposure causes phenotypical changes in renal proximal tubule cells. Furthermore, both pCS and pCG seem to depend on BCRP activity for their urinary secretion. It is well known that BCRP and MRP4 are of key importance for clearing a wide range of drugs and metabolites into the urine, and under uremic conditions their function may be affected, causing toxins to accumulate [16]. We also describe how exposure to pCG up-regulates BCRP activity, which may indicate an inducible response mechanism to cope with increased toxin levels that otherwise could lead to cellular stress. These results underline the importance of ABC transporters in the excretion of uremic toxins by proximal tubule cells and demonstrate how inactivity of these transporters may contribute to tubular damage and CKD progression by promoting pCS and pCG accumulation [37]. Moreover, the present findings provide the first support that pCG exposure results in EMT and cell stress, as was demonstrated by increased vimentin and Bcl-2 expression as well as decreased E-cadherin expression, accompanied by a change in morphology. Furthermore, we observed reduced levels of snail1 mRNA, whereas protein levels where unaffected by pCG. These results are in line with reports showing that in human kidney (HK-2) cells undergoing cyclosporine A-induced EMT, snail gene expression decreased while protein expression increased due to a reduced rate of protein degradation [38]. Furthermore, pCG caused a reduction in the expression of KIM-1, a marker associated with acute injury due to an inflammatory response [39], supporting the notion that pCG promotes EMT rather than inducing nephrotoxicity.

In contrast to the marked impact of pCG on ciPTEC phenotype, no changes were observed following exposure to pCS. Which is in stark disparity with previous results obtained in leucocytes [18]. This suggests that the toxicity of both *p*-cresol metabolites is cell type-specific. Also, in previous studies, the models used to investigate the impact of pCS on human renal cells, such as HK-2 cells, have limited physiological resemblance to proximal tubules [40]. Moreover, other reports showing that pCS evokes EMT in proximal tubules use murine models [41]. Still, it should be noted that the pCS and pCG concentrations used were rather high as compared to plasma concentrations. This was done because the ciPTEC used in the present study do not express the influx transporters OAT1 and OAT3 [42] that play a role in the uptake of both solutes [35, 37], yet we did clearly observe intracellular accumulation of both solutes in the cells. Furthermore, we believe that pCS and pCG mainly exert toxicity via an intracellular mechanism and until now the intracellular concentrations of both solutes during uremia are unknown. Nevertheless, we demonstrate that pCG might promote CKD progression by inducing phenotypical changes in human proximal tubule cells.

The paradigm of *p*-cresol as a uremic toxin of major importance was an artifact from strong acidification of plasma samples for deproteinization resulting in hydrolysis of both pCS and pCG [5]. In this study, PCA extraction was used as a method for protein removal, still the pCS levels determined are similar to the concentrations reported by several groups using different deproteinization methods including methanol extraction, enzymatic degradation or heating [7, 8, 17, 18]. Reports on pCG concentrations in CKD patients are scarce and considerably diverse. Our results corroborate the findings by Meert *et al*., suggesting that pCG levels are much higher than previously reported [18]. The observed change in glucuronidation might be caused by secondary factors, such as exposure to drugs or smoking, and warrants further clinical investigation beyond the scope of the present study. Taken together, our results indicate that acidification can be successfully used as a deproteinization method when measuring plasma levels of pCS and pCG; yet one has to take into account that the *p*-cresol conjugates are prone to hydrolysis.

In addition to endogenous metabolites, it is widely known that also drug disposition is altered in CKD patients [43, 44]. This includes the kinetics of drugs solely cleared via phase II metabolism [45-47], whose enzymes catalyze conjugation reactions such as glucuronidation, acetylation and sulfation. Recently, using ciPTEC, our group demonstrated that uremic toxins could diminish renal UDP-glucuronosyl transferase functionality, probably through interference with mitochondrial succinate dehydrogenase activity and by reducing the reserve capacity of the energy-generating oxidative phosphorylation system [12]. Simard *et al*., showed that exposing rat hepatocytes to uremic serum resultes in a decreased expression of N-acetyltransferases (NAT)1 and NAT2 [48]. Expression of both enzymes was also lower in the liver of CKD rats, which was accompanied by a reduction in NAT2-mediated acetylation of *p*-aminobenzoic acid [48]. However, little is known about sulfotransferase (SULT) expression and functionality in patients with renal failure. Together with the observed shift in *p*-cresol metabolite profile in this study, these findings provide the first evidence that SULT-mediated *p*-cresol metabolism is saturated or reduced in dialysis patients. Of interest, Sugimura *et al*. reported that SULT1C2 gene expression was reduced in an acquired polycystic kidney disease model in rats [49]. Still, the link between CKD and SULTs requires further investigation and may contribute to our understanding of the pathophysiology of CKD.

In conclusion, in this study the retention of pCS and pCG in CKD patients is reported and we provide the first evidence for the interference of both solutes with MRP4 and BCRP activity, with potential consequences for renal solute clearance. Moreover, we demonstrated active transport of pCS and pCG by BCRP. Furthermore, our results demonstrated that pCS does not exert nephrotoxic effects, whereas pCG induced cell stress and EMT, as well as changes in transporter expression in ciPTEC. These findings offer novel insights in the diverse contributions of both *p*-cresol conjugates to CKD pathophysiology.

ACKNOWLEDGEMENTS

This work was supported by the Dutch Kidney Foundation (grant number IK08.03; www.nierstichting.nl) and the FP7-PEOPLE-2011-ITN Nephrotools. M.J.G. Wilmer was supported by a grant from the Dutch Foundation (grant nr. 11.0023) and J.G. Hoenderop was supported by the Netherlands Organization for Scientific Research (016.130.668).

REFERENCES

1. Duranton F, Cohen G, De Smet R, Rodriguez M, Jankowski J, Vanholder R, et al. Normal and Pathologic Concentrations of Uremic Toxins. JAmSocNephrol. 2012;23(7):1258-70.
2. Vanholder R, De Smet R, Glorieux G, Argiles A, Baurmeister U, Brunet P, et al. Review on uremic toxins: classification, concentration, and interindividual variability. Kidney Int. 2003;63(5):1934-43. doi: 10.1046/j.1523-1755.2003.00924.x. PubMed PMID: 12675874.
3. Jourde-Chiche N, Dou L, Cerini C, gnat-George F, Vanholder R, Brunet P. Protein-bound toxins--update 2009. SeminDial. 2009;22(4):334-9.
4. Vanholder R, Van Laecke S, Glorieux G. What is new in uremic toxicity? PediatrNephrol. 2008;23(8):1211-21. PubMed PMID: 1557.
5. Vanholder R, Bammens B, de LH, Glorieux G, Meijers B, Schepers E, et al. Warning: the unfortunate end of p-cresol as a uraemic toxin. NephrolDialTransplant. 2011;26(5):1464-7.
6. Liabeuf S, Glorieux G, Lenglet A, Diouf M, Schepers E, Desjardins L, et al. Does p-cresylglucuronide have the same impact on mortality as other protein-bound uremic toxins? PloS one. 2013;8(6):e67168. doi: 10.1371/journal.pone.0067168. PubMed PMID: 23826225; PubMed Central PMCID: PMC3691113.
7. de Loor H, Bammens B, Evenepoel P, De Preter V, Verbeke K. Gas chromatographic-mass spectrometric analysis for measurement of p-cresol and its conjugated metabolites in uremic and normal serum. Clinical chemistry. 2005;51(8):1535-8. Epub 2005/07/26. doi: 10.1373/clinchem.2005.050781. PubMed PMID: 16040852.
8. Martinez AW, Recht NS, Hostetter TH, Meyer TW. Removal of P-cresol sulfate by hemodialysis. Journal of the American Society of Nephrology : JASN. 2005;16(11):3430-6. Epub 2005/08/27. doi: 10.1681/ASN.2005030310. PubMed PMID: 16120820.
9. Schepers E, Meert N, Glorieux G, Goeman J, Van der Eycken J, Vanholder R. P-cresylsulphate, the main in vivo metabolite of p-cresol, activates leucocyte free radical production. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2007;22(2):592-6. Epub 2006/10/17. doi: 10.1093/ndt/gfl584. PubMed PMID: 17040995.
10. Schepers E, Glorieux G, Vanholder R. The gut: the forgotten organ in uremia? Blood Purif. 2010;29(2):130-6.
11. Aronov PA, Luo FJ, Plummer NS, Quan Z, Holmes S, Hostetter TH, et al. Colonic Contribution to Uremic Solutes. JAmSocNephrol. 2011;22(9):1769-76.
12. Mutsaers HA, Wilmer MJ, Reijnders D, Jansen J, van den Broek PH, Forkink M, et al. Uremic toxins inhibit renal metabolic capacity through interference with glucuronidation and mitochondrial respiration. BiochimBiophysActa. 2013;1832(1):142-50.
13. Watanabe H, Sakaguchi Y, Sugimoto R, Kaneko KI, Iwata H, Kotani S, et al. Human organic anion transporters function as a high-capacity transporter for p-cresyl sulfate, a uremic toxin. Clinical and experimental nephrology. 2013. Epub 2013/11/05. doi: 10.1007/s10157-013-0902-9. PubMed PMID: 24185403.
14. Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, et al. Membrane transporters in drug development. NatRevDrug Discov. 2010;9(3):215-36.
15. Masereeuw R, Russel FG. Therapeutic implications of renal anionic drug transporters. Pharmacology & therapeutics. 2010;126(2):200-16. Epub 2010/03/17. doi: 10.1016/j.pharmthera.2010.02.007. PubMed PMID: 20227439.

16. Mutsaers HA, van den Heuvel LP, Ringens LH, Dankers AC, Russel FG, Wetzels JF, et al. Uremic toxins inhibit transport by breast cancer resistance protein and multidrug resistance protein 4 at clinically relevant concentrations. PloS one. 2011;6(4):e18438. Epub 2011/04/13. doi: 10.1371/journal.pone.0018438. PubMed PMID: 21483698; PubMed Central PMCID: PMC3070735.

17. Meijers BK, de LH, Bammens B, Verbeke K, Vanrenterghem Y, Evenepoel P. p-Cresyl sulfate and indoxyl sulfate in hemodialysis patients. ClinJAmSocNephrol. 2009;4(12):1932-8.

18. Meert N, Schepers E, Glorieux G, Van LM, Goeman JL, Waterloos MA, et al. Novel method for simultaneous determination of p-cresylsulphate and p-cresylglucuronide: clinical data and pathophysiological implications. NephrolDialTransplant. 2012;27(6):2388-96.

19. Poveda J, Sanchez-Nino MD, Glorieux G, Sanz AB, Egido J, Vanholder R, et al. p-Cresyl sulphate has pro-inflammatory and cytotoxic actions on human proximal tubular epithelial cells. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2014;29(1):56-64. Epub 2013/10/30. doi: 10.1093/ndt/gft367. PubMed PMID: 24166466.

20. Wu IW, Hsu KH, Hsu HJ, Lee CC, Sun CY, Tsai CJ, et al. Serum free p-cresyl sulfate levels predict cardiovascular and all-cause mortality in elderly hemodialysis patients--a prospective cohort study. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2012;27(3):1169-75. Epub 2011/09/06. doi: 10.1093/ndt/gfr453. PubMed PMID: 21891772.

21. Koppe L, Pillon NJ, Vella RE, Croze ML, Pelletier CC, Chambert S, et al. p-Cresyl sulfate promotes insulin resistance associated with CKD. JAmSocNephrol. 2013;24(1):88-99.

22. Kim SH, Yu MA, Ryu ES, Jang YH, Kang DH. Indoxyl sulfate-induced epithelial-to-mesenchymal transition and apoptosis of renal tubular cells as novel mechanisms of progression of renal disease. Lab Invest. 2012;92(4):488-98.

23. van Zuilen AD, Bots ML, Dulger A, van der Tweel I, van Buren M, Ten Dam MA, et al. Multifactorial intervention with nurse practitioners does not change cardiovascular outcomes in patients with chronic kidney disease. Kidney Int. 2012;82(6):710-7. doi: 10.1038/ki.2012.137. PubMed PMID: 22739979.

24. Feigenbaum J, Neuberg CA. Simplified Method for the Preparation of Aromatic Sulfuric Acid Esters. Journal of the American Chemical Society. 1941;63(12):3529-30. doi: doi: 10.1021/ja01857a508.

25. Van der Eycken E, Terryn N, Goeman JL, Carlens G, Nerinckx W, Claeyssens M, et al. Sudan-+¦-d-glucuronides and their use for the histochemical localization of +¦-glucuronidase activity in transgenic plants. Plant Cell Reports. 2000;19(10):966-70.

26. El-Sheikh AA, van den Heuvel JJ, Koenderink JB, Russel FG. Interaction of nonsteroidal anti-inflammatory drugs with multidrug resistance protein (MRP) 2/ABCC2- and MRP4/ABCC4-mediated methotrexate transport. JPharmacolExpTher. 2007;320(1):229-35.

27. Dankers AC, Sweep FC, Pertijs JC, Verweij V, van den Heuvel JJ, Koenderink JB, et al. Localization of breast cancer resistance protein (Bcrp) in endocrine organs and inhibition of its transport activity by steroid hormones. Cell Tissue Res. 2012;349(2):551-63.

28. Wittgen HG, van den Heuvel JJ, van den Broek PH, nter-Heidorn H, Koenderink JB, Russel FG. Cannabinoid type 1 receptor antagonists modulate transport activity of multidrug resistance-associated proteins MRP1, MRP2, MRP3, and MRP4. Drug Metab Dispos. 2011;39(7):1294-302.

29. Imai Y, Asada S, Tsukahara S, Ishikawa E, Tsuruo T, Sugimoto Y. Breast cancer

resistance protein exports sulfated estrogens but not free estrogens. MolPharmacol. 2003;64(3):610-8.

30. Wilmer MJ, Saleem M, Masereeuw R, Ni L, van der velden T, Russel FG, et al. A novel conditionally immortalized human proximal tubule cell line expressing functional influx and efflux transporters. Cell Tissue Res. 2010;339(2):449-57. PubMed PMID: 1433.

31. Vanholder R, Schepers E, Pletinck A, Nagler EV, Glorieux G. The uremic toxicity of indoxyl sulfate and p-cresyl sulfate: a systematic review. Journal of the American Society of Nephrology : JASN. 2014;25(9):1897-907. Epub 2014/05/09. doi: 10.1681/ASN.2013101062. PubMed PMID: 24812165; PubMed Central PMCID: PMC4147984.

32. Zeisberg M, Neilson EG. Biomarkers for epithelial-mesenchymal transitions. JClinInvest. 2009;119(6):1429-37.

33. Huo W, Zhang K, Nie Z, Li Q, Jin F. Kidney injury molecule-1 (KIM-1): a novel kidney-specific injury molecule playing potential double-edged functions in kidney injury. TransplantRev(Orlando). 2010;24(3):143-6.

34. Hosohata K, Ando H, Fujimura A. Urinary vanin-1 as a novel biomarker for early detection of drug-induced acute kidney injury. JPharmacolExpTher. 2012;341(3):656-62.

35. Mutsaers HA, Wilmer MJ, van den Heuvel LP, Hoenderop JG, Masereeuw R. Basolateral transport of the uraemic toxin p-cresyl sulfate: role for organic anion transporters? Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2011;26(12):4149. Epub 2011/10/26. doi: 10.1093/ndt/gfr562. PubMed PMID: 22025116.

36. Masereeuw R, Russel FG. Regulatory pathways for ATP-binding cassette transport proteins in kidney proximal tubules. The AAPS journal. 2012;14(4):883-94. Epub 2012/09/11. doi: 10.1208/s12248-012-9404-z. PubMed PMID: 22961390; PubMed Central PMCID: PMC3475864.

37. Masereeuw R, Mutsaers H, Toyohara T, Abe T, Jhawar S, Sweet D, et al. The Kidney and Uremic Toxin Removal: Glomerulus or Tubule? Seminars in Nephrology. 2014;34(2):191. Epub 2014.

38. Berzal S, Alique M, Ruiz-Ortega M, Egido J, Ortiz A, Ramos AM. GSK3, snail, and adhesion molecule regulation by cyclosporine A in renal tubular cells. ToxicolSci. 2012;127(2):425-37.

39. Wilkinson R, Wang X, Kassianos AJ, Zuryn S, Roper KE, Osborne A, et al. Laser capture microdissection and multiplex-tandem PCR analysis of proximal tubular epithelial cell signaling in human kidney disease. PloS one. 2014;9(1):e87345. doi: 10.1371/journal.pone.0087345. PubMed PMID: 24475278; PubMed Central PMCID: PMC3903679.

40. Jenkinson SE, Chung GW, van Loon E, Bakar NS, Dalzell AM, Brown CD. The limitations of renal epithelial cell line HK-2 as a model of drug transporter expression and function in the proximal tubule. Pflugers Archiv : European journal of physiology. 2012;464(6):601-11. doi: 10.1007/s00424-012-1163-2. PubMed PMID: 23014881.

41. Sun CY, Hsu HH, Wu MS. p-Cresol sulfate and indoxyl sulfate induce similar cellular inflammatory gene expressions in cultured proximal renal tubular cells. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2013;28(1):70-8. doi: 10.1093/ndt/gfs133. PubMed PMID: 22610984.

42. Jansen J, Schophuizen CM, Wilmer MJ, Lahham SH, Mutsaers HA, Wetzels JF, et al. A morphological and functional comparison of proximal tubule cell lines established from human urine and kidney tissue. Exp Cell Res. 2014;323(1):87-

99. doi: 10.1016/j.yexcr.2014.02.011. PubMed PMID: 24560744.

43. Dreisbach AW, Lertora JJ. The effect of chronic renal failure on drug metabolism and transport. ExpertOpinDrug Metab Toxicol. 2008;4(8):1065-74.

44. Sun H, Frassetto L, Benet LZ. Effects of renal failure on drug transport and metabolism. PharmacolTher. 2006;109(1-2):1-11.

45. Verbeeck RK. Glucuronidation and disposition of drug glucuronides in patients with renal failure. A review. Drug Metab Dispos. 1982;10(1):87-9.

46. Osborne R, Joel S, Grebenik K, Trew D, Slevin M. The pharmacokinetics of morphine and morphine glucuronides in kidney failure. ClinPharmacolTher. 1993;54(2):158-67.

47. Kim YG, Shin JG, Shin SG, Jang IJ, Kim S, Lee JS, et al. Decreased acetylation of isoniazid in chronic renal failure. ClinPharmacolTher. 1993;54(6):612-20.

48. Simard E, Naud J, Michaud J, Leblond FA, Bonnardeaux A, Guillemette C, et al. Downregulation of hepatic acetylation of drugs in chronic renal failure. JAmSocNephrol. 2008;19(7):1352-9.

49. Sugimura K, Tanaka T, Tanaka Y, Takano H, Kanagawa K, Sakamoto N, et al. Decreased sulfotransferase SULT1C2 gene expression in DPT-induced polycystic kidney. Kidney Int. 2002;62(3):757-62.

CHAPTER 5

Cetuximab prevents methotrexate-induced nephrotoxicity through epidermal growth factor dependent regulation of renal drug transporters

Pedro Caetano-Pinto[1], Amer Jamalpoor[1], Janneke Ham[2], Anastasia Goumenou[1], Monique Mommersteeg[3], Dirk Pijnenburg[3], Rob Ruijtenbeek[3], Bertrand van Zelst[4], Sandra Heil[4], Jitske Jansen[1], Martijn J. Wilmer[5], Carla M.L. van Herpen[2] and Rosalinde Masereeuw[1]

[1]Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University, The Netherlands
[2]Department of Oncology, Radboud university medical center, Nijmegen, The Netherlands
[3]PamGene, 's-Hertogenbosch, The Netherlands
[4]Department of Clinical Chemistry, ErasmusMC, Rotterdam, The Netherlands
[5]Department of Pharmacology and Toxicology, Radboud Institute of Molecular Life Sciences, Radboudumc, Nijmegen, The Netherlands

Submitted for publication: Molecular Pharmaceutics

ABSTRACT

The combination of methotrexate with epidermal growth factor receptor (EGFR) recombinant antibody, cetuximab, is currently being investigated in treatment of head and neck carcinoma. As methotrexate is cleared by renal excretion, we studied the effect of cetuximab on renal methotrexate handling. We used conditionally immortalized proximal tubule epithelial cells over-expressing organic anion transporter 1 (ciPTEC-OAT1) to examine OAT1, and the efflux pumps breast cancer resistance protein (BCRP), multidrug resistance protein 4 (MRP4) and P-glycoprotein (P-gp) in methotrexate handling upon EGF and/or cetuximab treatment. Protein kinase microarrays and knowledge-based pathway analysis were used to predict EGFR-mediated transporter regulation. Nephrotoxic effects of methotrexate were evaluated using the dimethylthiazol bromide (MTT) viability assay. Methotrexate inhibited OAT1-mediated fluorescein uptake, and decreased efflux of Hoechst33342 and glutathione-methylfluorescein (GS-MF), suggesting involvement of OAT1, BCRP and MRP4 in transepithelial transport, respectively. Cetuximab reversed the EGF-increased expression of OAT1 and BCRP, as well as their basolateral membrane expressions and transport activities, while MRP4 and P-gp were increased. Pathway analysis predicted cetuximab-induced modulation of PKC and PI3K pathways downstream of ERGF/ERBB2/PLCg. Pharmacological inhibition of ERK decreased expression of OAT1 and BCRP, while P-gp and MRP4 were increased. AKT inhibition reduced all transporters. Exposure of ciPTEC-OAT1 to methotrexate for 24 hours led to a decreased viability, an effect that was reversed by cetuximab.

In conclusion, cetuximab downregulates OAT1 and BCRP while upregulating P-gp and MRP4 through an EGFR-mediated regulation of PI3K-AKT and MAPKK-ERK pathways. Consequently, cetuximab attenuates methotrexate-induced nephrotoxicity, which may open possibilities for nephroprotective co-medication therapies.

Key words

Combination therapy, drug disposition, kinase signaling, drug transporters, renal proximal tubule.

INTRODUCTION

Current cancer treatments often rely on the administration of multiple chemotherapeutic drugs to improve overall survival. These combination therapies can overcome the limitations of single target drugs, as they exploit different action mechanisms and can be administered under several regimens, for curative and palliative purposes. Recurrent or metastatic squamous cell carcinoma of the head and neck (SCCHN) yields a poor prognosis, with limited treatment options, and a novel phase I/IIb trial (Identifier: NCT02054442; ClinicalTrials.gov) is set to investigate the efficacy and safety of methotrexate in combination with cetuximab.

Methotrexate is an established antifolate, and one of the most extensively used anticancer agents [1, 2]. It inhibits the enzyme dihydrofolate reductase, disrupting DNA synthesis [3], and can be administered in both high and low doses for the treatment of autoimmune diseases and cancers [4, 5]. High-dose methotrexate (i.e. 12,000 mg/m^2) is used in the treatment of malignancy, focusing on the prevention and management of toxicity, for example in osteosarcoma and in patients with high-risk lymphoma, while low-dose methotrexate (i.e. 40 mg/m^2) is used in metastatic SCCHN and in various nonmalignant immune-mediated disorders [6, 7]. Cetuximab is an anti-epidermal growth factor receptor (EGFR) recombinant monoclonal antibody (IgG1) [8] that displays beneficial clinical outcomes in patients with recurrent or metastatic SCCHN, and does not exert renal adverse effects [9]. It binds to the extracellular domain of EGFR and hinders the ligand-induced tyrosine kinase activation [10]. When active, multiple EGFR tyrosine-kinase domains can trigger down-stream signal transduction cascades, more specifically the phosphoinositide 3-kinase (PI3K)-AKT and mitogen activated protein kinase (MAPK) pathways [11]. These pathways are responsible for a series of intracellular regulatory processes such as cell cycle progression, neovascularization, migration, differentiation, proliferation and immunogenic responses [12].

The kidney, through active tubular secretion, clears a great variety of chemotherapeutics and among those is methotrexate. During this process, methotrexate accumulates within the kidney and, especially at high-dose, the drug can promote extensive necrosis of the proximal tubular epithelial cells (PTEC) [13, 14]. This is due to transporters expressed in PTEC that are involved in the excretion process of methotrexate. The drug is efficiently taken up from the blood compartment by the organic anion transporters 1 and 3 (OAT1-3) [15] and excreted into the tubular lumen by the multidrug resistance proteins (MRP) 2 and 4 [16] and breast cancer resistance protein (BCRP) [17]. In general, transmembrane transporters are key to renal function and their activity and expression can be regulated, amongst others via EGFR [18]. In the kidney, EGFR is expressed in PTEC, and activated by EGF and EGF-like hormones [19]. Furthermore, EGFR can stimulate renal epithelial regeneration in response to kidney injury [20].

Despite the wide use of methotrexate, a potential pharmacokinetic interaction with cetuximab when combined in therapy is not known. The aim of this study was to investigate

the effects of cetuximab on renal methotrexate handling using conditionally immortalized human PTEC overexpressing OAT1 (ciPTEC-OAT1) [21]. Previously, we demonstrated ciPTEC as a representative human proximal tubule cell line with preserved features, including cell polarization, monolayer organization, expression of tight junction proteins, as well as xenobiotic transporter and metabolic enzyme activities [22, 23]. Using this cell line, we revealed that cetuximab downregulates OAT1 and BCRP while it upregulates MRP4 via EGFR signaling, thereby reducing renal methotrexate uptake as well as its nephrotoxic potential. This appeared to be a common regulatory pathway, as cisplatin nephrotoxicity was counteracted as well, and opens possibilities for nephroprotective co-medication therapy.

METHODS

Chemicals

All chemicals were obtained from Sigma (Zwijndrecht, The Netherlands) unless stated otherwise. Stock solutions of all compounds used for transport assays were prepared according to specification in either dimethyl sulfoxide (DMSO) or dH_2O. Cetuximab (Erbitux®) was obtained from Merck Serono (Darmstadt, Germany).

Cell culture

The ciPTEC-OAT1 were cultured in phenol red-free DMEM/F12 (Invitrogen, Breda, The Netherlands), as previously described [24]. Cells were seeded at a density of 63,000 cell/cm^2, grown for 24 hours at 33°C and subsequently at 37°C for 7 days. ciPTEC required a temperature shift in order mature and grow into fully differentiated epithelial cells forming a tight monolayer, prior to each assay.

Cetuximab, methotrexate and cisplatin treatments

To study the effects of cetuximab, matured ciPTEC were treated for 24 or 48 hours by incubation with or without cetuximab (500 µg/mL) in culture medium, in the presence or absence of EGF (10 ng/mL). After cetuximab exposure, cells were incubated with either increasing concentrations of methotrexate or cisplatin (up to 100 µM) for 24 hours, using standard ciPTEC culture medium (CM; containing 10% fetal calf serum) or serum-free medium (SFM). Under normal conditions, ciPTEC medium contained EGF which was considered as control condition. Composition of ciPTEC medium is described in the supplementary information.

Fluorescent functional assays

The retention of fluorescent substrates was used to determine the changes in activity of membrane transporters. Fluorescein, Hoechst33342, calcein-AM, 5-chloromethyl fluorescein diacetate (CMFDA) and (4-(4-(dimethylamino) styryl)-*N*-methylpyridinium

(ASP^+) were used to evaluate the function of OAT1, BCRP, P-gp and MRP4 and OCT2, respectively, as described [21, 25]. To study the effect of methotrexate on these transporters, cells were exposed to a single concentration of the substrates (fluorescein and calcein-AM: 1 μM; Hoechst33342 and CMFDA: 1.25 μM), separately, or together with methotrexate. Model inhibitors probenecid, KO143, MK571 and PSC833 were used to validate transporter involved. To investigate cetuximab influence on transport function, a two-fold step dilution was performed to obtain a concentration gradient of the fluorescent substrates with maximum concentrations of 25 μM each. The calcein-AM assay was performed in the presence of 2.0 μM of inhibitor PSC833 and CMFDA in the presence of 5.0 μM of MK571. Cells were washed twice before incubation at 37°C for 10 minutes (fluorescein), 30 minutes (CMFDA, Hoechst33342 and ASP^+) or 60 minutes (calcein-AM), as described previously . Afterwards plates were washed twice and cells were lysed with either 0.1 M NaOH (fluorescein) or Triton-X100 1% (calcein-AM and CMFDA). Subsequently, fluorescence was acquired via an Ascent Fluoroskan FL microplate reader (Appropriate filter settings - wavelengths: fluorescein, calcein and glutathione-methyl fluorescein (GS-MF; the end-metabolites of calcein-AM and CMFDA, resp.); excitation: 494 nm, emission: 512 nm. Hoechst33342; excitation: 350 nm, emission: 461 nm. ASP^+; excitation: 470 nm, emission: 590 nm)

Gene expression

Transporters gene expression profiling was performed by isolating total RNA from cells grown in 6 well plates, using an RNeasy Mini kit (Qiagen, Venlo, The Netherlands), according to the manufacturers specifications. Subsequently, cDNA was synthesized using the Omniscript RT-kit (Qiagen). Subsequently quantitative PCR was performed in a CFX96 Real-Time PCR detection system (Bio-rad, Veenendaal, The Netherlands). GAPDH was used as housekeeping gene for normalization and relative expression levels were calculated as fold change using the $2^{-\Delta\Delta CT}$ method. The primer-probe sets were obtained from Applied Biosystems: GAPDH - hs99999905_m1; BCRP - hs00184979_m1; MRP4 - hs00195260_m1 and Pgp - hs00184500_m1; OAT1 - hs00537914.

Western Blot analysis

The protein levels of OAT1 and BCRP were determined by Western blotting using 9% (W/V) sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE). Cell samples were homogenized in ice-cold Tris-Sucrose (TS) buffer (10 mM Tris-HEPES and 250 mM sucrose, pH 7.4) containing protease inhibitors (PMSF 100 μM, Aprotinin 5 μg/ml, Leupeptin 5 μg/ml, Pepstatin 1 μg/ml, E64 10 μM). Membrane fractions were obtained by high shear passage using a microfluidizer LV1 (Microfluidics™, Westwood, AM, USA), and cell lysates were span for 20 and 90 minutes at 4,000 and 25,000 rcf at 4˚C, respectively. Membranes were incubated with mouse anti-BCRP (1:200 dilution; Abcams®, Cambridge, UK) and rabbit anti-OAT1 antibody (1:100 dilution; Abcams®, Cambridge,

UK) over night at 4˚C. As a loading control, rabbit anti-Na, K-ATPase antibody (α-subunit, 1:4,000 dilution, C356-M09, [26]) was used. Secondary antibodies, Alexa fluor® 680 goat anti-rabbit IgG (1:10,000 dilution; Life Technologies Europe BV), streptavidin Alexa fluor® 680 (1:10,000 dilution; Life Technologies Europe BV) and IRDye 800 goat anti-rabbit IgG (1:10,000 dilution; Rockland, PA). Fluorescence was detected using the Odyssey scanner CLx (Li-Cor Biosciences, USA). Data were normalized to protein expression levels of the loading control using ImageJ software (imagej.nih.gov).

Serine/Threonine and Tyrosine Kinase microarrays and pathway analysis

To investigate downstream cetuximab effects on ciPTEC, the serine/threonine kinase (STK) and protein tyrosine kinase (PTK) activity profiles were determined with 3D dynamic peptide microarrays. Using the PamStation12 platform and PamChip® microarrays, containing 4 identical arrays each, with 142 either STK or PTK immobilized phosphorylation sites, cetuximab treated ciPTEC samples were analyzed as previously described [27]. Cells were grown in 6 well plates, after maturation, cells were washed and incubated with cetuximab either in the presence or absence of EGF for 15 minutes in Hank's balanced salt solution (HBSS). Cell pellets were lysed using mammalian protein extraction reagent (MPER) in the presence of HALT™ phosphatase and protease inhibitors (Pierce) according to manufacturer instructions. Resulting cell lysates were profiled as described previously [28, 29]. The data was analyzed using Evolve and BioNavigator (signal acquisition and data analysis software, respectively; Pamgene) to generate heat maps and differential kinase activity hits by comparing ratios over the control condition (+EGF). Significantly relevant peptide phosphorylations (targets/hits) were selected and used to generate canonical pathway signaling hypotheses by performing an unbiased analysis using the Genego MetaCore™ (thomsonreuters.com) software and data base resources.

Intracellular methotrexate metabolite quantification by LC-MS/MS

In order to determine methotrexate uptake by ciPTEC, the intracellular levels of methotrexate polyglutamates were analyzed following 48 hours cetuximab pre-treatment and 24 hours methotrexate exposure in both CM and SFM. Cells were washed, harvested and cell pellets were frozen in liquid nitrogen and stored at -80°C prior to analysis. Samples were analyzed as previously described [30]. Briefly, 16% perchloric acid was used to precipitate protein, after a centrifugation step, 10 µL (per sample) of supernatant was analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS) using a Waters Acquity BEH C18 column and a 5-100% gradient of 10 mM ammonium bicarbonate pH10 and methanol on a classic Acquity UPLC (Waters Instruments). Detection was performed during a 6 minute run by positive electrospray ionization using a Quattro Premier XE (Waters Instruments).

Cell cycle analysis

For cell cycle analysis, cells were grown in 6 well plates and following treatment cells were harvested using accutase, washed and centrifuged before fixation with cold 70% (v/v) ethanol, for 1 hour. Subsequently the cells, in suspension, were stained with a propidium iodide (PI) solution containing 40 µg/mL PI, 100 µg/mL RNase A and 0.1% (v/v) Triton X-100, for 45 minutes. The data was retrieved using a BD FACSCanto II flow cytometer and analyzed using the FlowLogic® software (Chromocyte, Sheffield, UK).

Cell viability

Cell survival upon methotrexate or cisplatin exposure was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were washed twice and incubated with 5 mg/mL MTT (100µL p/well) for a minimum of 2 hours at 37°C, after washing wells are dissolved in 200µL DMSO (p/well) and absorbance read via a BioRad iMark microplate reader (absorbance: 550-600 nm). Cisplatin toxicity was also evaluated by the Presto-blue (PB) assay, cells were incubated for 1 hour with 1:10 dilution PB solution (100 µL p/well) at 37°C. Subsequently the supernatant was transferred to a 96 well plate and absorbance read using Jasco FP8300 Spectrophotometer (excitation wavelength: 560 nm, emission wavelength: 590 nm).

Data analysis

Transport activity was calculated by normalizing fluorescence intensity, expressed in arbitrary units (a.u.), to baseline values (no inhibitor) after background subtraction, as described [25]. Inhibition of efflux activity led to increased total fluorescence, therefore, efflux activity is depicted as the inverse of the fold increase in fluorescence. Non-linear analysis according to Michaelis-Menten kinetics was performed using GraphPad Prism 5.02 (GraphPad software, San Diego, CA, USA). Differences between groups were considered to be statistically significant when $p<0.05$ using a one-tailed Student's t-test. All data are presented as mean ± SEM.

RESULTS

Renal excretion of methotrexate is mediated by OAT1, BCRP and MRP4

Methotrexate clearance has been studied in humans and rodents *in vivo,* and in cell lines expressing a single transporter; however, its handling in a renal cell model containing multiple relevant transporters has not been performed. We first examined the renal excretion route for methotrexate in ciPTEC-OAT1 in an indirect way by measuring the interaction of methotrexate with marker substrates fluorescein, GS-MF and Hoechst33342 for OAT1, MRP4 and BCRP, respectively [21, 25]. Model inhibitors were used as positive control and the fluorescence levels without inhibition were normalized to

100%. Upon methotrexate exposure, OAT1, BCRP and MRP's activities were significantly reduced (Fig. 1A-C), whereas P-gp activity was not affected (Fig. 1D), confirming a renal excretion pathway previously suggested (Fig. 1E).

Cetuximab regulates tubular xenobiotic transport

Next, renal drug transporter expression and its activity were determined after treating ciPTEC with cetuximab. Exposure to cetuximab for 48 hours resulted in an altered expression and function of all major transport systems present in ciPTEC-OAT1 as compared to the standard culture conditions in presence of EGF. The gene expression of OAT1 was reduced in the absence of EGF and/or in presence of cetuximab, which was also observed for BCRP (Fig. 2A and C). In agreement, protein expression levels of OAT1 and BCRP were reduced in the absence of EGF (Fig. 2A and C). Both MRP4 and P-gp mRNA levels were increased in the absence of EGF as well as in the presence of cetuximab (Fig. 2E and G), for which we did not determine their protein expression. The accumulation of fluorescein, Hoechst33342, calcein and GS-MF were reduced upon cetuximab exposure both in the presence or absence of EGF (Fig. 2B, D, F and H). This effect was similar to that observed after culturing cells for 48 hours in absence of EGF. When cetuximab was given in absence of EGF, no further alterations were observed. Functional changes were evidenced from the accumulation of the fluorescent probes, in which a higher intensity yielded more accumulation of the probe. For efflux pumps, a higher activity yielded a reduction in substrate accumulation given an increased rate of removal, as was observed for GS-MF and calcein. The reduction in BCRP was not reflected by an increased Hoechst33342 retention, most likely due to the increased expression in P-gp which can also secrete Hoechst33342 [25]. After nonlinear regression analysis according to Michaelis-Menten kinetics, parameters were calculated and presented in Table 1. No significant changes in IC_{50} values were found for all conditions tested (Table 1), implicating that the results observed relate to changes in expression levels of the transporters rather than changes in affinities.

Cetuximab activates serine/tyrosine signaling down-stream of EGFR

To investigate the down-stream effects of cetuximab-EGFR signaling in ciPTEC, kinase activity profiling using peptide microarrays comprising either 144 PTK or STK phosphosite was performed (PamChip®), and used to generate an interaction network. Cells were treated with cetuximab for 15 minutes in the presence and absence of EGF. Overall, cetuximab activated PTK and STK. In the presence of EGF, STK activity decreased, an effect that was counteracted by cetuximab co-incubation (Fig. 3A). Subsequently, significantly modulated targets (phosphorylated peptides) were selected and evaluated with the MetaCore™ software. Analysis provided a list of known pathways that most significantly overlap with the PTK and STK targets determined for cetuximab. Results

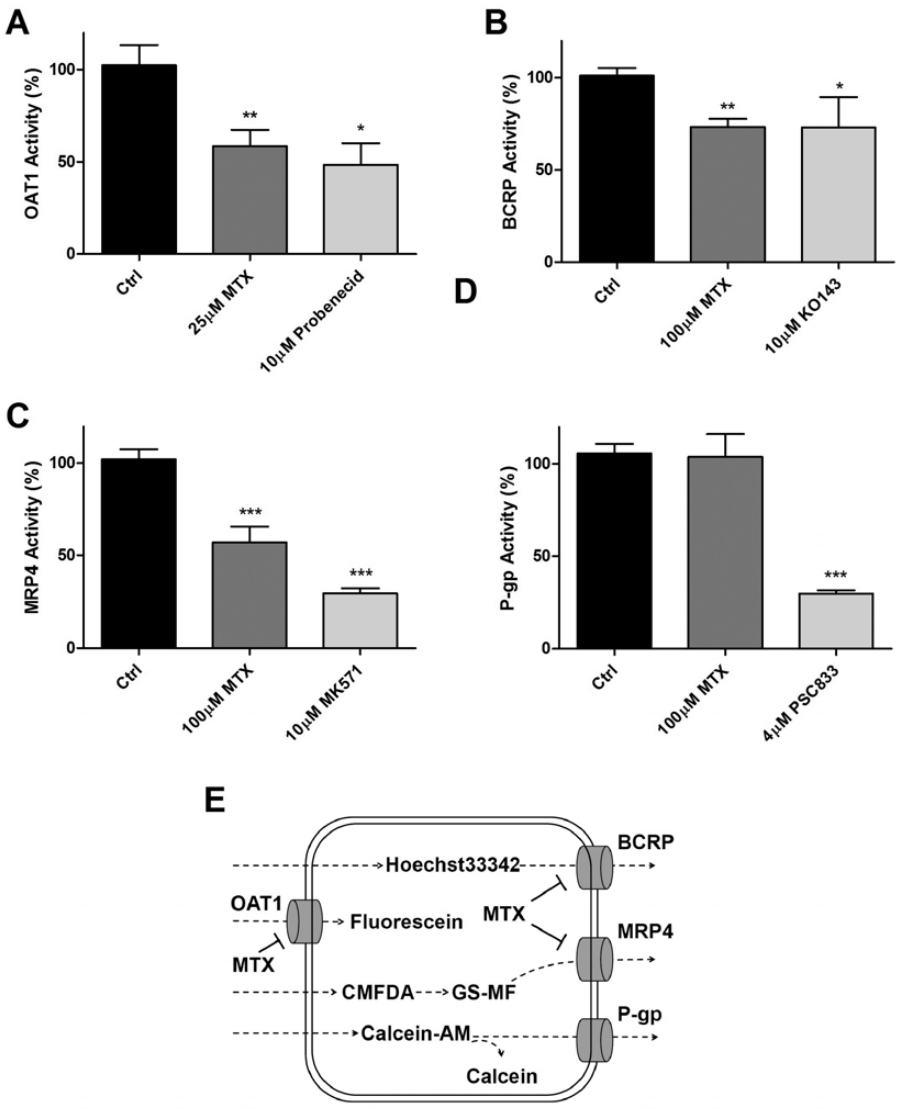


Figure 1. Methotrexate uptake in ciPTEC-OAT1. Methotrexate (MTX) significantly reduced the uptake of fluorescein (A) and inhibited the efflux of Hoechst33342 (B) and GS-MF (C). Calcein efflux was not blocked by methotrexate (D). Data are presented as mean values +/- SEM. Statistical analysis was performed via unpaired Student's *t*-test. *$p < 0.05$ and ***$p < 0.01$ compared to control (CTRL). A schematic depiction of the potential interactions (E).

show that the signaling pathways triggered by cetuximab mainly involve tyrosine kinases, while EGF mostly affects serine kinases, underlining the stark differences between both conditions. An in-depth scrutiny into the predicted pathways (Fig. 3C) revealed that in the control condition (+EGF) the key regulatory elements are: cAMP-dependent protein kinase A (PKA), serine/threonine specific kinase C (PKC), downstream transcription factor

Creb1 (cAMP responsive element binding protein 1) and the nuclear factor kappa light polypeptide gene enhancer (NF-kβ1). On the other hand, cetuximab exposure reduced EGFR, receptor tyrosine-protein kinase (Erbb2) and PKC activity, while enhancing phospholipase C-γ (PLCγ), phosphatidylinositol-4,5-bisphosphate 3-kinase (P13K) and phosphoinositide dependent protein kinase (PDK). There was also a direct link between EGFR inhibition and MRP4 enhanced phosphorylation. Additional results from the kinomic and pathway analyses are presented as supplementary information. Further down, EGFR signal transduction targeted the AKT and MAPK/ERK pathways implicated in regulating the expression of OAT1, BCRP, P-gp and MRP4. When cells were exposed to LY294002, an AKT inhibitor, the expression of all transporters was reduced (Fig. 3D). Whereas the ERK inhibitor U-0126 reduced the expression of OAT1 and BCRP, and upregulated the expression of P-gp and MRP4 (Fig. 3B).

Cetuximab reduces methotrexate uptake and ameliorates nephrotoxicity

Although high-dose methotrexate appears to be nephrotoxic *in vivo*, ciPTEC were only moderately sensitive to methotrexate, with a reduction in cell viability of about 70-80% at the highest concentration tested (Fig. 4). In agreement with its effect on the functional expression of the renal drug transporters, a 24 hours cetuximab pre-treatment effectively reduced the intracellular concentration of methotrexate-polyglutamates 1 (Fig. 4A). Cell cycle analysis showed that after 48 hours cetuximab treatment, G0/G1 phase (resting phase) increased from 75 ± 4% to 81 ± 6% (Fig. 4B). A similar effect as observed when cells were cultured in the absence of EGF (Fig. 4C). When exposed to increasing concentrations of methotrexate, ciPTEC viability reduced as measured by mitochondrial activity (Fig. 4D). However, when cells were exposed for 24 hours to 100 μM methotrexate followed by a 24 hours recovery period, the reduction in cell viability was reversed. This was independent of cetuximab treatment (Fig. 4E). In cells treated with cetuximab for 24 hours prior to a 48 hours methotrexate treatment the nephrotoxic effects of methotrexate could be prevented. A similar effect was observed after 24 hours pre-treatment with the ERK inhibitor, U-0126 (Fig. 4F).

EGFR-mediated signaling is also involved in cisplatin-induced nephrotoxicity

To investigate whether the attenuation in nephrotoxicity by cetuximab was specific for methotrexate or a more common phenomenon in tubule epithelial cells, we investigated the role of EGFR signaling on cisplatin-induced nephrotoxicity. Cisplatin is another agent used in SCCHN combination therapy that, unlike methotrexate, is taken up by PTEC via the organic cation transporter 2 (OCT2) and can lead to programmed cell death [31]. Similar to the effect observed for methotrexate, pretreatment with cetuximab for 24 hours ameliorated cisplatin-induced toxicity. Cisplatin treatment alone reduced viability to

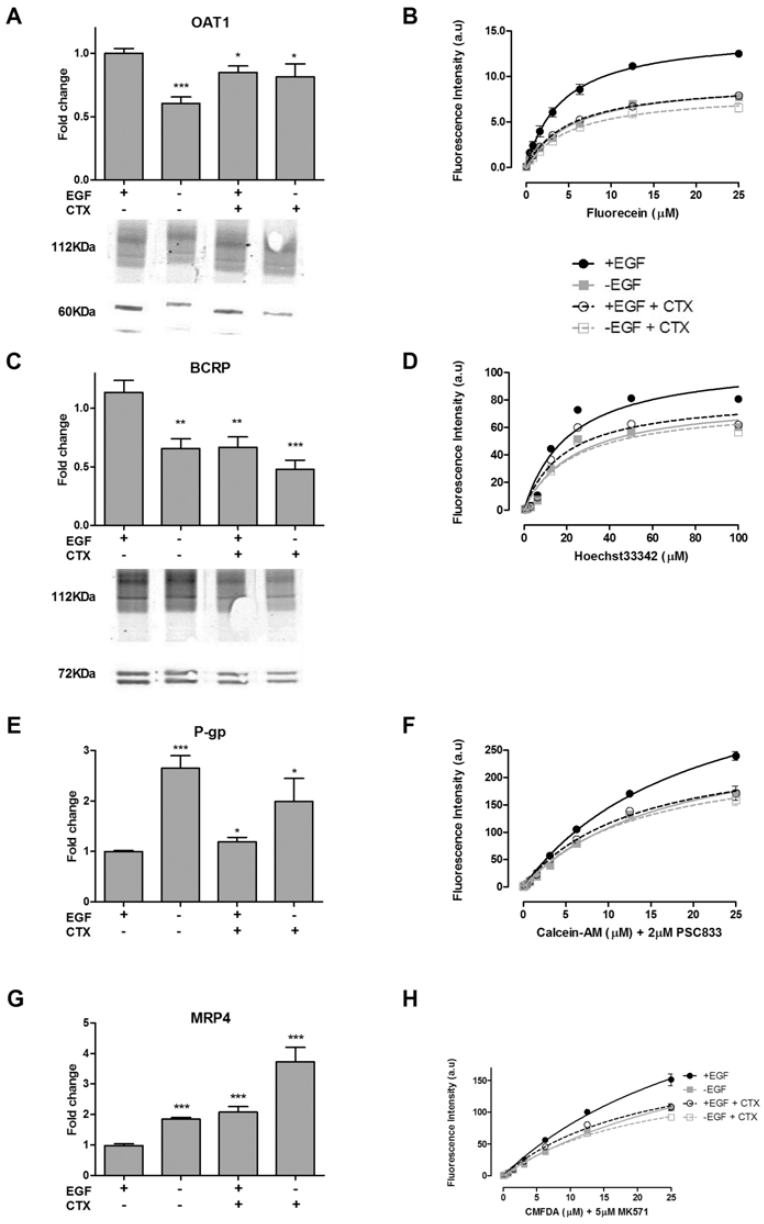


Figure 2. Expression and function of renal drug transporters upon Cetuximab pre-treatment. Cells were pre-treated with cetuximab (CTX) before gene and protein expression was determined. The activity of renal drug transporter was evaluated using fluorescent substrates (OAT1, fluorescein; BCRP, Hoechst33342; P-gp, calcein and MRP4; GS-MF, respectively). CTX effectively reduced the expression and activity of OAT1 (A, B) and BCRP (C,D), while increasing the expression and activity of P-gp (E,F) and MRP4 (G,H). Protein expression was determined for OAT1 (60 kDa) and BCRP (72 kDa) (A, C lower panel), Na,K-ATPase was used as loading control (112 kDa). Data are presented as mean values +/- SEM, n=3. Statistical analysis was performed via unpaired Student's *t*-test. *$p < 0.05$ and ***$p<0.01$ compared to control (+EGF condition).

Table1. Nonlinear regression analysis of transport activity of fluorescent substrates after 48 hours exposure to cetuximab.

	+EGF		-EGF		+EGF+CTX		-EGF+CTX	
	Km (μM)	Vmax (a.u.)	Km (μM)	Vmax (a.u.)	Km (a.u.)	Vmax (a.u.)	Km (μM)	Vmax (a.u.)
Fluorescein	4.3 ± 0.5	14.7±0.5	5.6±0.5	9.6±0.3	5.1±0.6	9.4±0.4	5.4±0.8	8.2±0.5
Hoechst 33342	21.3±4.3	108.7±8.1	23.8±4.6	81.0±6.0	19.15±4.3	82.7±6.6	22.4±4.3	76.2±5.5
Calcein-AM	36.8±6.3	378.7±43.6	38.4±6.7	272.8±32.4	22.4±3.3	209±18.0	22.7±3.8	178.3±17.5
CMFDA	20.2±1.5	436.5±18.1	17.5±1.6	296.6±14.8	13.0±1.6	267.2±15.8	13.5±1.4	250.4±13.1

Data is expressed as mean values +/- SEM of a minimum of 2 independent assays performed in triplo. A.u. indicates arbitrary units.

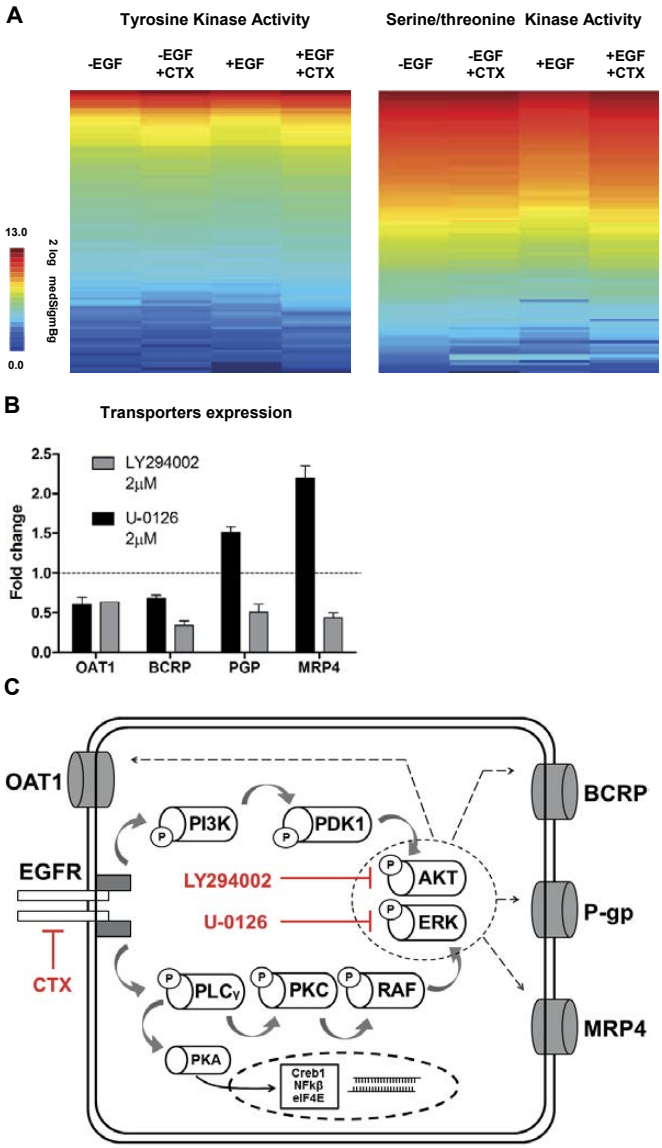


Figure 3. Cetuximab mediated regulation of EGFR downstream signaling. Cetuximab (CTX) promoted the activation of STK and phospholipases leading to PI3K-AKT and MAPK-ERK regulation of renal drug transporters. Data in B is expressed as mean values +/- SEM of a minimum of 2 independent assays performed in triplo.

64±5% and when preceded by cetuximab, viability was maintained at 91±10% (Fig. 5A). Increasing concentrations of cisplatin resulted in a severe loss of mitochondrial activity in ciPTEC-OAT1, where a maximum concentration of 100 μM resulted in near total activity loss after 6 hours exposure followed by a 72 hours recovery period. Cetuximab pre-

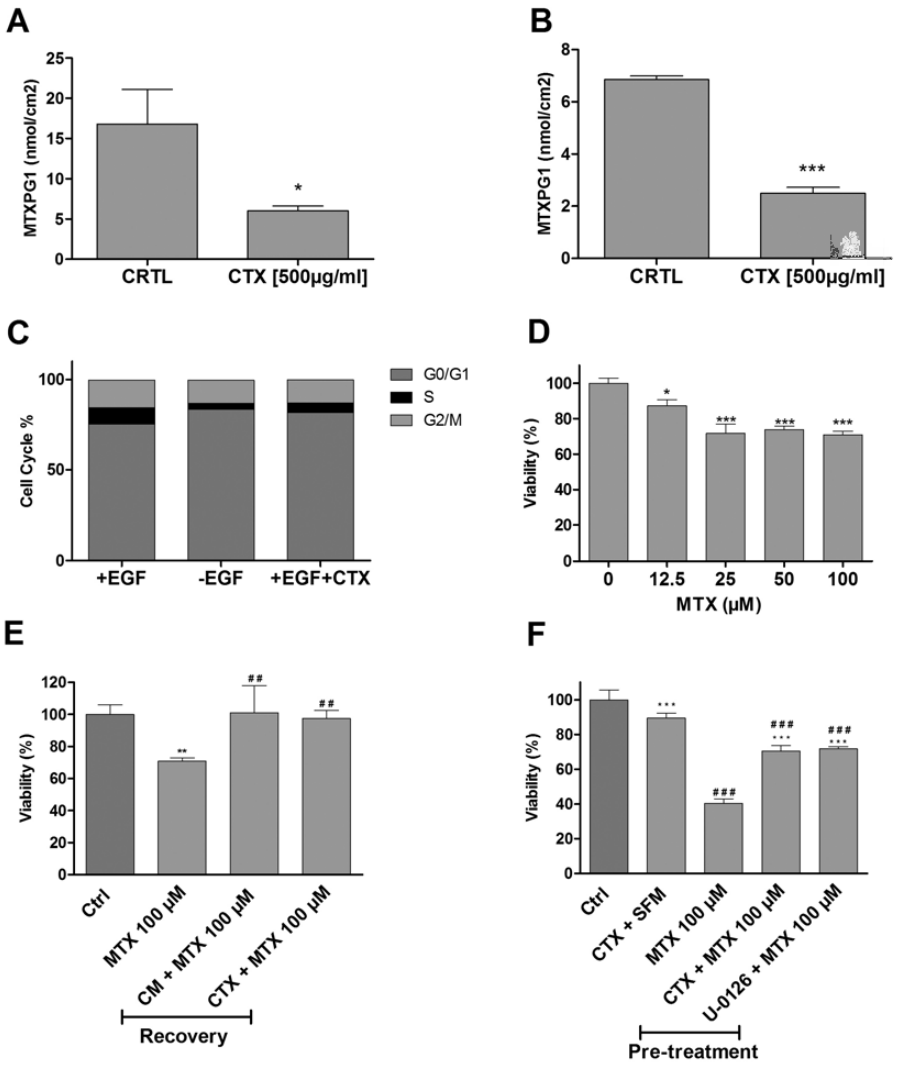


Figure 4. Cetuximab reduced methotrexate-induced nephrotoxicity. Exposure to cetuximab (CTX) for 24h reduced the uptake of methotrexate (MTX) in both CM (A) and SFM (B). CTX pre-treatment effectively rests cell cycle progression (C). For cell viability assessments, cells were exposed to methotrexate (0-100 µM) for 24h in SFM (D), MTX (100 µM) for 24h in SFM, followed by 24h of treatment with CM (E) or CTX. Cells were preconditioned for 24h in SFM with CTX or U-0126 (2 µM), followed by 48h of exposure to MTX (100 µM) (F). MTT assay was then performed. Results are expressed in viability (%) compared to control (i.e. SFM-treated cells). CTX pre-treatment effectively reduced MTX uptake and toxicity. Values are shown as mean ± SEM of minimally two independent experiments performed in triplicates. * significantly different from control ($p < 0.05$). # significantly different from MTX (100 µM; $p < 0.05$).

treatment did not exert any tangible effect on cell viability. On the other hand, the absence of EGF resulted in reduced cisplatin toxicity compared to the condition with EGF, revealing TC_{50} values of 44±10 μM compared to 32±18 μM, respectively (Fig. 5B). Furthermore the uptake of ASP^+, a well-known OCT2 model substrate [32], decreased upon cetuximab treatment (Fig. 5C), suggesting the transporter is regulated through EGFR signaling as well.

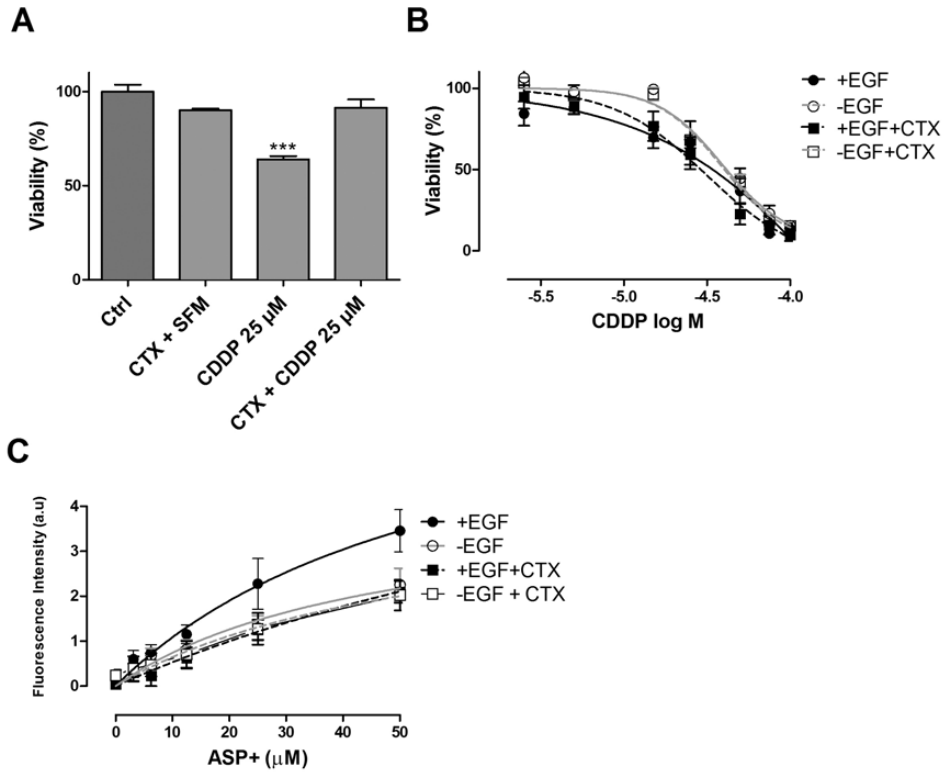


Figure. 5. Cetuximab reduced cisplatin-induced nephrotoxicity. Cell viability in the presence of cisplatin (CDDP), was determined after cetuximab (CTX) pre-treatment for 24hours in SFM, followed by exposure to CDDP (25 μM) for 24hours. MTT assay was then performed (A). Preconditioning of cells with CTX decreased CDDP nephrotoxicity. Results are expressed in viability (%) compared to control (i.e. SFM-treated cells). CDDP toxicity after 72hours recovery in CM and CTX was determined after 48 hours CTX pre-treatment followed by 6hours of increasing CDDP concentrations (0-100μM) (B) CDDP recovery was performed in CM to minimize cell stress. The absence of EGF ameliorated CDDP toxicity. The accumulation of ASP^+ ($OCT2$ substrate) was reduced upon CTX pre-treatment(C). Values are shown as mean ± SEM of minimally two independent experiments performed in triplicates. * significantly different from control ($p<0.05$).

DISCUSSION

In the present study, we revealed the mechanistic interactions between methotrexate and cetuximab affecting renal methotrexate transport activity and cytotoxic potency. Our results show that cetuximab changes drug transporter function through EGFR signaling in proximal tubule cells. The changes in transport activity attenuated the nephrotoxic effects of methotrexate and cisplatin. These findings shed light on the potential of using EGFR signaling modulation as a target to prevent renal toxicity in combination chemotherapy.

Nephrotoxicity is a major concern in various chemotherapeutic regimens, and often a dose-limiting factor. The delayed elimination of methotrexate in the course of high-dose methotrexate chemotherapy leads to a considerable accumulation of the drug in proximal tubules, greatly exceeding plasma levels up to 100-fold [33]. Severe renal toxicity is hence derived from the intra-tubular precipitation of methotrexate crystals, despite its anti-folate properties [34].

In proximal tubules, xenobiotic transporters play a key role in drug excretion and disposition. Acting as selective carriers, they remove the majority of xenobiotics and protein-bound drugs from the blood stream [35]. We demonstrated competition between methotrexate and fluorescein for OAT1-mediated uptake. Upon entering the cells, methotrexate is metabolized through the addition of glutamate residues by folylpolyglutamate-synthetase [36], of which methotrexate-polyglutamate 1 was measured intracellularly. Furthermore, we confirmed that BCRP as well as MRP4 are involved in cellular excretion of methotrexate, demonstrating that its renal tubular excretion pathway suggested can be mimicked *in vitro* using ciPTEC [37].

Cetuximab influences the activity of renal uptake as well as efflux transporters involved in methotrexate excretion. Cetuximab downregulated OAT1 and BCRP, while upregulating MRP4 and P-gp, predominantly through ERK mediated signaling downstream EGFR. The receptor is highly expressed in renal tubular cells and key to their physiology, as demonstrated by its involvement in *e.g.* electrolyte homeostasis by adjusting trans-epithelial resistance and tight-function configuration, and controlling sodium and magnesium reabsorption [38]. Changes in gene expression of the transporters after cetuximab exposure are reflected by their protein levels and, subsequently, at the functional level as a result of a transcriptional regulation of the transport systems. Moreover, cetuximab treatments showed parallels with the effects determined in the absence of EGF, further specifying the role of EGFR in renal xenobiotic transporter regulation.

By kinomic and pathway analysis [39] we determined that under standard culturing conditions (in the presence of EGF), the main signaling pathways involve STK activity resulting in the regulation of PKC and PKA. In line with our findings, activation of PKC has previously been shown to affect cellular distribution of OAT1 by promoting ubiquitination of the transport protein. This results in an accelerated internalization of the transporter from cell surface to intracellular compartments and a reduction in Vmax [40-42]. Similarly, OCT2 activity can be modulated by a phosphor-tyrosine switch and stimulation by EGF

is abolished by MAPK and PKA inhibition [43, 44]. Moreover, BCRP expression can be regulated by the axis EGFR – AKT – ERK – CREB, whereas P-gp function and membrane traffic is dependent on PKC and PKA-mediated phosphorylation [45, 46].

Cetuximab treatment led to an extensive shift in the kinase-mediated signaling cascades, most dominantly on PTK activity (thus upstream or initial signaling). Pathway analysis of the significant activity modulations indicated the involvement of PLC and PI3K, underlying the role of phospholipases in this pathway, leading to modulation of PKC and PDK. Here, the PI3K-AKT pathway reporting phosphosites indicated downregulation of the mechanistic target of rapamycin (mTOR) kinase and eukaryotic translation initiation factor 4E (eIF4E). In a seemingly reductant route, PLC-mediated PKC downregulation also results in RAS-mediated ERK and MAPK downregulation. The network of interactions predicted for cetuximab action in ciPTEC-OAT1 are in line with what has been previously described in cancer models [47]. Inhibition of AKT and ERK changed the expression of OAT1, BCRP, MRP4 and P-gp in the same fashion as when cells were exposed to cetuximab, further implicating these pathways in regulating the activity of renal drug transporters (Fig. 3C). The modulation of transcription factors (Creb1, NFKβ, eIF4E) revealed in our analysis could hypothetically account for the transcriptional changes in expression observed after 48 hours exposure to cetuximab.

Although high-methotrexate doses have been reported nephrotoxic, methotrexate toxicity in proximal tubule cells *in vitro* is limited. This is partly explained by the absence of a tubular lumen in which methotrexate can precipitate leading to tubular obstruction and eventually tubulopathy [34]. Moreover, ciPTEC-OAT1 are matured differentiated cells that show little proliferation which contributes further to the reduced sensitivity to methotrexate, as the drug preferentially targets proliferating and actively dividing cells. Nonetheless, cetuximab pre-treatment prevented methotrexate-induced toxicity in cells grown without serum. Under serum deprivation conditions cell cycle progression can be stimulated [48], which sensitized the cells to methotrexate. Remarkably pre-treatment with U-0126 also prevented methotrexate-related toxicity, re-enforcing the role EGFR mediated MAPK/ERK signaling plays in nephrotoxicity by regulating transport activity in the proximal tubule. Noteworthy, ciPTEC-OAT1 are immortalized and transduced to overexpress OAT1, which may have implications for its active regulatory pathways in this cell model. Nonetheless, our pathway analysis is consistent with previously reported results with respect to the regulation of drug transporters.

The consequences of EGFR cascades inhibition with positive outcomes for renal damage have been reported previously in rats, *in vivo* [49]. Here, we provide direct evidence implicating EGFR in drug transport regulation through PKA and PKC signaling, thereby suppressing methotrexate and cisplatin nephrotoxicity *in vitro*. While cetuximab combined with cisplatin and 5-fluorouracil improves cancer treatment outcome [50], improved efficacy of cetuximab combined with low-dose methotrexate is under investigation. As regimens of methotrexate used in the treatment of SCCHN can potentially lead to

nephrotoxicity, but especially nephrotoxicity of high-dose methotrexate in the curative treatment of osteosarcoma, this study underscores the potential of cetuximab and EGFR inhibition in nephro-protection. Further implications for drug distribution are underlined by the pronounced changes in transport activity promoted by cetuximab, in particular at the efflux side of proximal tubule cells.

ACKNOWLEDGMENTS

This work was supported by the Nephrotools consortium under the scope of EU FP7 Marie Curie actions (grant nr. 289754) and a Dutch Kidney Foundation grant (grant nr. KJPB 11.023). We thank Milos Mihajlovic and Sam Hariri for their insights and assistance with the FACS analysis performed for this study.

REFERENCES

1. Jolivet J, Cowan KH, Curt GA, Clendeninn NJ, Chabner BA. The pharmacology and clinical use of methotrexate. The New England journal of medicine. 1983;309(18):1094-104. Epub 1983/11/03. doi: 10.1056/nejm198311033091805. PubMed PMID: 6353235.
2. Bleyer WA. Methotrexate: clinical pharmacology, current status and therapeutic guidelines. Cancer treatment reviews. 1977;4(2):87-101. Epub 1977/06/01. PubMed PMID: 329989.
3. Rajagopalan PT, Zhang Z, McCourt L, Dwyer M, Benkovic SJ, Hammes GG. Interaction of dihydrofolate reductase with methotrexate: ensemble and single-molecule kinetics. Proceedings of the National Academy of Sciences of the United States of America. 2002;99(21):13481-6. Epub 2002/10/03. doi: 10.1073/pnas.172501499. PubMed PMID: 12359872; PubMed Central PMCID: PMCPmc129699.
4. Murakami T, Mori N. Involvement of Multiple Transporters-mediated Transports in Mizoribine and Methotrexate Pharmacokinetics. Pharmaceuticals. 2012;5(8):802-36. PubMed PMID: doi:10.3390/ph5080802.
5. Bright RD. Methotrexate in the treatment of psoriasis. Cutis. 1999;64(5):332-4. Epub 1999/12/03. PubMed PMID: 10582158.
6. Holmboe L, Andersen AM, Morkrid L, Slordal L, Hall KS. High dose methotrexate chemotherapy: pharmacokinetics, folate and toxicity in osteosarcoma patients. Br J Clin Pharmacol. 2012;73(1):106-14. doi: 10.1111/j.1365-2125.2011.04054.x. PubMed PMID: 21707700; PubMed Central PMCID: PMCPMC3248260.
7. Ashtari F, Savoj MR. Effects of low dose methotrexate on relapsing-remitting multiple sclerosis in comparison to Interferon beta-1alpha: A randomized controlled trial. J Res Med Sci. 2011;16(4):457-62. PubMed PMID: 22091259; PubMed Central PMCID: PMCPMC3214348.
8. Ciardiello F, Tortora G. EGFR Antagonists in Cancer Treatment. New England Journal of Medicine. 2008;358(11):1160-74. doi: doi:10.1056/NEJMra0707704. PubMed PMID: 18337605.
9. Cosmai L, Gallieni M, Porta C. Renal toxicity of anticancer agents targeting HER2 and EGFR. J Nephrol. 2015;28(6):647-57. doi: 10.1007/s40620-015-0226-9. PubMed PMID: 26341657.
10. Hynes NE, Lane HA. ERBB receptors and cancer: the complexity of targeted inhibitors. Nature reviews Cancer. 2005;5(5):341-54. Epub 2005/05/03. doi: 10.1038/nrc1609. PubMed PMID: 15864276.
11. Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. Nature reviews Molecular cell biology. 2006;7(7):505-16. Epub 2006/07/11. doi: 10.1038/nrm1962. PubMed PMID: 16829981.
12. Pozzi C, Cuomo A, Spadoni I, Magni E, Silvola A, Conte A, et al. The EGFR-specific antibody cetuximab combined with chemotherapy triggers immunogenic cell death. Nat Med. 2016;22(6):624-31. doi: 10.1038/nm.4078. PubMed PMID: 27135741.
13. Zhao S, Chen C, Liu S, Zeng W, Su J, Wu L, et al. CD147 promotes MTX resistance by immune cells through up-regulating ABCG2 expression and function. Journal of dermatological science. 2013;70(3):182-9. Epub 2013/04/30. doi: 10.1016/j.jdermsci.2013.02.005. PubMed PMID: 23622764.
14. Dabak DO, Kocaman N. Effects of silymarin on methotrexate-induced nephrotoxicity in rats. Renal failure. 2015;37(4):734-9. Epub 2015/02/25. doi: 10.3109/0886022x.2015.1012984. PubMed PMID: 25707519.
15. Takeda M, Khamdang S, Narikawa S, Kimura H, Hosoyamada M, Cha SH, et

al. Characterization of methotrexate transport and its drug interactions with human organic anion transporters. J Pharmacol Exp Ther. 2002;302(2):666-71. doi: 10.1124/jpet.102.034330. PubMed PMID: 12130730.

16. Garcia R, Franklin RA, McCubrey JA. EGF induces cell motility and multi-drug resistance gene expression in breast cancer cells. Cell cycle (Georgetown, Tex). 2006;5(23):2820-6. Epub 2006/12/19. PubMed PMID: 17172846.

17. Meyer zu Schwabedissen HE, Grube M, Dreisbach A, Jedlitschky G, Meissner K, Linnemann K, et al. Epidermal growth factor-mediated activation of the map kinase cascade results in altered expression and function of ABCG2 (BCRP). Drug metabolism and disposition: the biological fate of chemicals. 2006;34(4):524-33. Epub 2006/01/18. doi: 10.1124/dmd.105.007591. PubMed PMID: 16415123.

18. Liu N, Wang L, Yang T, Xiong C, Xu L, Shi Y, et al. EGF Receptor Inhibition Alleviates Hyperuricemic Nephropathy. Journal of the American Society of Nephrology : JASN. 2015. Epub 2015/03/20. doi: 10.1681/asn.2014080793. PubMed PMID: 25788532.

19. Tang J, Liu N, Zhuang S. Role of epidermal growth factor receptor in acute and chronic kidney injury. Kidney international. 2013;83(5):804-10. Epub 2013/01/18. doi: 10.1038/ki.2012.435. PubMed PMID: 23325080.

20. Flamant M, Bollee G, Henique C, Tharaux PL. Epidermal growth factor: a new therapeutic target in glomerular disease. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2012;27(4):1297-304. Epub 2012/04/03. doi: 10.1093/ndt/gfs030. PubMed PMID: 22467748.

21. Nieskens TT, Peters JG, Schreurs MJ, Smits N, Woestenenk R, Jansen K, et al. A Human Renal Proximal Tubule Cell Line with Stable Organic Anion Transporter 1 and 3 Expression Predictive for Antiviral-Induced Toxicity. AAPS J. 2016;18(2):465-75. doi: 10.1208/s12248-016-9871-8. PubMed PMID: 26821801; PubMed Central PMCID: PMCPMC4779111.

22. Mutsaers HA, Wilmer MJ, Reijnders D, Jansen J, van den Broek PH, Forkink M, et al. Uremic toxins inhibit renal metabolic capacity through interference with glucuronidation and mitochondrial respiration. Biochim Biophys Acta. 2013;1832(1):142-50. doi: 10.1016/j.bbadis.2012.09.006. PubMed PMID: 23017367.

23. Jansen J, Schophuizen CM, Wilmer MJ, Lahham SH, Mutsaers HA, Wetzels JF, et al. A morphological and functional comparison of proximal tubule cell lines established from human urine and kidney tissue. Exp Cell Res. 2014;323(1):87-99. doi: 10.1016/j.yexcr.2014.02.011. PubMed PMID: 24560744.

24. Wilmer MJ, Saleem MA, Masereeuw R, Ni L, van der Velden TJ, Russel FG, et al. Novel conditionally immortalized human proximal tubule cell line expressing functional influx and efflux transporters. Cell Tissue Res. 2010;339(2):449-57. doi: 10.1007/s00441-009-0882-y. PubMed PMID: 19902259; PubMed Central PMCID: PMCPMC2817082.

25. Caetano-Pinto P, Janssen MJ, Gijzen L, Verscheijden L, Wilmer MJ, Masereeuw R. Fluorescence-Based Transport Assays Revisited in a Human Renal Proximal Tubule Cell Line. Mol Pharm. 2016;13(3):933-44. doi: 10.1021/acs.molpharmaceut.5b00821. PubMed PMID: 26871298.

26. Koenderink JB, Geibel S, Grabsch E, De Pont JJ, Bamberg E, Friedrich T. Electrophysiological analysis of the mutated Na,K-ATPase cation binding pocket. The Journal of biological chemistry. 2003;278(51):51213-22. Epub 2003/10/09. doi: 10.1074/jbc.M306384200. PubMed PMID: 14532287.

27. Noe G, Bellesoeur A, Thomas-Schoemann A, Rangarajan S, Naji F, Puszkiel A, et al. Clinical and kinomic analysis identifies peripheral blood mononuclear cells as a potential pharmacodynamic biomarker in metastatic renal cell carcinoma patients treated with sunitinib. Oncotarget. 2016. doi: 10.18632/oncotarget.11686. PubMed PMID: 27589830.

28. Hilhorst R, Houkes L, Mommersteeg M, Musch J, van den Berg A, Ruijtenbeek R. Peptide microarrays for profiling of serine/threonine kinase activity of recombinant kinases and lysates of cells and tissue samples. Methods Mol Biol. 2013;977:259-71. doi: 10.1007/978-1-62703-284-1_21. PubMed PMID: 23436369.

29. Lemeer S, Jopling C, Naji F, Ruijtenbeek R, Slijper M, Heck AJ, et al. Protein-tyrosine kinase activity profiling in knock down zebrafish embryos. PLoS One. 2007;2(7):e581. doi: 10.1371/journal.pone.0000581. PubMed PMID: 17611617; PubMed Central PMCID: PMCPMC1895888.

30. den Boer E, Meesters RJ, van Zelst BD, Luider TM, Hazes JM, Heil SG, et al. Measuring methotrexate polyglutamates in red blood cells: a new LC-MS/MS-based method. Anal Bioanal Chem. 2013;405(5):1673-81. doi: 10.1007/s00216-012-6581-7. PubMed PMID: 23239179.

31. Miller RP, Tadagavadi RK, Ramesh G, Reeves WB. Mechanisms of Cisplatin nephrotoxicity. Toxins (Basel). 2010;2(11):2490-518. doi: 10.3390/toxins2112490. PubMed PMID: 22069563; PubMed Central PMCID: PMCPMC3153174.

32. Holle SK, Ciarimboli G, Edemir B, Neugebauer U, Pavenstadt H, Schlatter E. Properties and regulation of organic cation transport in freshly isolated mouse proximal tubules analyzed with a fluorescence reader-based method. Pflugers Arch. 2011;462(2):359-69. doi: 10.1007/s00424-011-0969-7. PubMed PMID: 21523352.

33. Yang SL, Zhao FY, Song H, Shen DY, Xu XJ. Methotrexate Associated Renal Impairment Is Related to Delayed Elimination of High-Dose Methotrexate. ScientificWorldJournal.2015;2015:751703. doi: 10.1155/2015/751703. PubMed PMID: 26185782; PubMed Central PMCID: PMCPMC4491404.

34. Hagos Y, Wolff NA. Assessment of the role of renal organic anion transporters in drug-induced nephrotoxicity. Toxins (Basel). 2010;2(8):2055-82. doi: 10.3390/toxins2082055. PubMed PMID: 22069672; PubMed Central PMCID: PMCPMC3153278.

35. Yin J, Wang J. Renal drug transporters and their significance in drug-drug interactions. Acta Pharm Sin B. 2016;6(5):363-73. doi: 10.1016/j.apsb.2016.07.013. PubMed PMID: 27709005.

36. Schmeling H, Horneff G, Benseler SM, Fritzler MJ. Pharmacogenetics: can genes determine treatment efficacy and safety in JIA? Nat Rev Rheumatol. 2014;10(11):682-90. doi: 10.1038/nrrheum.2014.140. PubMed PMID: 25112604.

37. Morrissey KM, Stocker SL, Wittwer MB, Xu L, Giacomini KM. Renal transporters in drug development. Annu Rev Pharmacol Toxicol. 2013;53:503-29. doi: 10.1146/annurev-pharmtox-011112-140317. PubMed PMID: 23140242.

38. Melenhorst WB, Mulder GM, Xi Q, Hoenderop JG, Kimura K, Eguchi S, et al. Epidermal growth factor receptor signaling in the kidney: key roles in physiology and disease. Hypertension. 2008;52(6):987-93. doi: 10.1161/HYPERTENSIONAHA.108.113860. PubMed PMID: 18981331.

39. Ferguson BD, Tan YH, Kanteti RS, Liu R, Gayed MJ, Vokes EE, et al. Novel EPHB4 Receptor Tyrosine Kinase Mutations and Kinomic Pathway Analysis in Lung Cancer. Sci Rep. 2015;5:10641. doi: 10.1038/srep10641. PubMed PMID: 26073592; PubMed Central PMCID: PMCPMC4466581.

40. Zhang Q, Hong M, Duan P, Pan Z, Ma J, You G. Organic anion transporter OAT1 undergoes constitutive and protein kinase C-regulated trafficking through a dynamin- and clathrin-dependent pathway. The Journal of biological chemistry. 2008;283(47):32570-9. doi: 10.1074/jbc.M800298200. PubMed PMID: 18818201; PubMed Central PMCID: PMCPMC2583290.

41. Barros SA, Srimaroeng C, Perry JL, Walden R, Dembla-Rajpal N, Sweet DH, et al. Activation of protein kinase Czeta increases OAT1 (SLC22A6)- and OAT3 (SLC22A8)-mediated transport. The Journal of biological chemistry. 2009;284(5):2672-9. doi: 10.1074/jbc.M808078200. PubMed PMID: 19028678; PubMed Central PMCID: PMCPMC2631969.

42. Li S, Zhang Q, You G. Three ubiquitination sites of organic anion transporter-1 synergistically mediate protein kinase C-dependent endocytosis of the transporter. Mol Pharmacol. 2013;84(1):139-46. doi: 10.1124/mol.113.086769. PubMed PMID: 23640180; PubMed Central PMCID: PMCPMC3684823.

43. Soodvilai S, Chatsudthipong A, Chatsudthipong V. Role of MAPK and PKA in regulation of rbOCT2-mediated renal organic cation transport. American journal of physiology Renal physiology. 2007;293(1):F21-7. doi: 10.1152/ajprenal.00043.2007. PubMed PMID: 17327500.

44. Sprowl JA, Ong SS, Gibson AA, Hu S, Du G, Lin W, et al. A phosphotyrosine switch regulates organic cation transporters. Nat Commun. 2016;7:10880. doi: 10.1038/ncomms10880. PubMed PMID: 26979622; PubMed Central PMCID: PMCPMC4799362.

45. Xie Y, Nakanishi T, Natarajan K, Safren L, Hamburger AW, Hussain A, et al. Functional cyclic AMP response element in the breast cancer resistance protein (BCRP/ABCG2) promoter modulates epidermal growth factor receptor pathway- or androgen withdrawal-mediated BCRP/ABCG2 transcription in human cancer cells. Biochim Biophys Acta. 2015;1849(3):317-27. doi: 10.1016/j.bbagrm.2015.01.003. PubMed PMID: 25615818; PubMed Central PMCID: PMCPMC4336604.

46. Wojtal KA, de Vries E, Hoekstra D, van Ijzendoorn SC. Efficient trafficking of MDR1/P-glycoprotein to apical canalicular plasma membranes in HepG2 cells requires PKA-RIIalpha anchoring and glucosylceramide. Mol Biol Cell. 2006;17(8):3638-50. doi: 10.1091/mbc.E06-03-0230. PubMed PMID: 16723498; PubMed Central PMCID: PMCPMC1525225.

47. Stegeman H, Kaanders JH, Wheeler DL, van der Kogel AJ, Verheijen MM, Waaijer SJ, et al. Activation of AKT by hypoxia: a potential target for hypoxic tumors of the head and neck. BMC Cancer. 2012;12:463. doi: 10.1186/1471-2407-12-463. PubMed PMID: 23046567; PubMed Central PMCID: PMCPMC3517352.

48. Langan TJ, Chou RC. Synchronization of mammalian cell cultures by serum deprivation. Methods Mol Biol. 2011;761:75-83. doi: 10.1007/978-1-61779-182-6_5. PubMed PMID: 21755442.

49. Wada Y, Iyoda M, Matsumoto K, Shindo-Hirai Y, Kuno Y, Yamamoto Y, et al. Epidermal growth factor receptor inhibition with erlotinib partially prevents cisplatin-induced nephrotoxicity in rats. PLoS One. 2014;9(11):e111728. Epub 2014/11/13. doi: 10.1371/journal.pone.0111728. PubMed PMID: 25390346; PubMed Central PMCID: PMCPmc4229108.

50. Sacco AG, Cohen EE. Current Treatment Options for Recurrent or Metastatic Head and Neck Squamous Cell Carcinoma. J Clin Oncol. 2015;33(29):3305-13. doi: 10.1200/JCO.2015.62.0963. PubMed PMID: 26351341.

SUPPLEMENTARY INFORMATION

Table 1. ciPTEC culture medium formulation

		Distributor	Concentration
Medium Supplements	DMEM-F12 HEPES, no phenol red	Thermo Fisher-Gibco	-
	Insulin	Sigma-Aldrich	5µg/ml
	Transferrin	Sigma-Aldrich	5µg/ml
	Sodium	Sigma-Aldrich	5ng/ml
	Hydrocortisone	Sigma-Aldrich	36ng/ml
	Epidermal growth factor	Sigma-Aldrich	10ng/ml
	Tri-iodothyonine	Sigma-Aldrich	40pg/ml
	FCS	Greiner Bio-one	10% (v/v)

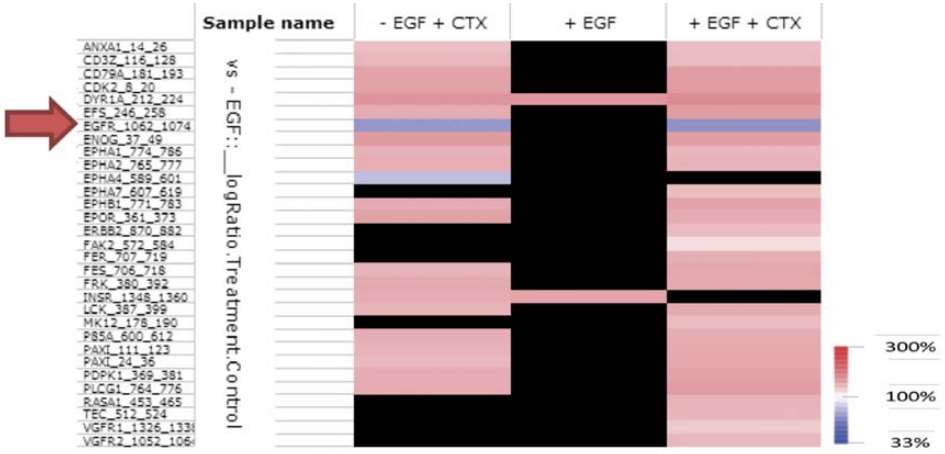


Figure 1. Cetuximab inhibition of EGFR phosphosite.

Table 2. Pathways identified as significant matches to the kinase phosphorylation sites obtained from the PTK/STK PamChip® array. The list of pathways was obtained based on statistical analysis of the phosphorylation targets and overlap with pathway targets retrieved from associated databases. Pathways with –log(pvalue) above 4 were considered significant.

#	Maps	0 2 4 6 -log(pvalue)
1	Development c-Kit ligand signaling pathway during hemopoiesis	
2	Development growth factors in regulation of oligodendrocyte precursor cell proliferation	
3	Immune response inhibitory PD-1 signaling in T-cells	
4	Role of α-6/β-4 integrins in carcinoma progression	
5	Signal transduction PKA signaling	
6	Oxidative stress activation of NADPH	
7	Development EGFR signaling pathway	
8	Development gastrin in cell growth and proliferation	
9	Main growth factor signaling cascades in multiple myeloma cells	
10	Development VEGF signaling via VEGFR2 – generic cascades	

Conditions: +EGF (orange-line1); +EGF+CTX (blue-line2); +CTX (red-line3)

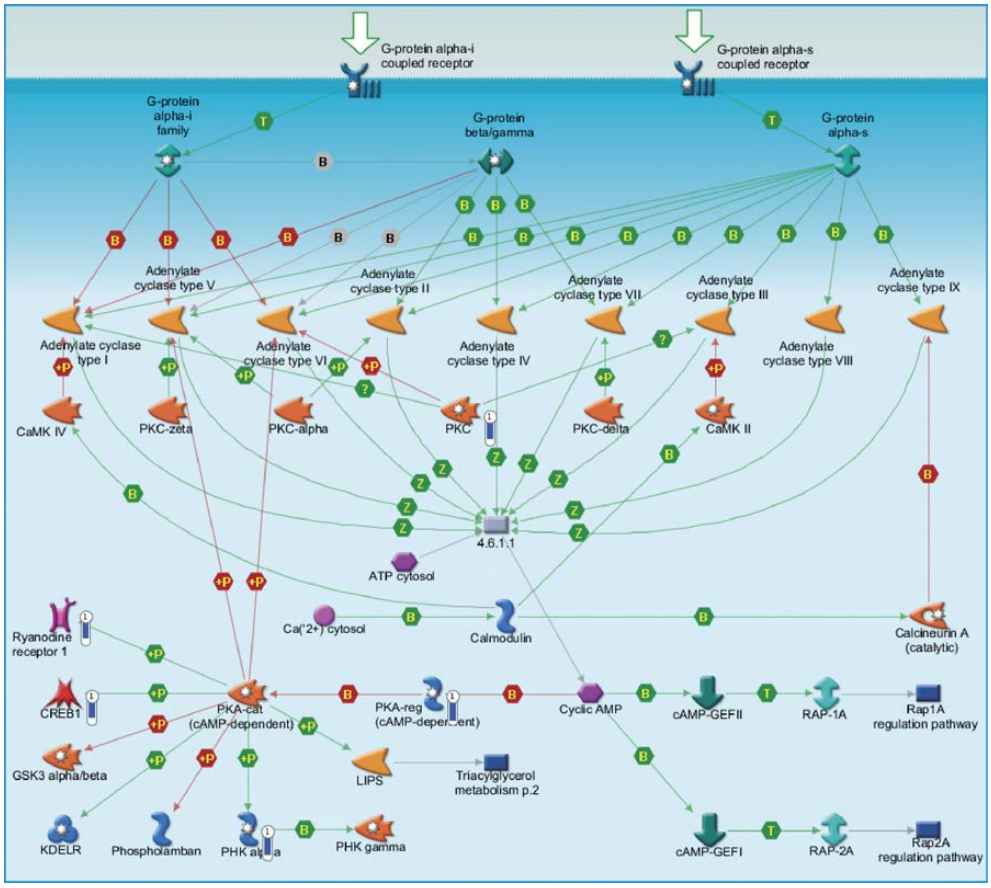


Figure 2. Metacore knowledge-based pathway analysis of the effect of EGF (control condition) on ciPTEC-OAT1.

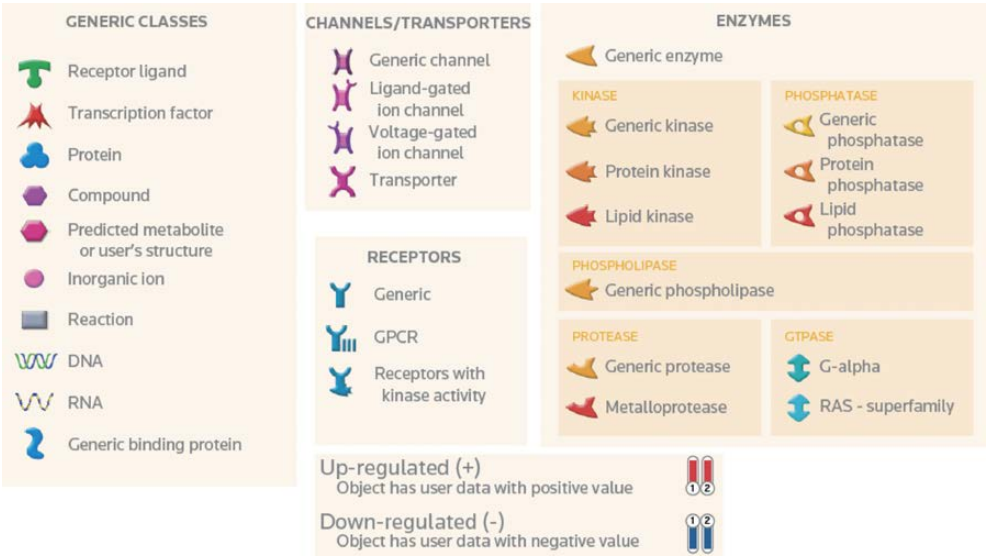


Figure 3. Pathway analysis symbols key. (adapted from: Metacore quick reference guide, Thomson Reuters)

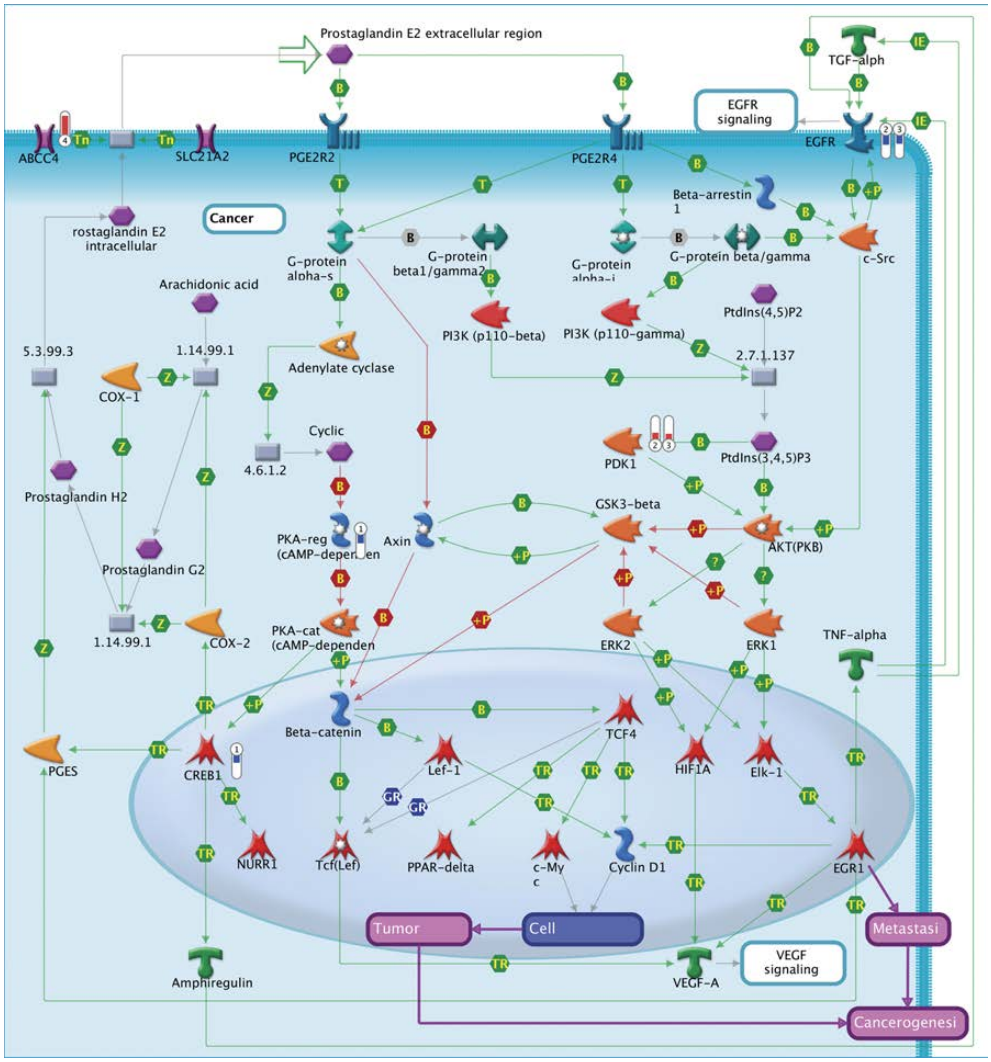


Figure 4. Metacore knowledge-based pathway analysis of the effect of EGF together with Cetuximab on ciPTEC-OAT1.

CHAPTER 6

Human derived fetal kidney progenitor cells differentiated towards functionally active proximal tubule epithelial cells

Pedro Caetano-Pinto[1], Chiara Mari[2], Marieke Wensween[1], Karen Price[2], Paul Winyard[2] and Rosalinde Masereeuw[1]

[1]Division of Pharmacology, Utrecht Institute for Pharmaceutical Sciences, The Netherlands
[2]Institute of Child Health, University College London. United Kingdom

ABSTRACT

Cell-based therapies to treat end-stage renal disease are still experimental, despite the demonstrated potency of stem cells to aid repair of damaged tissue. In the present study, we evaluated the potential of human fetal kidney-specific progenitor cells (KSPC) to differentiate into proximal tubule epithelial cells (PTEC), comparing lines derived from 8-10 week gestation that were either positive or negative for the presumptive renal stem cell markers CD24 and CD133. We examined both expression of proximal tubule markers and function *in vitro*. Cells were cultured in stem cell and PTEC medium followed by a characterization of PTEC renal phenotypical markers and xenobiotic transporters expression over passage (P) numbers – from P6 to P9. Fluorescent substrates were used to investigate the function of transporters expressed in mature PTEC. In addition, cells were grown in matrigel (BD matrigel™) to determine 3-dimensional differentiation. Our data shows that the cells acquired a proximal tubule-like phenotype from P6 to P9, based on marker gene expression, including the key tubular transporters: Multidrug resistance protein 4 (MRP4), P-glycoprotein (P-gp) and Breast cancer resistance protein (BCRP). Activity assays revealed that MRP4 and P-gp activity increased from P6 to P9 in both populations when cultured in PTEC medium. On the other hand, BCRP appeared functionally inactive despite its gene expression. When grown in matrigel, both populations form tubule-like structures after two days in culture, an effect only observed when cultured in PTEC medium suggesting differentiation. These results show that KSPC are functionally active and, though immature, retain PTEC characteristics in culture.

Key words

Kidney ontogeny, renal function, progenitor stem cells, xenobiotic transporter, proximal tubule

INTRODUCTION

During nephrogenesis, the precursor of the adult kidney (the metanephros) begins forming from cells of the intermediate mesoderm around week five of gestation. The metanephros is generated as reciprocal signaling between the ureteric bud and the metanephric mesenchyme triggers successive branching events that lead to the formation of collecting ducts and nephrons [1, 2]. These cells will proliferate and differentiate into the complex tubular architecture of the nephron, comprising glomeruli, proximal tubules, loop of Henle and distal tubules [3]. Nephrogenesis ceases before birth at about week 34, with about one million nephrons per kidney complete (Figure 1). New nephrons are never generated after this timepoint.

Different types of stem cells have been described as potential kidney progenitors and for renal regeneration purposes [4, 5], including bone marrow derived stem cell (BMSC) [6-8] and adult kidney progenitor cells [9, 10]. Even though BMSC are recruited upon kidney damage, most studies indicated that repopulation is mostly done by residing renal cells [11, 12]. The existence of adult renal stem cells has been subject of debate [13], and in the past decade their existence was reported in several independent studies [14-17]. The common denominator appeared to be the presence of the cluster differentiation markers (CD) - CD24 and CD133 by which these cell populations could be distinguished and isolated.

CD24 and CD133 double positive (DP) cells are reported to be present in adult kidney tissue and capable to differentiated towards renal lineages. These cells are also upregulated in tubules of patients diagnosed with acute kidney injury (AKI) compared to those with normal kidney function [4, 17, 18]. This raises the possibility that the DPs are dedifferentiated kidney cells that could proliferate and differentiate into PTEC, thereby recovering the loss of cells.

In the same way, these cells could replenish tubular cells that are shed in urine [16]. As a result, a small amount of DPs can be found in the tubules of kidneys from healthy adults. Further evidence suggesting that these dedifferentiated cells can proliferate and differentiate into PTECs is based on animal studies. For example, injection of human DPs in mice suffering from glycerol-induced AKI improved renal function repair [17].

PTEC express a broad range of membrane transporters that handle endo- and xenobiotics, and are key to the kidney's blood purification function. These pumps take up free and protein-bound solutes at the basolateral side and subsequently efflux them across the apical membrane of the PTEC into the pre-urine. Additionally, PTEC reabsorb many vital nutrients back into the bloodstream [19-21]. The role of a particular class of xenobiotic transporters belonging to the ATP binding cassette family (ABC) is now reasonably well established in the adult kidney. A great variety of solutes, including metabolic wastes, are removed by the efflux pumps BCRP (*ABCG2*), P-gp (*ABCB1*) and *MRP4 (ABCC4)* [22-24]. These prominent transporters are a hallmark of PTEC activity and can serve as determinants for its phenotype [25, 26]. While adult PTEC function and

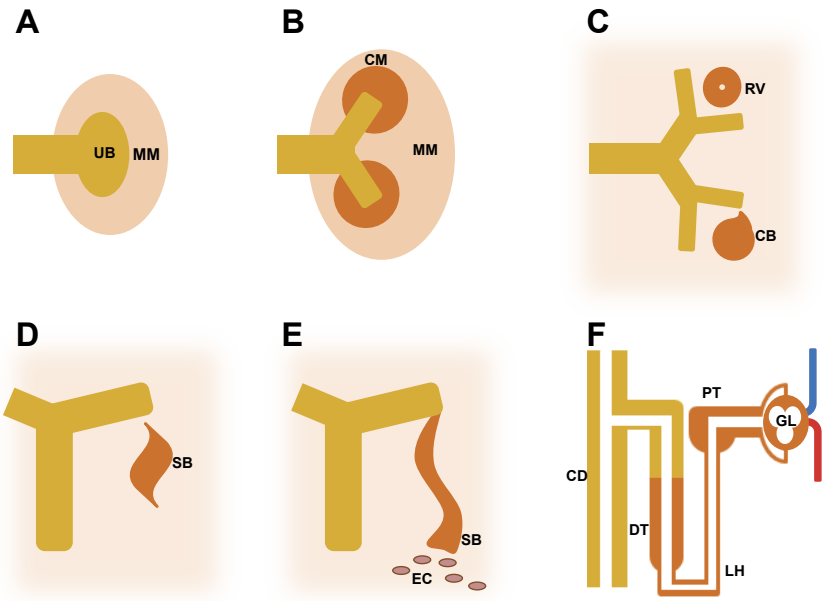


Figure 1. Kidney development. (A) Signal exchange between epithelial cells from the ureteric bud (UB) and mesenchymal cells from the metanephric mesenchyme (MM) initiates nephron growth. (B) The UB invades the MM and subpopulations of mesenchyme cells condense around the UB branching tips, forming the cap mesenchyme (CM). This CM is believed to accommodate self-renewing progenitor cells that give rise to the population that undergoes mesenchymal to epithelial transformation creating (C) increasing complex structures, renal vesicles (RV), comma-shaped bodies (CB) and S-shaped bodies (SB) (D). Elongating SB connect to the UB distally (E) resulting in distal segment and collecting duct development respectively (CD) while the proximal ends become invaded by endothelial cells (EC), giving rise to the glomeruli and nephron vasculature (F). Further maturation generates the distal tubules (DT), loop of Henle (LH), proximal tubules (PT) and glomerulus (GL).

transport activity have been extensively characterized, their ontogeny from embryonic status remains unclear. Additionally, little is known about the potential use of developing kidney cells in regenerative and replacement therapies for adult kidneys [27]. In the present study, we set to determine the differentiation of 8-10 week old embryonic human KSPC. Cells either positive or negative for CD24 and CD133 were isolated from the cap mesenchyme into two populations (DP-double positive, DN-double negative). Phenotypical marker gene expression, 3-dimensional structure formation in matrigel and transporter function were investigated while cultured in media designed to promote proximal differentiation over sequential passages *in vitro*, to determine how transport activity evolves in early nephron formation.

MATERIALS AND METHODS

Chemicals

All chemicals were obtained from Sigma (Zwijndrecht, Yhe Netherlands) unless stated differently. Stock solutions were dissolved according to manufacturing recommendation, in either dimethyl sulfoxide (DMSO) or dH_2O.

Cells and cell culture

Kidney tissues were obtained from the ethically-approved MRC-Wellcome Human Developmental Biology Resource at University College London (http://www.hdbr.org/). These were enzymatically dissociated and cultured for one week KSPC medium consisting of DMEM/F12 (50/50) (Invitrogen, Breda, the Netherlands) supplemented with 10% (v/v) fetal calf serum (FCS; Greiner Bio-One, Alphen a/d Rijn, The Netherlands) containing insulin (5 µg/mL), transferrin (5 µg/mL), selenium (5 ng/mL), prostaglandin E1 (50 µg/mL), dexamethasone (20 µg/mL), holo-transferin (10 µg/mL) and tri-iodothyrionine (20 pg/mL), with 1% pen/strep (Invitrogen, Breda, The Netherlands). The cells were then sorted for CD133 and CD24, generating $CD133^+CD24^+$ double positive (DP) and $CD133^-CD24^-$ double negative (DN) lines. The first four passages were cultured in KSPC medium, then either KSPC medium or PTEC medium between passage 5 and 10. PTEC medium consists of phenol red free DMEN/F12 (Invitrogen, Breda, The Netherlands) supplemented with 10% (v/v) FCS (Greiner Bio-One, Alphen a/d Rijn, The Netherlands), containing insulin (5 µg/mL), transferrin (5 µg/mL), selenium (5 ng/mL), hydrocortisone (36 ng/mL), epithelial growth factor (10 ng/mL), and tri-iodothyrionine (40 pg/mL), with 1% pen/strep (Invitrogen, Breda, The Netherlands). The seeding cell density was 15,000 cells/cm². Cells were cultured at 37°C and 5% v/v CO2 till confluency (5-7 days).

mRNA expression of transporters and kidney cell markers

Total mRNA was isolated using the RNA isolation kit (Qiagen, Hilden, Germany) from DPs and DNs cultured in either KSPC or PTEC medium at different passages (P6 and P9). cDNA was synthesized using MMLV transcriptase (Invitrogen, Breda, The Netherlands) in two separate experiments. Afterwards, mRNA expression was evaluated using gene-specific primer probe sets: GAPDH (hs99999905_m1), BCRP (hs00184979_m1), MRP4 (hs00195260_m1), P-gp (hs00184500), PODXL (hs01574644_m1) and AQP1 (hs01028916_m1). TaqMan Universal PCR Master Mix (Applied Biosystems) was used and the qPCR reactions were performed using MyCycler Thermal Cycler (Biorad).

Matrigel cell culture and structure imaging

KSPCs were cultured in matrigel to investigate 3-dimensional structure formation. Different number of cells (10000 cells - 100000 cells) were combined with 25 µL medium and 25µL matrigel per well. The mixed solution was incubated overnight in a black,

ultra-thin, clear bottom, 96 well plate (Corning Incorporated, New York, USA) at 37°C, with 5% v/v CO_2 to polymerize the gel. Subsequently, 100µL medium (either PTEC medium or KSPC medium) was added per well. Medium was refreshed every other day. Growth was monitored daily using bright-field microscopy. When tubule-like structures were formed in matrigel, cells were stained with 10 µM Hoechst (nuclear staining) in medium. Optimal staining was achieved overnight at 37°C with 5% v/v CO2. The next day, the matrigel was washed and incubated with 2µM CMFDA or 2 µM calcein-AM for 30 minutes, in the presence or absence of 50µM MK571 or 5µM PSC833 as inhibitors of MRP4 and P-gp, respectively. Live uptake images were acquired using a CV7000S imager (Yokogawa, Tokyo, Japan).

Fluorescence-based functional efflux assays

The function of efflux transporters was quantified using fluorescent substrates, which can accumulate intracellularly in combination with competitor inhibitors as described by Caetano-Pinto *et al.* [25]. In short, to evaluate the function of MRP4, 1.25 µM CMFDA (Life Technologies, Carlsbad, CA, USA) was added to the cells with a two-fold dilution range of MK571 (maximum concentration: 50µM). After 30 minutes incubation at 37°C with CMFDA and MK571, cells were washed and afterwards, 1% Triton X100 was added to permeabilize the cell membrane, the plates were kept for an additional 60 minutes at 37°C before fluorescence was read (excitation: 492nm, emission: 517nm). To test the function of P-gp, cells were incubated for 60 minutes at 37°C with 1 µM calcein-AM (Life Technologies, Carlsbad, CA, USA), in the presence of PSC833. A two-fold dilution range was used for PSC833, with a maximum concentration of 6 µM. Subsequently, cells were washed with HBSS buffer and incubated at 37°C with 1% Triton X100 buffer for another 30 minutes. Afterwards, fluorescence was read (excitation: 492nm, emission: 517nm). Each solution was prepared in HBSS buffer. To examine the BCRP activity, cells were incubated with (or without) 10 µM KO143 (an inhibitor for BCRP) and a two-fold dilution range of the substrate Hoechst (maximum concentration: 100 µM). After 30 minutes incubation at 37°C, cells were washed with HBSS buffer and HBSS was put on top. Fluorescence was read immediately after (excitation: 350 nm, emission: 460 nm) using a Fluoroskan Ascent™ plate reader (ThermoFisher).

Data analysis

The efflux activity was calculated by analyzing fluorescence intensity values obtained from the functional assays. Data is presented as the inverse of the fold increase in fluorescence, as described by Caetano-Pinto et al. [25]. Gene expression was plotted using MS Exel. Functional data was fitted using nonlinear regression with a variable slope or with a Michaelis-Menten fit in GraphPad Prism 5.03. All functional data is presented as mean ± SEM, unless stated otherwise.

RESULTS

KSPC numbers were expanded (by serial passage) until sufficient cells for functional assays were obtained; starting at P3. Cells were monitored daily and cultures were terminated for assay, when cell replication rates fell substantially or when they detached from the culture flasks, usually at P10. Maximum passage number utilized here was P9. Results are shown for sequential passages of both populations (CD24/CD133 either positive or negative; DP or DN) cultured in KSPC or PTEC medium.

Tubular markers and renal transporters expression levels

Gene expression of relevant transporters and phenotypical markers were measured for P6 and P9 for all conditions tested. The podocyte marker podocalyxin is expressed and its expression increased over passage number in both cell types when cultured in PTEC medium (Figure 2A). Podocalyxin was less expressed when the DP and DN were cultured in KSPC medium. Proximal tubule marker aquaporin 1 (AQP1) is expressed under all conditions tested, but lowest in DP grown in PTEC medium (Figure 2B). Furthermore, both populations lack the expression of nephrin (podocyte marker) and the glucose transporter SGLT2 (proximal tubule marker). The expression of two prominent proximal tubular transporters, organic anion transporter 1 and 3 (OAT1 and 3), is also absent. These findings suggest that both DP and DN show, at least, a mixed podocyte and PTEC phenotype upon culturing. With regard to renal transporters, relative changes in expression from P6 to P9 in both KSPC and PTEC medium are presented in tables 1 and 2 (DN and DP, respectively).

3D structure formation in matrigel

DP and DN formed tube-like structures when grown for 1-5 days in BD matrigel™, cultured in PTEC medium (Figure 3). The optimal cell seeding concentration determined for DN was established as 100,000 cells/50 µL, where tube-like structures were observed after 2 days in culture (Figure 3A). The optimal seeding density of DP was 45,000 cells/50 µL (Figure 3B). Both DN and DP (P9) grown in KSPC medium did not organize well. After 7 days, unstructured monolayers were formed on the bottom of the well (Figure 4C-D), very different from the tube-like structures from DN grown in PTEC (Figure 3A-B).

Structural organization was visualized by nuclear staining with Hoechst 33342, and cytoplasmic labelling using CMFDA, which is converted into the glutathione conjugate, GS-MF, intracellularly [25]. In terms of activity, in the presence of the inhibitor MK571, intracellular GS-MF accumulation increased, the same effect was observed for calcein in the presence of the inhibitor PSC833. These findings point towards the presence of functionally active MRP and P-gp transporters, respectively (Figure 5).

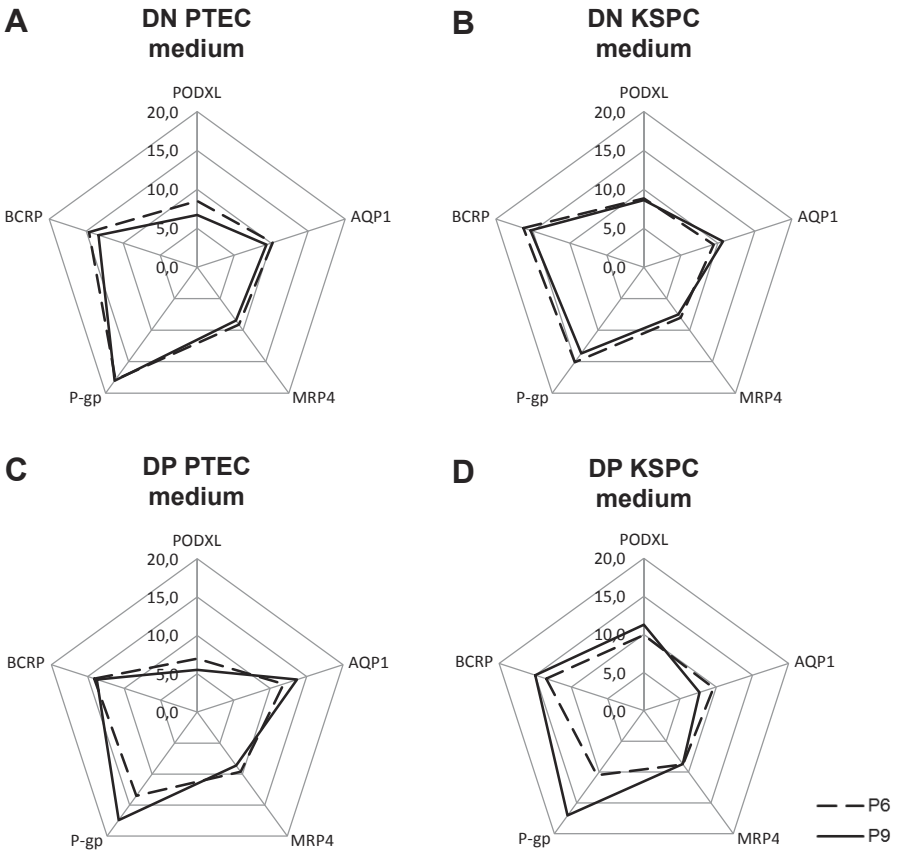


Figure 2. Gene expression of representative markers. Renal tubule developmental markers podocalyxin (PODXL) and aquaporin-1 (AQP1) and tubular transporters MRP4, P-gp and BCRP are depicted in radar graphs representing DP and DN cells cultured in PTEC medium (A,C) and KSPC medium (B,D) at P6 and P9. Expression is presented as ΔCt, relative to GAPDH (mean Ct=20), a decrease in ΔCt indicates an increase in gene expression. Values are presented as mean ± SEM, of two independent assays performed in triplo.

MRP4 is expressed and active both CD24/CD133 positive and negative populations

To evaluate MRP4 function, cells were incubated with CMFDA in the presence of MK571. The IC_{50} values of MK571 decreased over passage numbers (Table 1), indicating an increase of MRP4 activity when the cells were expanded in culture. DP cultured in KSPC medium showed a 5-fold increase in IC_{50} value from P6 to P8. Moreover, DP cultured in PTEC medium, showed a 9-fold increase in IC_{50} over identical passages. For the DN a similar pattern was observed in both types of medium; an increase of 7-fold in IC_{50} from P6 to P10 in PTEC and KSPC medium. The results of the dose response effect of GS-MF retention are depicted in Figure 6.

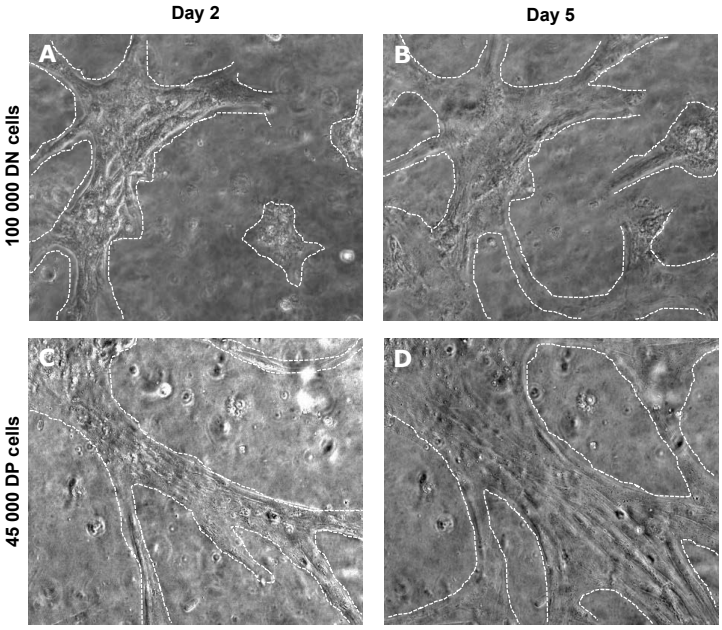


Figure 3. Representative images of cell growth patterns of the DN (A,C)) and DP (B,D) over 5 days of culture in Matrigel using PTEC medium. Organized structures are visibly present from day 2.

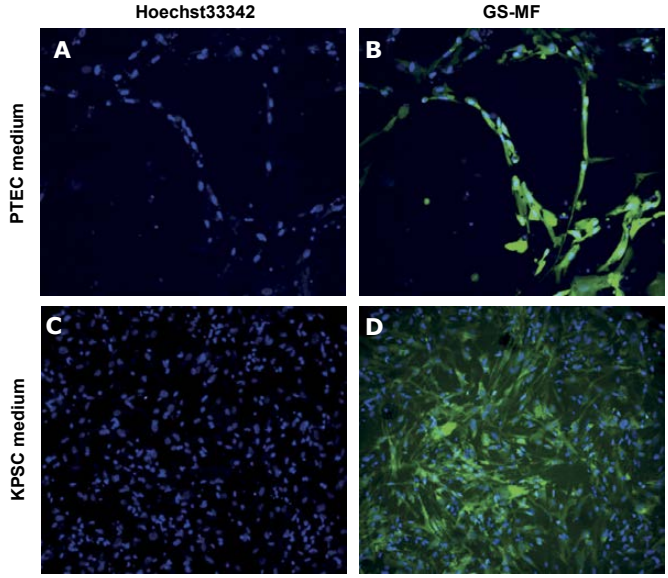


Figure 4. DN cells (P9) grown in either PTEC (A,B) medium or KSPC (C,D) medium were stained for Hoechst (blue) and CMFDA, which is converted into GS-MF (green). Hoechst staining was performed at day 6, while the GS-MF staining was examined in live cells at day 7. Similar patterns were observed for DP cells.

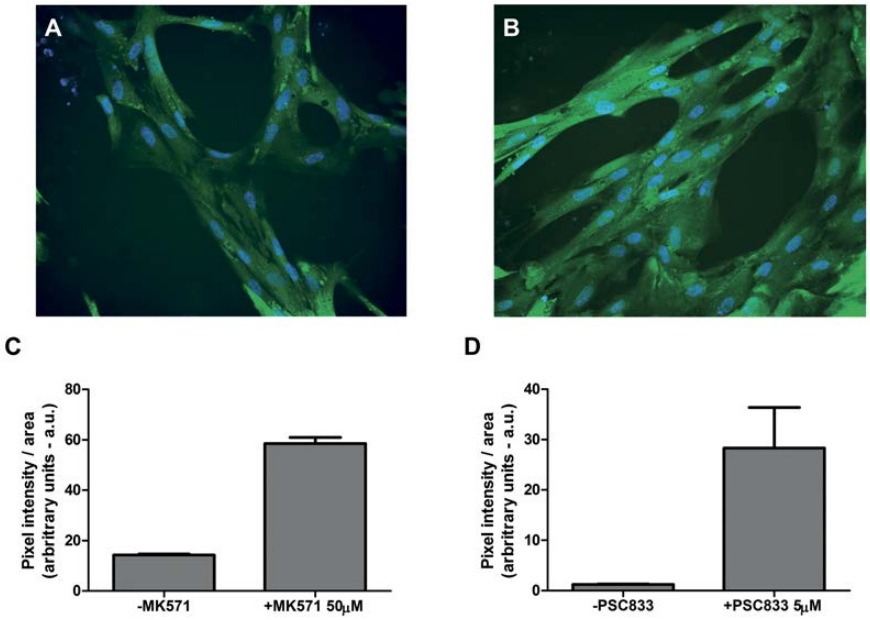


Figure 5. Fluorescent substrate retention in the presence or absence of inhibitors in organized structures formed by DN in culture in PTEC medium (A, B). The presence of MK571 intensifies the accumulation of GS-MF (C), indicating MRP4 activity, a similar effect is observed for calcein in the presence of PSC833, indicating P-gp activity (D). Substrate accumulation was determined using ImageJ software by marking a selected area in different frames (corresponding to the conditions with and without inhibitor) and defining the pixel intensity by the area depicted. Values are presented as mean ± SEM, of two independent experiments.

P-gp is expressed and active in both CD24/CD133 positive and negative populations

To evaluate P-gp function, cells were incubated with calcein-AM in the presence of a PSC833 gradient. In DN function increased over passage number, in contrast to the expression levels (Figure 2D). This is also confirmed by the reduction in IC_{50} values from P6 to P9. In the conditions tested, calcein efflux was not detectable at P6. DP in KSPC medium showed a 9-fold decrease in IC_{50} over passages (P7 to P9). Furthermore, the IC_{50} for PSC833 in DP cultured in PTEC medium was also reduced. The DN in KSPC medium only showed calcein efflux at P7 (Figure 7C). The DN in PTEC medium showed a slight reduction in IC_{50} values from P7 to P9, and at a higher passage number no activity could be determined.

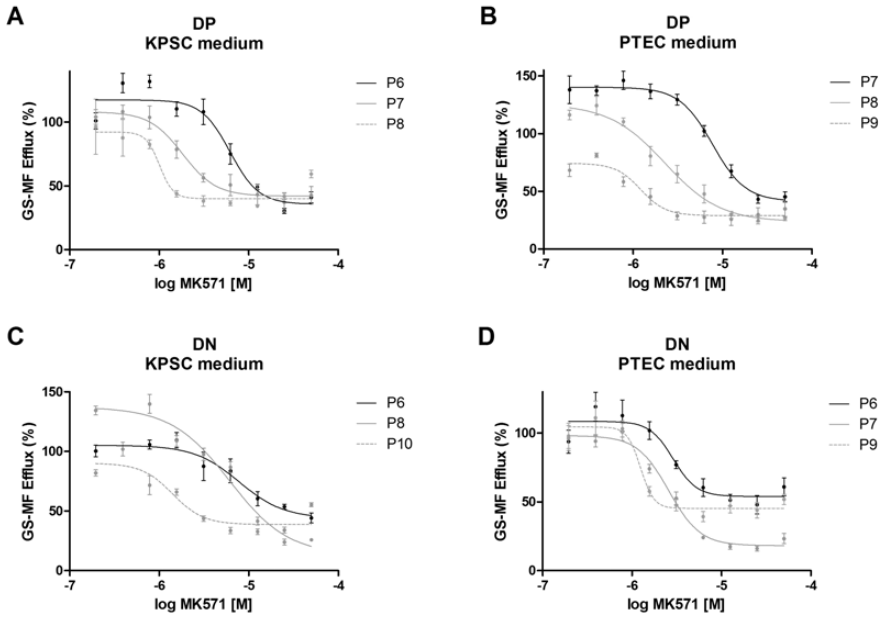


Figure 6. Functional activity of MRP4 in CD133+CD24+ (DP) cells cultured in KSPC medium (A), DP cells cultured in PTEC medium (B), CD133-CD24- (DN) cells cultured in KSPC medium (C) and DN cells cultured in PTEC medium. The functional activity in the four experimental conditions was evaluated at least at three different passages (P): low passage (black line), intermediate passage (grey line) and high passage (dotted line). Cells were incubated with 1.25 μM CMFDA, in the presence of a concentration range of the MRP4 inhibitor MK571. Values are presented as mean ± SEM, of two independent experiments performed in triplo.

BCRP is expressed but not active in both CD24/CD133 positive and negative populations

To evaluate BCRP function, cells were incubated with a gradient of Hoechst33342 in the presence or absence of KO143. In contrast to our expectation, we did not observe an accumulation of Hoechst33342 in all conditions tested (Figure 8).

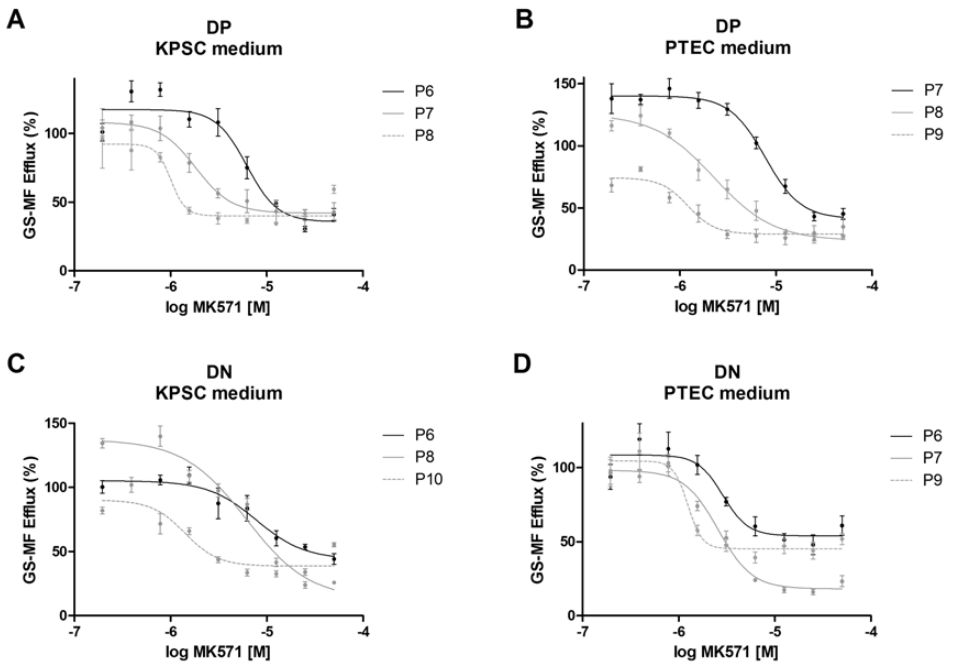


Figure 7. Functional activity of P-gp in CD133+CD24+ (DP) cells cultured in KSPC medium (A), DP cells cultured in PTEC medium (B), CD133-CD24- (DN) cells cultured in KSPC medium (C) and DN cells cultured in PTEC medium (D). The functional activity in the four experimental conditions was evaluated at least at three different passages (P): low passage (black line), intermediate passage (grey line) and high passage (dotted line). Cells were incubated with 1.0 μM calcein-AM, in the presence of a concentration range of the P-gp inhibitor PSC833. Values are presented as mean ± SEM, of two independent experiments performed in triplo.

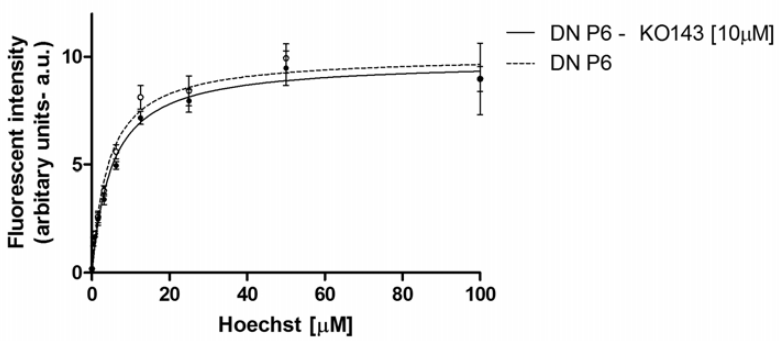


Figure 8. Representative graph of the functional efflux activity of BCRP, determined by Hoechst33342 accumulation in the presence or absence of 10 μM of the inhibitor KO143. Data shown for DN cultured in PTEC medium at P9, all conditions and passages showed similar pattern. Values are presented as mean ± SEM, of two independent experiments performed in triplo

Table 1. Inhibitory potencies of MK571 and PSC833 on MRP4 and P-gp, respectively, tested in DP and DN cultures in KSPC or PTEC medium. Values are presented as mean ± SEM, of two independent experiments performed in triplo

IC50 values (μM)			P6	P7	P8	P9
MRP4	DP	KSPC medium	5.8 ± 2.8	1.8 ± 0.3	1.1 ± 0.5	-
		PTEC medium	-	7.7 ± 0.9	3.2 ± 1.9	1.3 ± 0.4
	DN	KSPC medium	10.1 ± 1.7	-	6.6 ± 1.4	-
		PTEC medium	2.9 ± 1.1	2.6 ± 0.2	-	1.1 ± 0.5
P-gp	DP	KSPC medium	-	8.7 ± 7.3	0.3 ± 0.1	0.9 ± 0.1
		PTEC medium	-	2.3 ± 0.4	0.4 ± 0.2	0.1
	DN	KSPC medium	1.9 ± 0.6	-	-	-
		PTEC medium		0.7 ± 0.1	-	0.5 ± 0.3

DISCUSSION

The present study investigated the development of PTEC from human KSPC. To this end, phenotypic marker expression, 3-dimensional differentiation and functional assessments were performed for two populations, sorted for CD24 and CD133, over passages six to nine. To identify functional characteristics of human KSPC, we performed fluorescence-based activity assays and determined gene expression of key tubular transporters. A summary of the results is depicted in Figure 9.

The diverse marker expression found is a clear indication of a mixed phenotype at this stage. The increase in podocalyxin expression, a podocyte marker [28], is noteworthy. When DP and DN are cultured in PTEC medium, which should stimulate KSPC differentiation, our findings suggest that these cells are *de facto* immature expressing markers from different nephron segments. Transporter expression can vary among individuals and also different genetic variants can be present, which may account for variations observed between the KSPC populations and when compared to ciPTEC. Nonetheless, we show that cells from fetal kidneys between week eight and ten functionally express major tubular transporters, except for the OAT1/3 carriers. Tubular epithelial cells are known to lose these transporters expression when isolated and cultured *in vitro* [29], a phenomenon that can explain the lack of OATs in KSPCs.

On the other hand, MRP4 is the highest expressed PTEC-specific transporter, followed by P-gp and then BCRP. The same pattern was observed in ciPTEC a mature *in vitro* proximal tubule cell model [20] and in human renal tissue. A defining feature of the side population (SP) of mesenchymal stem cells is an enhanced expression of BCRP, therefore its functional presence was expected [30, 31]. Having a high expression of BCRP may protect SP, allowing the cells to efflux harmful substances [32]. However, BCRP overexpression partly blocks differentiation of the cells. To promote differentiation, BCRP is downregulated while P-gp expression is enhanced [30].

However, the mRNA levels we found in KSPCs are substantially lower compared to the ones observed in ciPTEC [25]. Mature ciPTEC show a low BCRP expression compared to proliferating cells, [33] and in the human renal cortex BCRP is also minor as compared to the other apical ABC transporters [34]. This evidence may point to a low BCRP expression as a trait of PTEC.

Cells grown in PTEC medium in matrigel organize into tube-like structures and are functionally active, while KSPC medium maintains cells in an undifferentiated state. This study highlights the impact culture conditions can have in cells, in particular in two populations believed to be inherently different. The cell growth was not consistent for every condition and passage, and beyond P10 cell replication rates were significantly delayed. This is common for primary cells in culture and a bottleneck in further exploring the cells behavior *in vitro*.

The activity of MRP4 and P-gp over passage number was increased, as determined by fluorescence assays. However fluorescent substrates retention does not accurately reflect transporter specificity, as previously demonstrated [25]. For instance, calcein-AM

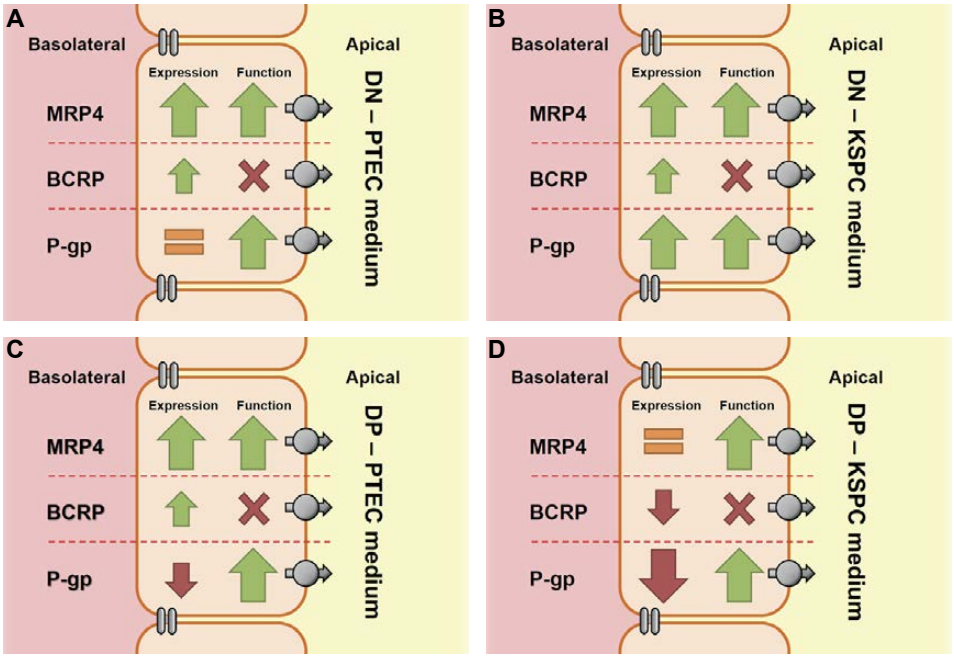


Figure 9. Overview of the changes in transporter activity and expression levels over passage (from lowest to highest passage evaluated). This was examined in four conditions: DNs cultured in PTEC medium (A), DNs cultured in KSPC medium (B), DPs cultured in PTEC medium (C) and DPs cultured in KSPC medium (D). In general, the MRP4 activity and gene expression increased over passage, the BCRP was not active, while a small increase in gene expression was found over passage. Additionally, P-gp increased in function, while in most cases the P-gp mRNA expression stayed similar or decreased over passage.

is mainly transported via P-gp, however, its product, calcein, can also be effluxed by MRP transporters. Therefore, the expression and function of P-gp can be similar over passage, whereas a limited accumulation of calcein can be a reflection of the activity of other transporters. This effect could have accounted for the increase in calcein efflux in DN grown in PTEC medium as MRP4 increased in both activity and function. Post-transcriptional regulatory mechanisms such as transporters shuttling to the membrane may play a role in the increase in activity as observed (given that MRP4 and P-gp expression does not follow activity). When we compared the function of KSPC at higher passages with that of ciPTEC [25], the activity of the MRP4 and P-gp transporters was similar. This could mean that over passage KSPC resemble the function of PTECs, except for BCRP activity at this point, a fact that may implicate that functional BCRP as a discriminative marker for differentiated proximal tubule cells (given that BCRP is expressed but not functional). From the data in hand is it evident that the differences between both DP and DN populations are slim, both with comparable functional activities upon culturing. Our data showed that KSPC retain their differentiation ability *in vitro*, in line with immunohistochemistry presented by van Kalken *et al.* demonstrating that P-gp is expressed in the kidney upward of week 11 of gestation [35].

Further research into renal progenitor cell activity should account for more donors, and should point out whether the differences found in function and gene levels are caused by individual changes or by the presence and absence of CD133 and CD24. Our KSPC populations showed differentiation potential when grown in matrigel, therefore exploring novel culture materials and conditions can further shed light into the *in vitro* development of both KSPC populations without the limitation of sequential passages. Given the functional similarities between DP and DN populations, transporter activity does seem to discriminate both positive and negative CD24/CD133 cells. A recent study by da Sacco *et at.,* described the isolation of human kidney progenitor cells from the cap mesenchyme by using SIX2 and CITED1 as markers [36]. As the discussion surrounding which markers characterize nephron progenitors in the human developing kidney is still open, it would be of interest to also test the function of progenitor populations isolated using different procedures. True progenitor cells should yield comparable activities regardless of the isolation method used.

In the past decades, pluripotent stem cells have tantalized with the promise of tissue regeneration and replacement therapies [37]. However, the challenges and limitations are plentiful, the yield for efficient differentiation is low and there are hardly effective tests to evaluate the function of the differentiated cells [38-40]. For kidney regeneration, stem cell-derived therapies could be useful in acute and the early stages of chronic kidney disease (CKD), while at later stages of CKD, renal replacement therapies are still necessary [2]. For renal replacement, KSPC can provide a promising source of stem cells other than pluripotent cells, given their commitment to a renal lineage when isolated. Song *et al.* [41] succeeded in generating a partially functioning re-cellularized kidney from human endothelial cells and rat kidney progenitor cells. A reliable human source of progenitor

cells could potentially be used to generate re-cellularized kidneys for replacement therapy. However, aspects such as availability of cells, size of organ, immunogenicity, tumorigenic potential and directed and irreversible differentiation are just a few issues in a long list that need to be resolved [42].

KSPC are also a valuable research tool given their functional activity and potential to commit to different renal lineages. These cells can be more representative of renal physiology than currently available *in vitro* models of animal origin and/or transformed cell lines. The use of KSPC is hampered by their poor availability and limited expansion in culture, issues that can be bypassed by immortalizing freshly isolated cells. As current efforts to reduce animal experimentation and to find novel predictive tools in areas such as drug development and toxicology progress, KSPC grown in 3D cultures could provide relevant models as they mimic nephron structures.

In conclusion, we showed for the first time that KSPC are highly functional *in vitro,* forming organized structures when grown in appropriate medium. The cells express key tubular transporters with the exception of BCRP, which could be a signature feature at an early stage of development. While DNs and DPs populations did not differ substantially in function and expression, these features change when cells are expanded in culture. Overall KSPC cultures are promising as a model of early kidney development and for the study of transport function during development.

ACKNOWLEDGMENTS

This work was funded by the EU FP7 Marie Curie actions (grant nr. 289754). Thanks to the UK HDBR (http://www.hdbr.org/) for providing ethically approved, anonymized kidney tissues to generate the cell lines in this project.

REFERENCES

1. Winyard PJ, Price KL. Experimental renal progenitor cells: repairing and recreating kidneys? Pediatr Nephrol. 2014;29(4):665-72. doi: 10.1007/s00467-013-2667-5. PubMed PMID: 24221350.

2. Mari C, Winyard P. Concise Review: Understanding the Renal Progenitor Cell Niche In Vivo to Recapitulate Nephrogenesis In Vitro. Stem Cells Transl Med. 2015;4(12):1463-71. doi: 10.5966/sctm.2015-0104. PubMed PMID: 26494782; PubMed Central PMCID: PMCPMC4675508.

3. Bertram JF, Douglas-Denton RN, Diouf B, Hughson MD, Hoy WE. Human nephron number: implications for health and disease. Pediatr Nephrol. 2011;26(9):1529-33. doi: 10.1007/s00467-011-1843-8. PubMed PMID: 21604189.

4. Smeets B, Boor P, Dijkman H, Sharma SV, Jirak P, Mooren F, et al. Proximal tubular cells contain a phenotypically distinct, scattered cell population involved in tubular regeneration. The Journal of pathology. 2013;229(5):645-59. Epub 2012/11/06. doi: 10.1002/path.4125. PubMed PMID: 23124355; PubMed Central PMCID: PMCPMC3951144.

5. Lazzeri E, Crescioli C, Ronconi E, Mazzinghi B, Sagrinati C, Netti GS, et al. Regenerative potential of embryonic renal multipotent progenitors in acute renal failure. Journal of the American Society of Nephrology : JASN. 2007;18(12):3128-38. Epub 2007/11/06. doi: 10.1681/asn.2007020210. PubMed PMID: 17978305.

6. Lange C, Togel F, Ittrich H, Clayton F, Nolte-Ernsting C, Zander AR, et al. Administered mesenchymal stem cells enhance recovery from ischemia/reperfusion-induced acute renal failure in rats. Kidney international. 2005;68(4):1613-7. Epub 2005/09/17. doi: 10.1111/j.1523-1755.2005.00573.x. PubMed PMID: 16164638.

7. Morigi M, Imberti B, Zoja C, Corna D, Tomasoni S, Abbate M, et al. Mesenchymal stem cells are renotropic, helping to repair the kidney and improve function in acute renal failure. Journal of the American Society of Nephrology : JASN. 2004;15(7):1794-804. Epub 2004/06/24. PubMed PMID: 15213267.

8. Duffield JS, Park KM, Hsiao LL, Kelley VR, Scadden DT, Ichimura T, et al. Restoration of tubular epithelial cells during repair of the postischemic kidney occurs independently of bone marrow-derived stem cells. The Journal of clinical investigation. 2005;115(7):1743-55. Epub 2005/07/12. doi: 10.1172/jci22593. PubMed PMID: 16007251; PubMed Central PMCID: PMCPMC1159124.

9. Little MH. Regrow or repair: potential regenerative therapies for the kidney. Journal of the American Society of Nephrology : JASN. 2006;17(9):2390-401. Epub 2006/07/28. doi: 10.1681/asn.2006030218. PubMed PMID: 16870708.

10. Cantley LG. Adult stem cells in the repair of the injured renal tubule. Nature clinical practice Nephrology. 2005;1(1):22-32. Epub 2006/08/26. doi: 10.1038/ncpneph0021. PubMed PMID: 16932361.

11. Dekel B, Shezen E, Even-Tov-Friedman S, Katchman H, Margalit R, Nagler A, et al. Transplantation of human hematopoietic stem cells into ischemic and growing kidneys suggests a role in vasculogenesis but not tubulogenesis. Stem cells (Dayton, Ohio). 2006;24(5):1185-93. Epub 2006/01/18. doi: 10.1634/stemcells.2005-0265. PubMed PMID: 16410390.

12. Casiraghi F, Perico N, Cortinovis M, Remuzzi G. Mesenchymal stromal cells in renal transplantation: opportunities and challenges. Nat Rev Nephrol. 2016;12(4):241-53. doi: 10.1038/nrneph.2016.7. PubMed PMID: 26853275.

13. Al-Awqati Q, Oliver JA. Stem cells in the kidney. Kidney international. 2002;61(2):387-95. Epub 2002/02/19. doi: 10.1046/j.1523-1755.2002.00164.x. PubMed PMID: 11849378.

14. Challen GA, Bertoncello I, Deane JA, Ricardo SD, Little MH. Kidney side population reveals multilineage potential and renal functional capacity but also cellular heterogeneity. Journal of the American Society of Nephrology : JASN. 2006;17(7):1896-912. Epub 2006/05/19. doi: 10.1681/asn.2005111228. PubMed PMID: 16707564.

15. Sagrinati C, Netti GS, Mazzinghi B, Lazzeri E, Liotta F, Frosali F, et al. Isolation and characterization of multipotent progenitor cells from the Bowman's capsule of adult human kidneys. Journal of the American Society of Nephrology : JASN. 2006;17(9):2443-56. Epub 2006/08/04. doi: 10.1681/asn.2006010089. PubMed PMID: 16885410.

16. Bruno S, Bussolati B, Grange C, Collino F, di Cantogno LV, Herrera MB, et al. Isolation and characterization of resident mesenchymal stem cells in human glomeruli. Stem cells and development. 2009;18(6):867-80. Epub 2009/07/07. doi: 10.1089/scd.2008.0320. PubMed PMID: 19579288.

17. Angelotti ML, Ronconi E, Ballerini L, Peired A, Mazzinghi B, Sagrinati C, et al. Characterization of renal progenitors committed toward tubular lineage and their regenerative potential in renal tubular injury. Stem cells (Dayton, Ohio). 2012;30(8):1714-25. Epub 2012/05/26. doi: 10.1002/stem.1130. PubMed PMID: 22628275.

18. Romagnani P, Remuzzi G. CD133+ renal stem cells always co-express CD24 in adult human kidney tissue. Stem Cell Res. 2014;12(3):828-9. doi: 10.1016/j.scr.2013.12.011. PubMed PMID: 24467938.

19. Russel FG, Masereeuw R, van Aubel RA. Molecular aspects of renal anionic drug transport. Annual review of physiology. 2002;64:563-94. Epub 2002/02/05. doi: 10.1146/annurev.physiol.64.081501.155913. PubMed PMID: 11826280.

20. Wilmer MJ, Saleem MA, Masereeuw R, Ni L, van der Velden TJ, Russel FG, et al. Novel conditionally immortalized human proximal tubule cell line expressing functional influx and efflux transporters. Cell Tissue Res. 2010;339(2):449-57. doi: 10.1007/s00441-009-0882-y. PubMed PMID: 19902259; PubMed Central PMCID: PMCPMC2817082.

21. Vanholder R, De Smet R, Lameire N. Protein-bound uremic solutes: the forgotten toxins. Kidney international Supplement. 2001;78:S266-70. Epub 2001/02/13. doi: 10.1046/j.1523-1755.2001.59780266.x. PubMed PMID: 11169024.

22. Masereeuw R, Mutsaers HA, Toyohara T, Abe T, Jhawar S, Sweet DH, et al. The kidney and uremic toxin removal: glomerulus or tubule? Seminars in nephrology. 2014;34(2):191-208. Epub 2014/05/02. doi: 10.1016/j.semnephrol.2014.02.010. PubMed PMID: 24780473.

23. Mutsaers HA, van den Heuvel LP, Ringens LH, Dankers AC, Russel FG, Wetzels JF, et al. Uremic toxins inhibit transport by breast cancer resistance protein and multidrug resistance protein 4 at clinically relevant concentrations. PLoS One. 2011;6(4):e18438. doi: 10.1371/journal.pone.0018438. PubMed PMID: 21483698; PubMed Central PMCID: PMCPMC3070735.

24. Mutsaers HA, Caetano-Pinto P, Seegers AE, Dankers AC, van den Broek PH, Wetzels JF, et al. Proximal tubular efflux transporters involved in renal excretion of p-cresyl sulfate and p-cresyl glucuronide: Implications for chronic kidney disease pathophysiology. Toxicol In Vitro. 2015;29(7):1868-77. doi: 10.1016/j.tiv.2015.07.020. PubMed PMID: 26216510.

25. Caetano-Pinto P, Janssen MJ, Gijzen L, Verscheijden L, Wilmer MJ, Masereeuw

R. Fluorescence-Based Transport Assays Revisited in a Human Renal Proximal Tubule Cell Line. Mol Pharm. 2016;13(3):933-44. doi: 10.1021/acs.molpharmaceut.5b00821. PubMed PMID: 26871298.

26. Masereeuw R, Russel FG. Regulatory pathways for ATP-binding cassette transport proteins in kidney proximal tubules. The AAPS journal. 2012;14(4):883-94. Epub 2012/09/11. doi: 10.1208/s12248-012-9404-z. PubMed PMID: 22961390; PubMed Central PMCID: PMCPMC3475864.

27. Little MH, Kairath P. Regenerative medicine in kidney disease. Kidney international. 2016;90(2):289-99. doi: 10.1016/j.kint.2016.03.030. PubMed PMID: 27234568.

28. Kerjaschki D, Sharkey DJ, Farquhar MG. Identification and characterization of podocalyxin--the major sialoprotein of the renal glomerular epithelial cell. The Journal of cell biology. 1984;98(4):1591-6. Epub 1984/04/01. PubMed PMID: 6371025; PubMed Central PMCID: PMCPMC2113206.

29. Nieskens TT, Peters JG, Schreurs MJ, Smits N, Woestenenk R, Jansen K, et al. A Human Renal Proximal Tubule Cell Line with Stable Organic Anion Transporter 1 and 3 Expression Predictive for Antiviral-Induced Toxicity. AAPS J. 2016;18(2):465-75. doi: 10.1208/s12248-016-9871-8. PubMed PMID: 26821801; PubMed Central PMCID: PMCPMC4779111.

30. Zhou S, Schuetz JD, Bunting KD, Colapietro AM, Sampath J, Morris JJ, et al. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nature medicine. 2001;7(9):1028-34. Epub 2001/09/05. doi: 10.1038/nm0901-1028. PubMed PMID: 11533706.

31. Bunting KD. ABC transporters as phenotypic markers and functional regulators of stem cells. Stem cells (Dayton, Ohio). 2002;20(1):11-20. Epub 2002/01/18. doi: 10.1634/stemcells.20-3-274. PubMed PMID: 11796918.

32. Krishnamurthy P, Ross DD, Nakanishi T, Bailey-Dell K, Zhou S, Mercer KE, et al. The stem cell marker Bcrp/ABCG2 enhances hypoxic cell survival through interactions with heme. The Journal of biological chemistry. 2004;279(23):24218-25. doi: 10.1074/jbc.M313599200. PubMed PMID: 15044468.

33. Jansen J, Schophuizen CM, Wilmer MJ, Lahham SH, Mutsaers HA, Wetzels JF, et al. A morphological and functional comparison of proximal tubule cell lines established from human urine and kidney tissue. Exp Cell Res. 2014;323(1):87-99. doi: 10.1016/j.yexcr.2014.02.011. PubMed PMID: 24560744.

34. Prasad B, Johnson K, Billington S, Lee CA, Chung GW, Brown CD, et al. Abundance of drug transporters in the human kidney cortex as quantified by quantitative targeted proteomics. Drug Metab Dispos. 2016. doi: 10.1124/dmd.116.072066. PubMed PMID: 27621205.

35. van Kalken CK, Giaccone G, van der Valk P, Kuiper CM, Hadisaputro MM, Bosma SA, et al. Multidrug resistance gene (P-glycoprotein) expression in the human fetus. Am J Pathol. 1992;141(5):1063-72. PubMed PMID: 1359789; PubMed Central PMCID: PMCPMC1886663.

36. Da Sacco S, Thornton ME, Petrosyan A, Lavarreda-Pearce M, Sedrakyan S, Grubbs BH, et al. Direct Isolation and Characterization of Human Nephron Progenitors. Stem Cells Transl Med. 2016. doi: 10.5966/sctm.2015-0429. PubMed PMID: 27612517.

37. Takasato M, Er PX, Becroft M, Vanslambrouck JM, Stanley EG, Elefanty AG, et al. Directing human embryonic stem cell differentiation towards a renal lineage generates a self-organizing kidney. Nat Cell

Biol. 2014;16(1):118-26. doi: 10.1038/ncb2894. PubMed PMID: 24335651.

38. Pino CJ, Humes HD. Stem cell technology for the treatment of acute and chronic renal failure. Transl Res. 2010;156(3):161-8. doi: 10.1016/j.trsl.2010.07.005. PubMed PMID: 20801413; PubMed Central PMCID: PMCPMC2930898.

39. Eirin A, Lerman LO. Mesenchymal stem cell treatment for chronic renal failure. Stem Cell Res Ther. 2014;5(4):83. doi: 10.1186/scrt472. PubMed PMID: 25158205; PubMed Central PMCID: PMCPMC4097822.

40. Peired AJ, Sisti A, Romagnani P. Mesenchymal Stem Cell-Based Therapy for Kidney Disease: A Review of Clinical Evidence. Stem Cells Int. 2016;2016:4798639. doi: 10.1155/2016/4798639. PubMed PMID: 27721835.

41. Song JJ, Guyette JP, Gilpin SE, Gonzalez G, Vacanti JP, Ott HC. Regeneration and experimental orthotopic transplantation of a bioengineered kidney. Nature medicine. 2013;19(5):646-51. doi: 10.1038/nm.3154. PubMed PMID: 23584091; PubMed Central PMCID: PMCPMC3650107.

42. Dekel B, Reisner Y. Embryonic committed stem cells as a solution to kidney donor shortage. Expert Opin Biol Ther. 2004;4(4):443-54. doi: 10.1517/14712598.4.4.443. PubMed PMID: 15102595.

CHAPTER 7

General Discussion

INTRODUCTION

The kidneys are crucial for the removal of waste and toxicants from the blood stream. For this purpose, proximal tubule epithelial cells are equipped with a myriad of different membrane transporters responsible for the uptake and extrusion of a great variety of metabolic products and exogenous compounds (xenobiotics) [1]. Fundamental research into the mechanisms responsible for renal transport activity is key to our understanding of kidney function. Kidney disease progression and uremic solutes retention, as well as interactions affecting drug disposition are intrinsically related to transport activity [2, 3].

The model of choice for the present thesis was the conditionally immortalized human proximal tubule cell line - ciPTEC [4], a cell model that closely resembles human proximal tubule physiology. The model was improved with the expression of the OAT1 transporter [5], a transporter lost in epithelial cell cultures *in vitro* [6]. Previous studies have shown ciPTEC metabolic activity [7], and potential use in the development of bio-artificial membranes, with the purpose of improving current hemodialysis for patients with kidney failure [8-11]. With over 30 peer-reviewed publications (ncbi.gov) on the properties and applications of ciPTEC, this model is a proven platform for kidney research and the work in this thesis explored the activity and regulation of renal drug transporters [2]. In this final chapter, the overall implications of this work for drug discovery, kidney physiology and regenerative nephrology is being discussed.

TRANSPORT ACTIVITY AND FLUORESCENCE-BASED ASSAYS

Fluorescent techniques have become a cornerstone of molecular and cellular research. Currently there is a broad variety of fluorescent probes available for multiple uses, from staining cellular components such as the cytoskeleton to visualizing processes like apoptosis and mitochondrial activity [12, 13]. As non-physiological compounds, these probes are potential substrates for membrane transporters. Their recognition as key intervenients in multidrug resistance (MDR) brought them into the spotlight in the 70's of last century [14]. Since then, several drug transporters have been discovered and implicated in a multitude of physiological processes [15]. Early on research showed that cancer cells poorly retained fluorescent dyes, derived from their enhanced efflux activity, while the dyes accumulated in combination with pharmacological agents [16-18]. Hence the potential use of fluorescent substrates as tools to study transport activity *in vitro* has been long recognized.

Just as research progressed and the number of proteins characterized as membrane transporters expanded, so did the list of dyes described as substrates [19]. In the present thesis, fluorescence is a tool, transversal in all experimental work (Chapters 3-6). We used the known substrates Hoechst33342, calcein-AM, CMFDA/GS-MF and fluorescein, described for Breast cancer resistance protein (BCRP), P-glycoprotein (P-gp), Multidrug resistance proteins (MRPs) and organic anion transporters (OATs), respectively, to evaluate their function [3, 5].

Fluorescent assays revealed to be technically straightforward and a powerful tool capable of generating large data sets, as was evident in chapters 3 and 6. These assays are a valuable addition to other analytical methodologies widely applied to investigate membrane transporters function, such as scintillation counting when radiolabeled substrates are being used and mass-spectrometry of unlabeled compounds, as applied in chapter 4. A major benefit of fluorescent-based techniques lays in the use of compounds that do not require special handling or laborious sample preparation, and the analysis can be performed rapidly using a plate reader. This allows for multiple experimental conditions to be tested in parallel, and makes this approach less laborious than fluorescence acquisition via flow cytometry or confocal microscopy [20, 21]. Despite their advantages, the use of fluorescence-based transport assays requires certain considerations. Fluorescence can be influenced by changes in pH and temperature, quenching, and optical or luminescent properties of other compounds/drugs/substrates added simultaneously [22]. Autofluorescence of the cells can impair results, promoting unreliable measurements. This effect is derived from the excitation of endogenous fluorophores, such as the reducing agent NADH, in mitochondria and lysosomes [23]. The substrate concentration used in combination with a given blocker should be below the Michaelis-Menten constant - Km - value to avoid non-specific effects. Furthermore, long incubation times can lead to oversaturation, reduced viability and loss in fluorescent signal. Finally, the sensitivity of a typical plate reader can fall behind other quantification methods [24].

As illustrated by Jenkinson *et al.*, fluorescent assays were unable to determine function in a model that revealed poor transporter expression [25], in stark contrast to ciPTEC. Therefore, a comprehensive knowledge of the transporters expressed is essential to interpret functional results. The use of a specific transporter inhibitor in combination with fluorescent dyes results in considerable intracellular fluorescence, when blocking efflux transporters. However, caution is required given the protein homology, providing model inhibitors at different concentrations with the capacity to inhibit several transporters, as shown in chapter 3. The dyes used for investigating efflux transporters in ciPTEC were lipophilic and could diffuse across the cell membrane. Diffusion may also be involved in the substrates movement out of the cell; nonetheless this phenomenon should be largely overshadowed by the transporters activity. Certain substrates, such as calcein-AM and CMFDA, are fluorescent precursors and require enzymatic activation to acquire fluorescence. *In vitro* models lacking such enzymes (*e.g.* hydrolases and glucuronidases) are not suited for these assays. Conversely, substrates can be potentially rendered ineffective by the action of metabolic enzymes.

The efflux activity studies in ciPTEC (chapter 3), underline the complex interactions between drug transporters and substantial substrate promiscuity [26]. The interactions between uptake and efflux transporters can be studied using a substrate selective for both an uptake and efflux carrier (Figure 1). The bioluminescent substrate D-luciferin is a known BCRP substrate used *in vivo* [27]. When tested using ciPTEC-OAT1 cells, a ciPTEC-derived cell line overexpressing the renal uptake transporter OAT1 [5], we found

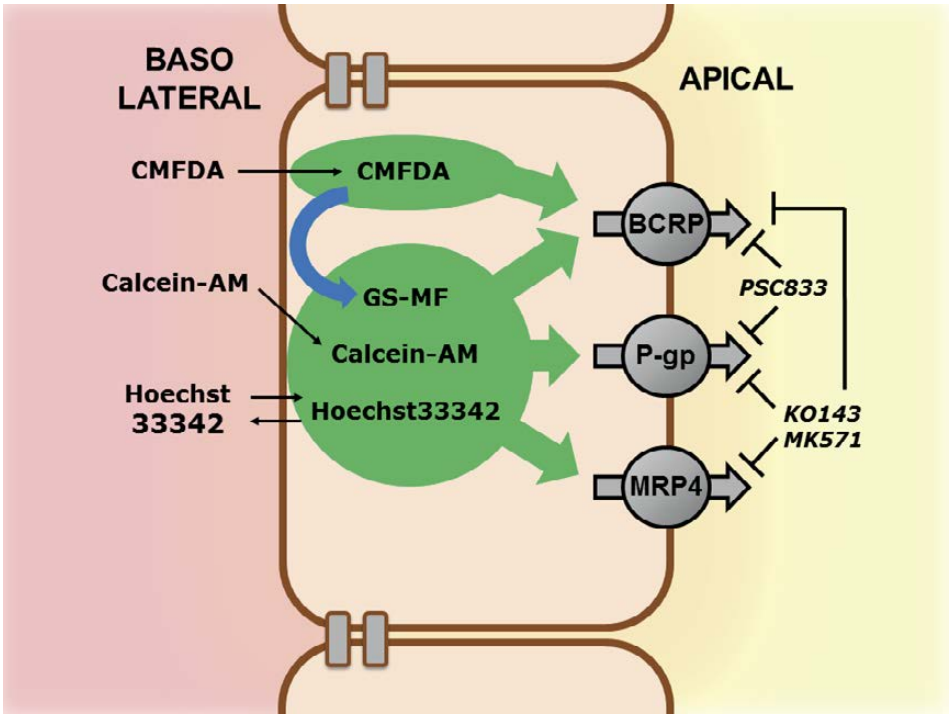


Figure 1. Fluorescent substrates uptake and efflux in proximal tubular cells. Summary of the interactions between fluorescent substrates and inhibitors in proximal tubular cells. Receptor mediated endocytosis can also be determined using fluorescent substrates and it is also depicted in the bottom of the picture.

that D-luciferin only accumulates in OAT1 expressing cells upon BCRP inhibition. This was not observed when cells do not overexpress OAT1, as demonstrated by the parent ciPTEC (Figure 2A). Further, OAT1 inhibition abolished D-Luciferin uptake, underlining OAT1 role in its handling. These findings prove a cooperative effect between drug uptake (OAT1) and efflux (BCRP) transporters [28, 29].

Overall fluorescent assays can be reliable and efficient approaches to obtain functional data and study pharmacological interactions mediated by membrane transporters *in vitro*. Nonetheless, it is imperative to determine the boundaries of such assays. As novel fluorescent dyes are being identified as substrates and the number of specific inhibitors expand, these type of assays have the potential to become mainstream in transporters research in drug development.

The challenges of renal function in regenerative nephrology

Tissue engineering together with stem cell research have paved the way for regenerative medicine, a collection of methodologies that allows to repair or replace damaged organs [30, 31]. Gaining momentum since the late 20th century, the field of regenerative medicine

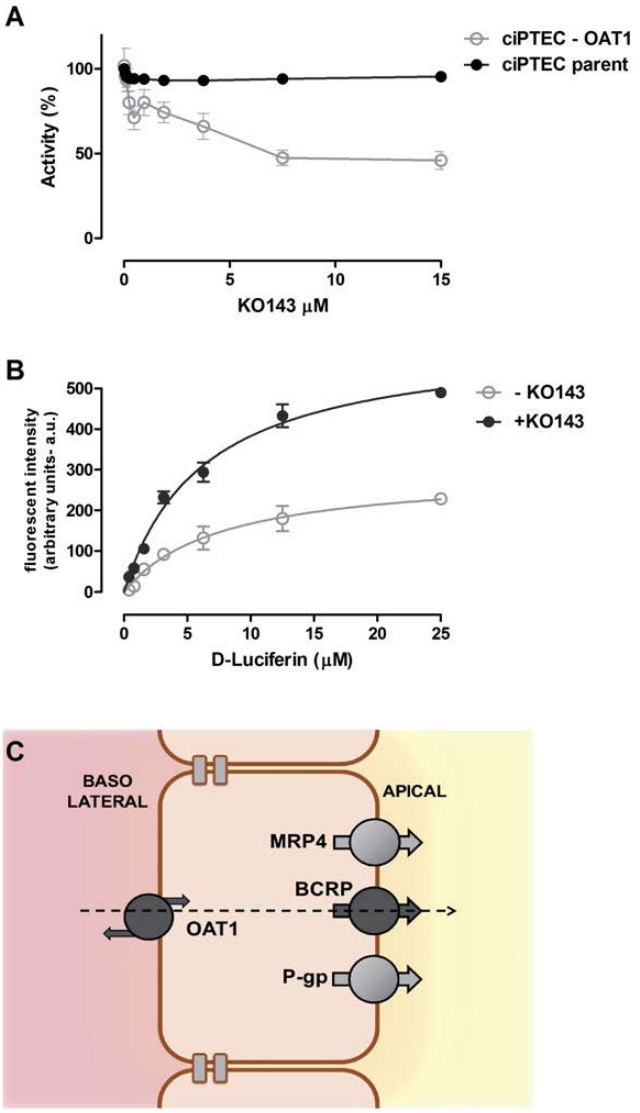


Figure 2. D-Luciferin uptake and efflux in ciPTEC-OAT1 cells. BCRP inhibitor KO143 only yields Luciferin accumulation in ciPTEC-OAT1 cells in contrast with wild type ciPTEC (ciPTEC 14.4) (A). D-Luciferin accumulates in the presence of 5 µM KO143 (B). Panel C depicts D-luciferin transport from the basolateral (blood side) to the apical side (pro-urine) of proximal tubule cells.

has exploded [32, 33]. Significant achievements have been made in the reconstruction of skin, bone, cartilage and muscle, leading to clinical applications [34-37], and continuous research efforts are seeking strategies to generate virtually every organ in the human body [38]. The success of regenerative medicine is built on the multi-disciplinary nature of combining advances in material science and bio-fabrication with know-how on cellular physiology and organ development/ontogeny together with clinical practices [39]. Nonetheless, the more complex the organ in hand, the more complex the challenges.

To date the major breakthrough in the treatment of chronic renal diseases has been replacement therapy, either by kidney transplantation or by hemodialysis [40]. Kidney transplants may offer a lifelong solution for patients, however, they are hampered by organ availability and histocompatibility issues, that require permanent monitoring and pharmacological intervention to prevent organ rejection [41]. On the other hand, hemodialysis can improve the quality of life, though it does not represent a permanent solution and carries a significant burden for patients and families [42, 43].

The so-called bio-artificial kidney (BAK) is a popular approach that uses synthetic fibers lined with cells. Arguably, it aims at replacing function and not regenerating the kidney. Nonetheless it restores renal function using elements from dialysis and kidney cells [44]. As discussed in Chapter 1, cell source is a pressing matter, and guarantying the proper cell phenotype and function are vital. The fluorescent tools described in Chapter 3 are a reliable methodology to verify the cellular activity of bio-engineered applications for kidney regeneration [10]. This became further evident in chapter 6 where kidney progenitor cells were tested for their differentiation capability towards proximal tubule epithelial cells. Upon passaging progenitor cells acquired a more differentiated phenotype with enhanced transporter activity. Fetal progenitor cells are a valuable research tool but with poor practical applicability [45]. The discovery of renal progenitors in the urine of preterm neonates [46] can abolish the source limitation of renal progenitors and can be used to exploit the regenerative potential of these cells.

Other promising strategies in renal regeneration rely on the of recellularization of kidney scaffolds, either de-cellularized or engineered by biofabrication [47-49]. Recreating kidneys *in vitro*, a method that builds on the immense know-how gathered from developmental nephrology [50], is also showing promising results. Both renal progenitors and induced pluripotent stem cells (iPS) were used successfully to generate phenotypically accurate kidney organoids [51, 52]. Though anatomically correct and expressing the desired renal markers, these renal constructs can only be useful when mimicking not only kidney structures but also function [53]. Currently, the organoids are still in an embryonic state [52, 54, 55].

Generating kidney organoids and testing renal progenitor cells *in vitro* present an opportunity not only for regenerative medicine, but also for the improvement and refinement of current experimental methods [56, 57]. Renal progenitors can be used to test the toxicity of a variety of compounds in cells cultured in three-dimensional structures and can be used as a "down-scaled" prototype for nephrotoxicity in developing structures.

Pre-clinical testing and experimentation refinement

The development of compounds intended to be administered to humans, either drugs for therapeutical purposes or molecules/tracers for diagnostics procedures, involves several meticulous testing phases [58]. Human testing is a tightly regulated, expensive and time-consuming enterprise. To reduce development costs and approval timeframes of human trials, pre-clinical results, often derived from animal models, must provide reliable results.

In recent years, the increasing awareness of the scientific community and general public towards laboratory animals welfare pushed for more restrictive legislation governing animal experimentation [59]. Research guidelines such as the 3R's – replacement, reducement and refinement of animal experimentation- have gained considerable momentum and novel models, techniques and study designs are being sought for pre-clinical testing with human predictive value [60].

In chapter 1, we reviewed renal cell models currently available as research tools, and these systems are of particular interest in drug development given the fact that a large number of drugs is cleared via the kidneys and current drug development guidelines enforce the testing of both drug transporters and clearance route of drugs in the research pipeline [61]. The experimental work laid out in chapters 2-5 evaluated the use of ciPTEC to investigate renal drug transport function. This work served the double purpose of further characterizing the ciPTEC as a human physiologically relevant model and to reveal the contours of pathophysiology, by studying uremic toxin handling and the renal toxicological effects of cancer medication.

As model able to recreate *in vitro* tubular excretion and reabsorption, the ciPTEC was an ideal candidate to screen novel tracers for *in vivo* renal function assessment, in an early testing phase. This was applied to novel compounds suitable for transcutaneous measurement of renal function (TMRF) by assessing the glomerular filtration rate (GFR) in real time [62, 63]. To meet this purpose, it is crucial that the probes are completely excreted into urine solely by filtration and without proximal tubular reabsorption or secretion. Other requirements include fluorescent stability, efficient glomerular filtration, metabolic inactivity and absence of overt toxicity. In the development of optimized fluorescently labeled cyclodextrin derivatives, several compounds were generated and extensively tested for TMRF. Screening for compound distribution, absorption and toxicity using a rat model requires considerable numbers of animals *per* compound tested. Therefore, a first screening was done using ciPTEC to determine their renal tubular cell handling and cytotoxic potential. In the end, candidate molecules were validated and the results were recently published [64]. Figure 3, depicts the uptake assays performed for three candidate cyclodextrin probes.

On the road to clinical implementation, the ciPTEC model can be used as a physiological prototype of the proximal tubules, with endogenous transport activity allowing drug testing. A significant number of compounds fail during trials at a later stage in drug development [65, 66]. This is often due to poor predictability of the models used in early testing phases and the variability of animal studies [67]. Comprehensive models can improve the quality of *in vitro* data and when allied with predictive tools that are able to simulate pharmacokinetic parameters [68] (*e.g*. bioinformatics), such models can greatly reduce the time-frames and the quality of medicines/therapies implemented into practice.

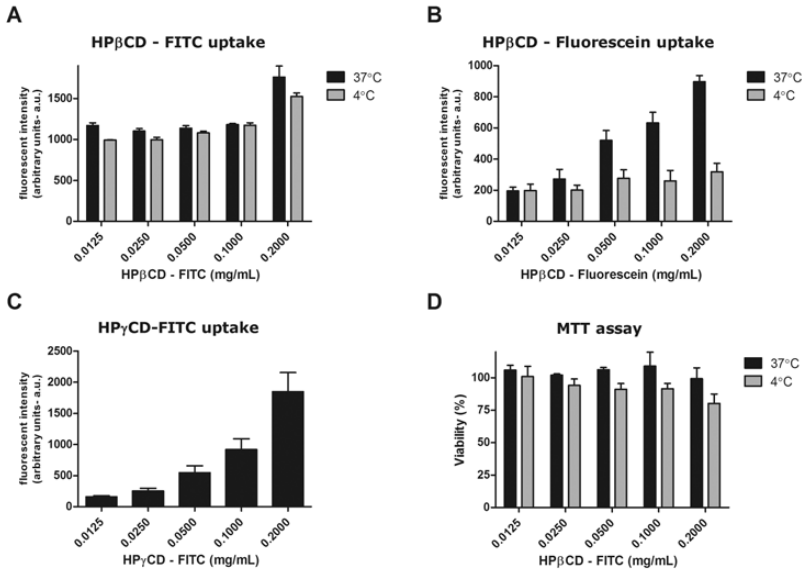


Figure 3. Uptake and toxicity examples of fluorescently labeled cyclodextrins in ciPTEC-OAT1. Dose-dependent uptake of the probes was performed at either 37°C or 4°C, as a negative control for diminished biological activity. The compound HPβCD conjugated with a FITC label yielded only an increase in fluorescence at the highest concentration tested (A) and no toxicity (D), while conjugated fluorescein HPβCD showed dose-dependent uptake at 37°C (B). Another compound, HPγCD conjugated with FITC, showed a dose dependent uptake at 37°C, fluorescence acquisition was not determined at 4°C (C). This procedure using ciPTEC allowed screening for compounds showing biological activity in proximal tubular cells.

RENAL DRUG TRANSPORTERS REGULATION AND CLINICAL IMPLICATIONS

Extensive research into MDR has deciphered the signaling mechanisms that govern the activity of distinct drug transporters and the effects of chemotherapeutical agents [69]. Cellular signaling pathways are responsible for controlling, virtually, all aspects of a cell's physiology, from development to death. Drugs excreted via the renal route can affect the activity of BCRP by modulating ubiquitous pathways, as shown in chapter 2. Drug transporter regulation can potentially impact drug disposition, a phenomenon widely described in MDR [70]. Understanding how membrane transporters are regulated under normal physiological conditions is crucial when investigating drug-drug interactions, predicting side-effects of medication and even determining the effectiveness of a drug [71, 72]. In chapter 5, a cancer combinatory therapy involving two agents and undergoing clinical trials (NCT02054442 - ClinicalTrials.gov) was investigated *in vitro* to determine consequences at the renal level. Our findings demonstrate that the non-nephrotoxic epidermal growth factor receptor (EGFR) inhibitor, cetuximab [73], extensively changes the expression of all major renal drug transporters, subsequently reducing methotrexate

nephrotoxicity [74]. Overall this evidence uncovered a seemingly beneficial off target effect of a EGFR inhibitor derived from its cellular regulatory properties.

Until know, chronic kidney disease (CKD) cannot be reversed, and current treatments aim at managing and relieving symptoms [40]. Furthermore, it is roughly estimated that 30% of patients undergoing cancer treatment are affected by renal insufficiency and that 12% suffer from acute kidney injury [75, 76]. Following renal replacement therapy, the risk of developing cancer increases by 3-fold [77]. The number of kidney patients undergoing co-medication therapies is increasing, and common chemotherapeutical drugs can be nephrotoxic [78, 79]. It is important to understand how drug-interactions affect renal physiology and by drawing evidence from both clinic and biomedical fundamental research novel therapies, using available drugs, can be developed to manage kidney and oncological disease [80-82]. The benefits of such approach can increase the efficacy of nephrotoxic drugs, such as cisplatin, if kidney damage can be limited or prevented. CKD progression can be minimized if potentially damaging drug combinations can be predicted and avoided.

PERSPECTIVES

A renewed wave of interest in transporter research came with the implementation of guidelines that include the testing of membrane transporters in drug interaction studies [83, 84]. Both academia and industry have committed a fair amount of resources exploring transporter mediated pharmacological interactions. Considering the high density of transporters in the kidney [85], further and more complex interactions could be uncovered in the future. The generalization of functional assays, high throughput screening strategies and precision analytical technologies (*e.g.* targeted proteomics, peptide arrays) is boosting research into all aspects of the transporter influence sphere. Present trends in research are shifting towards transporter regulation and the mechanisms behind transporter interactions. Concepts as remote sensing signaling and transport-mediated systemic regulation of solutes (*e.g.* drugs, metabolites and hormones) are emerging [86, 87]. The classical view of transporters as single entities operating alone is being abandoned. Absorption, distribution, metabolism and excretion (ADME) studies incorporate renal transport activity and expression of several transporters in their design [61], and the ontogeny and expression of renal drug transporters is now also recognized in pediatric pharmacology [88]. Bioinformatics can manage large data sets and bring transporters regulation, expression, activity and interactions together by the construction of potent perdition computational tools for ADME in drug development [89]. Overall, research into renal transport can be expected to become more integrative while it moves towards clinical significance.

Transport function is a highly dynamic physiological process and it is accepted that *in vitro* or end point animal experiments only take a snapshot. Quantifying transport activity can become a powerful diagnostic tool. Positron emission tomography has been successfully used to determine live transporter mediated drug disposition in humans

[90]. Although promising, this type of approach requires advanced equipment, it is labor intensive and involves substantial costs. A simpler and cost-effective solution can be found in real-time TMRF [62]. Novel probes, build on the present understanding of transporters function, could be used to measure renal transport-mediated excretion, in the same fashion used to determine filtration rate [64]. These strategies are being explored for clinical applications (medibeacon.com) and could potentially be implemented into practice, providing easy-access tools for comprehensive renal disease diagnostic and monitoring.

The use of advanced and well-characterized *in vitro* models in renal drug transport research is also expected to expand in the upcoming years. Nephrotoxicity screening and drug testing will be at the forefront of *in vitro* assays. The expansion of "organ-on-a-chip" technologies has seen the merger of cellular models with microfluidic nanofabrication technologies seeking to control the cellular micro-environment and offer high-throughput screening tools. The so-called 'kidney-on-a-chip' may soon become a commercially available reality, providing a platform for further investigating renal transport activity [91-93]. In terms of improving the cell model phenotype that resembles the physiological condition, the use of renal progenitors and iPS cells can be of additional value. Targeted differentiation combined with custom-made biomaterials could provide high quality renal cell prototypes. A novel technique, known as the clustered regularly interspaced short palindromic repeats and associated gene or CRISPR-CAS, allows for the precise gene manipulation of living cells. Genes can now be removed and inserted in precise locations, rendering existing techniques such as viral transfections obsolete [94-96]. Transporter genes can be added or removed from a cell line on-demand or mutated to represent a polymorphism; the opportunities for genetically modified models to study transporter interactions and regulation are enormous. Further the technique can be also be used *in vivo,* by manipulating germ cells, tailoring animals according to the experimental needs. It can be expected that the CRISPR-CAS technology will become ubiquitous in many biomedical research fields in the near future, providing better models for drug-interactions and transport regulation studies as well as kidney diseases.

The potential for translational and regenerative medicine of the methodologies exposed here is considerable. Mostly driven by principles of the 3R's, it is likely that tools and innovative approaches derived from kidney tissue engineering will find their way into fundamental renal pharmacological research. The use of CRISPR-CAS combined with iPS cell technology could provide us with a sustainable and reliable source of high quality cells for tissue repair and fundamental research.

All things considered, this thesis demonstrates the use of fluorescent substrates as a tool to investigate the activity of ABC and SLC transporters acting simultaneously; sheds light on the role played by ubiquitous cell signaling in renal drug excretion and shows that kidney fetal progenitors cells are functionally active. This is a step forward in understanding the activity of renal drug transporters and provides tools to further explore renal transport regulation.

REFERENCES

1. Nigam SK, Wu W, Bush KT, Hoenig MP, Blantz RC, Bhatnagar V. Handling of Drugs, Metabolites, and Uremic Toxins by Kidney Proximal Tubule Drug Transporters. Clin J Am Soc Nephrol. 2015;10(11):2039-49. doi: 10.2215/CJN.02440314. PubMed PMID: 26490509; PubMed Central PMCID: PMCPMC4633783.

2. Mutsaers HA, Caetano-Pinto P, Seegers AE, Dankers AC, van den Broek PH, Wetzels JF, et al. Proximal tubular efflux transporters involved in renal excretion of p-cresyl sulfate and p-cresyl glucuronide: Implications for chronic kidney disease pathophysiology. Toxicol In Vitro. 2015;29(7):1868-77. doi: 10.1016/j.tiv.2015.07.020. PubMed PMID: 26216510.

3. Caetano-Pinto P, Janssen MJ, Gijzen L, Verscheijden L, Wilmer MJ, Masereeuw R. Fluorescence-Based Transport Assays Revisited in a Human Renal Proximal Tubule Cell Line. Mol Pharm. 2016;13(3):933-44. doi: 10.1021/acs.molpharmaceut.5b00821. PubMed PMID: 26871298.

4. Wilmer MJ, Saleem MA, Masereeuw R, Ni L, van der Velden TJ, Russel FG, et al. Novel conditionally immortalized human proximal tubule cell line expressing functional influx and efflux transporters. Cell Tissue Res. 2010;339(2):449-57. doi: 10.1007/s00441-009-0882-y. PubMed PMID: 19902259; PubMed Central PMCID: PMCPMC2817082.

5. Nieskens TT, Peters JG, Schreurs MJ, Smits N, Woestenenk R, Jansen K, et al. A Human Renal Proximal Tubule Cell Line with Stable Organic Anion Transporter 1 and 3 Expression Predictive for Antiviral-Induced Toxicity. AAPS J. 2016;18(2):465-75. doi: 10.1208/s12248-016-9871-8. PubMed PMID: 26821801; PubMed Central PMCID: PMCPMC4779111.

6. Miller JH. Sodium-sensitive, probenecid-insensitive p-aminohippuric acid uptake in cultured renal proximal tubule cells of the rabbit. Proc Soc Exp Biol Med. 1992;199(3):298-304. PubMed PMID: 1347169.

7. Mutsaers HA, Wilmer MJ, Reijnders D, Jansen J, van den Broek PH, Forkink M, et al. Uremic toxins inhibit renal metabolic capacity through interference with glucuronidation and mitochondrial respiration. Biochimica et biophysica acta. 2013;1832(1):142-50. doi: 10.1016/j.bbadis.2012.09.006. PubMed PMID: 23017367.

8. Schophuizen CM, De Napoli IE, Jansen J, Teixeira S, Wilmer MJ, Hoenderop JG, et al. Development of a living membrane comprising a functional human renal proximal tubule cell monolayer on polyethersulfone polymeric membrane. Acta Biomater. 2015;14:22-32. doi: 10.1016/j.actbio.2014.12.002. PubMed PMID: 25527093.

9. Jansen J, De Napoli IE, Fedecostante M, Schophuizen CM, Chevtchik NV, Wilmer MJ, et al. Human proximal tubule epithelial cells cultured on hollow fibers: living membranes that actively transport organic cations. Sci Rep. 2015;5:16702. doi: 10.1038/srep16702. PubMed PMID: 26567716; PubMed Central PMCID: PMCPMC4644946.

10. Jansen J, Fedecostante M, Wilmer MJ, Peters JG, Kreuser UM, van den Broek PH, et al. Bioengineered kidney tubules efficiently excrete uremic toxins. Sci Rep. 2016;6:26715. doi: 10.1038/srep26715. PubMed PMID: 27242131; PubMed Central PMCID: PMCPMC4886219.

11. Chevtchik NV, Fedecostante M, Jansen J, Mihajlovic M, Wilmer M, Ruth M, et al. Upscaling of a living membrane for bioartificial kidney device. Eur J Pharmacol. 2016. doi: 10.1016/j.ejphar.2016.07.009. PubMed PMID: 27395800.

12. Nagano T. Development of fluorescent probes for bioimaging applications. Proc Jpn Acad Ser B Phys Biol Sci. 2010;86(8):837-47. PubMed PMID: 20948177; PubMed Central PMCID: PMCPMC3037519.

13. Terai T, Nagano T. Small-molecule fluorophores and fluorescent probes

for bioimaging. Pflugers Arch. 2013;465(3):347-59. doi: 10.1007/s00424-013-1234-z. PubMed PMID: 23412659.

14. Chen CJ, Chin JE, Ueda K, Clark DP, Pastan I, Gottesman MM, et al. Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. Cell. 1986;47(3):381-9. PubMed PMID: 2876781.

15. Nigam SK. What do drug transporters really do? Nat Rev Drug Discov. 2015;14(1):29-44. doi: 10.1038/nrd4461. PubMed PMID: 25475361; PubMed Central PMCID: PMCPMC4750486.

16. Neyfakh AA. Use of fluorescent dyes as molecular probes for the study of multidrug resistance. Exp Cell Res. 1988;174(1):168-76. PubMed PMID: 3335222.

17. Neyfakh AA, Dmitrevskaya TV, Serpinskaya AS. The membrane transport system responsible for multidrug resistance is operating in nonresistant cells. Exp Cell Res. 1988;178(2):513-7. PubMed PMID: 3169137.

18. Matsumoto Y, Sasaoka N, Tsuchida T, Fujiwara T, Nagao S, Ohmoto T. Fluorescent dye rhodamine 6G as a molecular probe to study drug resistance of C6 rat glioma cells. J Neurooncol. 1992;13(3):217-22. PubMed PMID: 1517798.

19. Fardel O, Le Vee M, Jouan E, Denizot C, Parmentier Y. Nature and uses of fluorescent dyes for drug transporter studies. Expert Opin Drug Metab Toxicol. 2015;11(8):1233-51. doi: 10.1517/17425255.2015.1053462. PubMed PMID: 26050735.

20. Masereeuw R, Russel FG, Miller DS. Multiple pathways of organic anion secretion in renal proximal tubule revealed by confocal microscopy. Am J Physiol. 1996;271(6 Pt 2):F1173-82. PubMed PMID: 8997391.

21. Lebedeva IV, Pande P, Patton WF. Sensitive and specific fluorescent probes for functional analysis of the three major types of mammalian ABC transporters. PLoS One. 2011;6(7):e22429. doi: 10.1371/journal.pone.0022429. PubMed PMID: 21799851; PubMed Central PMCID: PMCPMC3142157.

22. Sarder P, Maji D, Achilefu S. Molecular probes for fluorescence lifetime imaging. Bioconjug Chem. 2015;26(6):963-74. doi: 10.1021/acs.bioconjchem.5b00167. PubMed PMID: 25961514; PubMed Central PMCID: PMCPMC4482119.

23. Bartolome F, Abramov AY. Measurement of mitochondrial NADH and FAD autofluorescence in live cells. Methods Mol Biol. 2015;1264:263-70. doi: 10.1007/978-1-4939-2257-4_23. PubMed PMID: 25631020.

24. Eric Jones SM, and G. Sitta Sittampalam. Basics of Assay Equipment and Instrumentation for High Throughput Screening. National Center for Advancing Translational Sciences National Institutes of Health. 2012.

25. Jenkinson SE, Chung GW, van Loon E, Bakar NS, Dalzell AM, Brown CD. The limitations of renal epithelial cell line HK-2 as a model of drug transporter expression and function in the proximal tubule. Pflugers Arch. 2012;464(6):601-11. doi: 10.1007/s00424-012-1163-2. PubMed PMID: 23014881.

26. Wong K, Ma J, Rothnie A, Biggin PC, Kerr ID. Towards understanding promiscuity in multidrug efflux pumps. Trends Biochem Sci. 2014;39(1):8-16. doi: 10.1016/j.tibs.2013.11.002. PubMed PMID: 24316304.

27. Bakhsheshian J, Wei BR, Hall MD, Simpson RM, Gottesman MM. In Vivo Bioluminescent Imaging of ATP-Binding Cassette Transporter-Mediated Efflux at the Blood-Brain Barrier. Methods Mol Biol. 2016;1461:227-39. doi: 10.1007/978-1-4939-3813-1_19. PubMed PMID: 27424909.

28. VanWert AL, Gionfriddo MR, Sweet DH. Organic anion transporters: discovery, pharmacology, regulation and roles in pathophysiology. Biopharm Drug Dispos.

2010;31(1):1-71. doi: 10.1002/bdd.693. PubMed PMID: 19953504.

29. Mao Q, Unadkat JD. Role of the breast cancer resistance protein (BCRP/ABCG2) in drug transport--an update. AAPS J. 2015;17(1):65-82. doi: 10.1208/s12248-014-9668-6. PubMed PMID: 25236865; PubMed Central PMCID: PMCPMC4287283.

30. Bianco P, Robey PG. Stem cells in tissue engineering. Nature. 2001;414(6859):118-21. doi: 10.1038/35102181. PubMed PMID: 11689957.

31. Howard D, Buttery LD, Shakesheff KM, Roberts SJ. Tissue engineering: strategies, stem cells and scaffolds. J Anat. 2008;213(1):66-72. doi: 10.1111/j.1469-7580.2008.00878.x. PubMed PMID: 18422523; PubMed Central PMCID: PMCPMC2475566.

32. Berthiaume F, Maguire TJ, Yarmush ML. Tissue engineering and regenerative medicine: history, progress, and challenges. Annu Rev Chem Biomol Eng. 2011;2:403-30. doi: 10.1146/annurev-chembioeng-061010-114257. PubMed PMID: 22432625.

33. Power C, Rasko JE. Promises and challenges of stem cell research for regenerative medicine. Ann Intern Med. 2011;155(10):706-13, W217. doi: 10.7326/0003-4819-155-10-201111150-00010. PubMed PMID: 22084335.

34. Zhang Z, Michniak-Kohn BB. Tissue engineered human skin equivalents. Pharmaceutics. 2012;4(1):26-41. doi: 10.3390/pharmaceutics4010026. PubMed PMID: 24300178; PubMed Central PMCID: PMCPMC3834903.

35. Nowicki MA, Castro NJ, Plesniak MW, Zhang LG. 3D printing of novel osteochondral scaffolds with graded microstructure. Nanotechnology. 2016;27(41):414001. doi: 10.1088/0957-4484/27/41/414001. PubMed PMID: 27606933.

36. Zhao W, Tian LX, Zhou XR, Zhao FR. Clinical Experience of Zhao's Artificial Trachea. Chin Med J (Engl). 2016;129(1):95-7. doi: 10.4103/0366-6999.172602. PubMed PMID: 26712440; PubMed Central PMCID: PMCPMC4797551.

37. Orabi H, Bouhout S, Morissette A, Rousseau A, Chabaud S, Bolduc S. Tissue engineering of urinary bladder and urethra: advances from bench to patients. ScientificWorldJournal. 2013;2013:154564. doi: 10.1155/2013/154564. PubMed PMID: 24453796; PubMed Central PMCID: PMCPMC3886608.

38. Mao AS, Mooney DJ. Regenerative medicine: Current therapies and future directions. Proc Natl Acad Sci U S A. 2015;112(47):14452-9. doi: 10.1073/pnas.1508520112. PubMed PMID: 26598661; PubMed Central PMCID: PMCPMC4664309.

39. Terzic A, Pfenning MA, Gores GJ, Harper CM, Jr. Regenerative Medicine Build-Out. Stem Cells Transl Med. 2015;4(12):1373-9. doi: 10.5966/sctm.2015-0275. PubMed PMID: 26537392; PubMed Central PMCID: PMCPMC4675513.

40. Wouters OJ, O'Donoghue DJ, Ritchie J, Kanavos PG, Narva AS. Early chronic kidney disease: diagnosis, management and models of care. Nat Rev Nephrol. 2015;11(8):491-502. doi: 10.1038/nrneph.2015.85. PubMed PMID: 26055354; PubMed Central PMCID: PMCPMC4531835.

41. Filiopoulos V, Boletis JN. Renal transplantation with expanded criteria donors: Which is the optimal immunosuppression? World J Transplant. 2016;6(1):103-14. doi: 10.5500/wjt.v6.i1.103. PubMed PMID: 27011908; PubMed Central PMCID: PMCPMC4801786.

42. Cohen LM, Germain MJ. Caregiver burden and hemodialysis. Clin J Am Soc Nephrol. 2014;9(5):840-2. doi: 10.2215/CJN.02690314. PubMed PMID: 24721889; PubMed Central PMCID: PMCPMC4011438.

43. Thomas R, Kanso A, Sedor JR. Chronic kidney disease and its complications. Prim Care. 2008;35(2):329-44, vii. doi:

10.1016/j.pop.2008.01.008. PubMed PMID: 18486718; PubMed Central PMCID: PMCPMC2474786.

44. Jansen J, Fedecostante M, Wilmer MJ, van den Heuvel LP, Hoenderop JG, Masereeuw R. Biotechnological challenges of bioartificial kidney engineering. Biotechnol Adv. 2014;32(7):1317-27. doi: 10.1016/j.biotechadv.2014.08.001. PubMed PMID: 25135479.

45. Lodi D, Iannitti T, Palmieri B. Stem cells in clinical practice: applications and warnings. J Exp Clin Cancer Res. 2011;30:9. doi: 10.1186/1756-9966-30-9. PubMed PMID: 21241480; PubMed Central PMCID: PMCPMC3033847.

46. Arcolino FO, Zia S, Held K, Papadimitriou E, Theunis K, Bussolati B, et al. Urine of Preterm Neonates as a Novel Source of Kidney Progenitor Cells. J Am Soc Nephrol. 2016;27(9):2762-70. doi: 10.1681/ASN.2015060664. PubMed PMID: 26940093; PubMed Central PMCID: PMCPMC5004650.

47. Song JJ, Guyette JP, Gilpin SE, Gonzalez G, Vacanti JP, Ott HC. Regeneration and experimental orthotopic transplantation of a bioengineered kidney. Nat Med. 2013;19(5):646-51. doi: 10.1038/nm.3154. PubMed PMID: 23584091; PubMed Central PMCID: PMCPMC3650107.

48. Peloso A, Katari R, Patel T, Hemal S, Zambon JP, Salvatori M, et al. Considerations on the development of a model of kidney bioengineering and regeneration in rats. Expert Rev Med Devices. 2013;10(5):597-601. doi: 10.1586/17434440.2013.827528. PubMed PMID: 23972073.

49. Bajaj P, Schweller RM, Khademhosseini A, West JL, Bashir R. 3D biofabrication strategies for tissue engineering and regenerative medicine. Annu Rev Biomed Eng. 2014;16:247-76. doi: 10.1146/annurev-bioeng-071813-105155. PubMed PMID: 24905875; PubMed Central PMCID: PMCPMC4131759.

50. Chesney RW, Chevalier R. Thirty-three years of progress: the International Workshops on Developmental Nephrology and the role of IPNA. Pediatr Nephrol. 2014;29(4):499-504. doi: 10.1007/s00467-013-2750-y. PubMed PMID: 24452330.

51. Little MH, Takasato M. Generating a self-organizing kidney from pluripotent cells. Curr Opin Organ Transplant. 2015;20(2):178-86. doi: 10.1097/MOT.0000000000000174. PubMed PMID: 25856180.

52. Little MH. Growing Kidney Tissue from Stem Cells: How Far from "Party Trick" to Medical Application? Cell Stem Cell. 2016;18(6):695-8. doi: 10.1016/j.stem.2016.05.015. PubMed PMID: 27257757; PubMed Central PMCID: PMCPMC4967556.

53. Xinaris C, Benedetti V, Rizzo P, Abbate M, Corna D, Azzollini N, et al. In vivo maturation of functional renal organoids formed from embryonic cell suspensions. J Am Soc Nephrol. 2012;23(11):1857-68. doi: 10.1681/ASN.2012050505. PubMed PMID: 23085631; PubMed Central PMCID: PMCPMC3482737.

54. Eknoyan G. The artificial kidney: past, present, and future. Minerva Chir. 2008;63(4):293-9. PubMed PMID: 18607326.

55. Kooman JP, Joles JA, Gerritsen KG. Creating a wearable artificial kidney: where are we now? Expert Rev Med Devices. 2015;12(4):373-6. doi: 10.1586/17434440.2015.1053466. PubMed PMID: 26076370.

56. Huang L, Holtzinger A, Jagan I, BeGora M, Lohse I, Ngai N, et al. Ductal pancreatic cancer modeling and drug screening using human pluripotent stem cell- and patient-derived tumor organoids. Nat Med. 2015;21(11):1364-71. doi: 10.1038/nm.3973. PubMed PMID: 26501191; PubMed Central PMCID: PMCPMC4753163.

57. Fatehullah A, Tan SH, Barker N. Organoids as an in vitro model of human development and disease. Nat Cell Biol. 2016;18(3):246-

54. doi: 10.1038/ncb3312. PubMed PMID: 26911908.

58. Swinney DC, Anthony J. How were new medicines discovered? Nat Rev Drug Discov. 2011;10(7):507-19. doi: 10.1038/nrd3480. PubMed PMID: 21701501.

59. Hudson-Shore M. Statistics of Scientific Procedures on Living Animals 2014: A new format, and hopefully a new era of diminishing animal experimentation? Altern Lab Anim. 2016;44(1):71-83. PubMed PMID: 27031603.

60. Burden N, Mahony C, Muller BP, Terry C, Westmoreland C, Kimber I. Aligning the 3Rs with new paradigms in the safety assessment of chemicals. Toxicology. 2015;330:62-6. PubMed PMID: 25932488.

61. International Transporter C, Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, et al. Membrane transporters in drug development. Nat Rev Drug Discov. 2010;9(3):215-36. doi: 10.1038/nrd3028. PubMed PMID: 20190787; PubMed Central PMCID: PMCPMC3326076.

62. Huang J, Gretz N, Weinfurter S. Filtration markers and determination methods for the assessment of kidney function. Eur J Pharmacol. 2016. doi: 10.1016/j.ejphar.2016.06.060. PubMed PMID: 27377216.

63. Schock-Kusch D, Geraci S, Ermeling E, Shulhevich Y, Sticht C, Hesser J, et al. Reliability of transcutaneous measurement of renal function in various strains of conscious mice. PLoS One. 2013;8(8):e71519. doi: 10.1371/journal.pone.0071519. PubMed PMID: 23977062; PubMed Central PMCID: PMCPMC3747225.

64. Huang J, Weinfurter S, Pinto PC, Pretze M, Kranzlin B, Pill J, et al. Fluorescently labeled cyclodextrin derivatives as exogenous markers for real-time transcutaneous measurement of renal function. Bioconjug Chem. 2016. doi: 10.1021/acs.bioconjchem.6b00452. PubMed PMID: 27611623.

65. London AJ, Kimmelman J. Why clinical translation cannot succeed without failure. Elife. 2015;4:e12844. doi: 10.7554/eLife.12844. PubMed PMID: 26599839; PubMed Central PMCID: PMCPMC4657068.

66. Everett JR. Academic drug discovery: current status and prospects. Expert Opin Drug Discov. 2015;10(9):937-44. doi: 10.1517/17460441.2015.1059816. PubMed PMID: 26088126.

67. Akhtar A. The flaws and human harms of animal experimentation. Camb Q Healthc Ethics. 2015;24(4):407-19. doi: 10.1017/S0963180115000079. PubMed PMID: 26364776; PubMed Central PMCID: PMCPMC4594046.

68. Hamon J, Renner M, Jamei M, Lukas A, Kopp-Schneider A, Bois FY. Quantitative in vitro to in vivo extrapolation of tissues toxicity. Toxicol In Vitro. 2015;30(1 Pt A):203-16. doi: 10.1016/j.tiv.2015.01.011. PubMed PMID: 25678044.

69. Li W, Zhang H, Assaraf YG, Zhao K, Xu X, Xie J, et al. Overcoming ABC transporter-mediated multidrug resistance: Molecular mechanisms and novel therapeutic drug strategies. Drug Resist Updat. 2016;27:14-29. doi: 10.1016/j.drup.2016.05.001. PubMed PMID: 27449595.

70. Kathawala RJ, Gupta P, Ashby CR, Jr., Chen ZS. The modulation of ABC transporter-mediated multidrug resistance in cancer: a review of the past decade. Drug Resist Updat. 2015;18:1-17. doi: 10.1016/j.drup.2014.11.002. PubMed PMID: 25554624.

71. Relling MV, Evans WE. Pharmacogenomics in the clinic. Nature. 2015;526(7573):343-50. doi: 10.1038/nature15817. PubMed PMID: 26469045; PubMed Central PMCID: PMCPMC4711261.

72. Konig J, Muller F, Fromm MF. Transporters and drug-drug interactions: important determinants of drug disposition and effects. Pharmacol Rev. 2013;65(3):944-66. doi: 10.1124/pr.113.007518. PubMed PMID: 23686349.

73. Cosmai L, Gallieni M, Porta C. Renal toxicity of anticancer agents targeting HER2 and EGFR. J Nephrol. 2015;28(6):647-57. doi: 10.1007/s40620-015-0226-9. PubMed PMID: 26341657.

74. Chan AJ, Rajakumar I. High-dose methotrexate in adult oncology patients: a case-control study assessing the risk association between drug interactions and methotrexate toxicity. J Oncol Pharm Pract. 2014;20(2):93-9. doi: 10.1177/1078155213482602. PubMed PMID: 23698198.

75. Yang Y, Li HY, Zhou Q, Peng ZW, An X, Li W, et al. Renal Function and All-Cause Mortality Risk Among Cancer Patients. Medicine (Baltimore). 2016;95(20):e3728. doi: 10.1097/MD.0000000000003728. PubMed PMID: 27196494; PubMed Central PMCID: PMCPMC4902436.

76. Lameire N. Nephrotoxicity of recent anti-cancer agents. Clin Kidney J. 2014;7(1):11-22. doi: 10.1093/ckj/sft135. PubMed PMID: 25859345; PubMed Central PMCID: PMCPMC4389154.

77. Stengel B. Chronic kidney disease and cancer: a troubling connection. J Nephrol. 2010;23(3):253-62. PubMed PMID: 20349418; PubMed Central PMCID: PMCPMC4823382.

78. Pickering JW, Endre ZH. Bench to Bedside: The next steps for biomarkers in acute kidney injury. Am J Physiol Renal Physiol. 2016:ajprenal 00268 2016. doi: 10.1152/ajprenal.00268.2016. PubMed PMID: 27465990.

79. Perazella MA. Onco-nephrology: renal toxicities of chemotherapeutic agents. Clin J Am Soc Nephrol. 2012;7(10):1713-21. doi: 10.2215/CJN.02780312. PubMed PMID: 22879440.

80. Chen HH, AbouEzzeddine OF, Anstrom KJ, Givertz MM, Bart BA, Felker GM, et al. Targeting the kidney in acute heart failure: can old drugs provide new benefit? Renal Optimization Strategies Evaluation in Acute Heart Failure (ROSE AHF) trial. Circ Heart Fail. 2013;6(5):1087-94. doi: 10.1161/CIRCHEARTFAILURE.113.000347. PubMed PMID: 24046475; PubMed Central PMCID: PMCPMC3878083.

81. Ojo A. Addressing the global burden of chronic kidney disease through clinical and translational research. Trans Am Clin Climatol Assoc. 2014;125:229-43; discussion 43-6. PubMed PMID: 25125737; PubMed Central PMCID: PMCPMC4112688.

82. Schork NJ. Personalized medicine: Time for one-person trials. Nature. 2015;520(7549):609-11. doi: 10.1038/520609a. PubMed PMID: 25925459.

83. Zhang L, Strong JM, Qiu W, Lesko LJ, Huang SM. Scientific perspectives on drug transporters and their role in drug interactions. Mol Pharm. 2006;3(1):62-9. PubMed PMID: 16686370.

84. (CDER) USDoHaHS-FaDA-CfDEaR. Guidance for Industry - Drug Interaction Studies — Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations 2012.

85. Prasad B, Johnson K, Billington S, Lee CA, Chung GW, Brown CD, et al. Abundance of drug transporters in the human kidney cortex as quantified by quantitative targeted proteomics. Drug Metab Dispos. 2016. doi: 10.1124/dmd.116.072066. PubMed PMID: 27621205.

86. Wu W, Dnyanmote AV, Nigam SK. Remote communication through solute carriers and ATP binding cassette drug transporter pathways: an update on the remote sensing and signaling hypothesis. Mol Pharmacol. 2011;79(5):795-805. doi: 10.1124/mol.110.070607. PubMed PMID: 21325265; PubMed Central PMCID: PMCPMC3082935.

87. Bhatnagar V, Richard EL, Wu W, Nievergelt CM, Lipkowitz MS, Jeff J, et al. Analysis of ABCG2 and other urate transporters in uric acid homeostasis in chronic kidney disease:

potential role of remote sensing and signaling. Clin Kidney J. 2016;9(3):444-53. doi: 10.1093/ckj/sfw010. PubMed PMID: 27274832; PubMed Central PMCID: PMCPMC4886906.

88. Brouwer KL, Aleksunes LM, Brandys B, Giacoia GP, Knipp G, Lukacova V, et al. Human Ontogeny of Drug Transporters: Review and Recommendations of the Pediatric Transporter Working Group. Clin Pharmacol Ther. 2015;98(3):266-87. doi: 10.1002/cpt.176. PubMed PMID: 26088472; PubMed Central PMCID: PMCPMC4731327.

89. Montanari F, Ecker GF. Prediction of drug-ABC-transporter interaction--Recent advances and future challenges. Adv Drug Deliv Rev. 2015;86:17-26. doi: 10.1016/j.addr.2015.03.001. PubMed PMID: 25769815.

90. Bauer M, Romermann K, Karch R, Wulkersdorfer B, Stanek J, Philippe C, et al. Pilot PET Study to Assess the Functional Interplay Between ABCB1 and ABCG2 at the Human Blood-Brain Barrier. Clin Pharmacol Ther. 2016;100(2):131-41. doi: 10.1002/cpt.362. PubMed PMID: 26940368; PubMed Central PMCID: PMCPMC4979595.

91. Nieskens TT, Wilmer MJ. Kidney-on-a-chip technology for renal proximal tubule tissue reconstruction. Eur J Pharmacol. 2016. doi: 10.1016/j.ejphar.2016.07.018. PubMed PMID: 27401035.

92. Wilmer MJ, Ng CP, Lanz HL, Vulto P, Suter-Dick L, Masereeuw R. Kidney-on-a-Chip Technology for Drug-Induced Nephrotoxicity Screening. Trends Biotechnol. 2016;34(2):156-70. doi: 10.1016/j.tibtech.2015.11.001. PubMed PMID: 26708346.

93. Kim S, Takayama S. Organ-on-a-chip and the kidney. Kidney Res Clin Pract. 2015;34(3):165-9. doi: 10.1016/j.krcp.2015.08.001. PubMed PMID: 26484042; PubMed Central PMCID: PMCPMC4608869.

94. Ledford H. CRISPR: gene editing is just thebeginning. Nature. 2016;531(7593):156-9. doi: 10.1038/531156a. PubMed PMID: 26961639.

95. Tschaharganeh DF, Lowe SW, Garippa RJ, Livshits G. Using CRISPR/Cas to study gene function and model disease in vivo. FEBS J. 2016;283(17):3194-203. doi: 10.1111/febs.13750. PubMed PMID: 27149548.

96. Roy A, Goodman JH, Begum G, Donnelly BF, Pittman G, Weinman EJ, et al. Generation of WNK1 knockout cell lines by CRISPR/Cas-mediated genome editing. Am J Physiol Renal Physiol. 2015;308(4):F366-76. doi: 10.1152/ajprenal.00612.2014. PubMed PMID: 25477473; PubMed Central PMCID: PMCPMC4329490.

CHAPTER 8

Summary

Nederlandse Samenvatting

Resumo em Português

List of abbreviations

SUMMARY

The present thesis describes the investigations on the function and regulation of renal drug transporters in proximal tubule cells *in vitro,* including the development of reliable methodology using human cells representative of renal physiology. The kidneys are responsible for maintaining whole body homeostasis and the removal of waste products from systemic circulation. A number of energy dependent xenobiotic transporters present in the proximal tubule epithelial cells (PTEC) account for the kidneys excretory capacity. At the basolateral side, PTEC express the uptake transporters organic anion transporter 1 and 3 (OAT1 and 3) and organic cation transporter 2 (OCT2); at the apical site, amongst others, the efflux transporters breast cancer resistance protein (BCRP), P-glycoprotein (P-gp), multidrug resistance proteins 2 and 4 (MRP2 and MRP4) and multidrug and toxin extrusion proteins 1 and 2 (MATE1 and MATE2). Together these transporters remove a variety of solutes, including drugs and uremic toxins. The disruption of normal kidney function can often lead to development of chronic kidney disease (CKD), the progressive loss of function over a prolonged period of time. CKD is a major healthcare concern often leading to end stage renal disease, for which current treatments consist of dialysis and ultimately kidney transplantation. Understanding renal function is important to develop novel strategies to treat kidney disease. To date, several *in vitro* models representative of proximal tubules physiology are available. Nonetheless few cell lines retain native transport activity like the conditionally immortalized human proximal tubule cell line (ciPTEC) that endogenously expresses a number of important PTEC transporters. Chapter 1 gives an overview of the role of PTEC in kidney physiology and describes the rationale of using ciPTEC for studying xenobiotic transporters in more detail.

Chapter 2 reviews the importance of BCRP in renal function. Expression of BCRP is lower compared to other ABC – ATP binding cassette - transporters present in PTEC, nonetheless its activity accounts for the excretion of a number of endogenous compounds and widely used drugs, including chemotherapeutics. Incidentally, BCRP has been extensively associated with chemoresistance and cancer drugs themselves can influence its expression and function.

In chapter 3 assays for studying transport processes over the apical membrane of PTEC are described. Using fluorescence-based transport assays, the function of BCRP, P-gp and MRP4, as well as protein uptake through receptor-mediated endocytosis was determined. The substrates Hoechst33342, chloromethylfluorescein-diacetate (CMFDA/GS-MF) and calcein-AM were used in presence or absence of model inhibitors for BCRP (KO143), MRP's (MK571) or P-gp (PSC833), respectively. Receptor-mediated endocytosis was analyzed by determining the intracellular accumulation of fluorescently labeled receptor associated protein (RAP-GST), a ligand for the megalin-cubilin receptor complex. The retention of fluorescent Hoechst33342, GS-MF and calcein in the presence of KO143, MK571 and PSC833 revealed redundancy in handling of fluorescent substrates between the transporters. While KO143 and MK571 blocked BCRP, P-gp and MRP4, PSC833

blocked both BCRP and P-gp, but not MRP4. Furthermore, the cells accumulate RAP-GST in endosomal vesicles in a dose and time dependent manner. Overall, fluorescence-based assays appeared to be viable and reproducible tools to determine apical transport mechanisms, *in vitro.* Model substrates and inhibitors tested, appeared not specific for the designated transporters, reflecting the complex interactions that can take place *in vivo* in PTEC.

In **chapter 4,** the effects of uremic solutes *p*-cresyl sulfate (pCS) and *p*-cresyl glucuronide (pCG) on renal efflux transport and CKD progression are studied. In CKD patients, uremic solutes accumulate due to compromised renal excretion processes and can contribute to the progression of the disease. By exposing ciPTEC to pCS and pCG it was demonstrated that these solutes are substrates and can competitively inhibit the activity of both BCRP and MRP4. Further, pCS positively influenced the expression and function of BCRP. On the other hand, pCG promoted phenotypical changes by increasing the expression of vimentin while decreasing E-cadherin levels, indicating the induction of epithelial to mesenchymal transition. These findings support the previously suggested finding that uremic toxins may be involved in CKD progression by affecting renal tubule cell phenotype and functionality.

The pharmacological interaction between two different anticancer drugs – cetuximab, anti-epidermal growth factor receptor recombinant monoclonal antibody, and methotrexate, a chemotherapeutical and anti-inflammatory agent – was investigated at the renal cellular level, in **chapter 5**. The activity of OAT1, BCRP, P-gp and MRP4 was investigated using the fluorescence assays developed in ciPTEC overexpressing OAT1. Methotrexate inhibited OAT-mediated fluorescein uptake, and decreased BCRP- and MRP4-mediated Hoechst33342 and GS-MF efflux. After cetuximab exposure, the expression and activity of OAT1 and BCRP were downregulated, while the activity of P-gp and MRP4 was increased. The mechanisms implicated in transporter regulation were analyzed further by kinomic analysis using the Pamchip kinase microarrays and pathway predictions. Pathway analysis and subsequent pharmacological studies confirmed that cetuximab triggered EGFR-AKT-MEK signaling pathway leading to extensive changes in transporters expression and activity. Finally, it was demonstrated that cetuximab pretreatment reduced methotrexate uptake in ciPTEC and its cytotoxic potential. Taken together, cetuximab attenuates methotrexate-induced nephrotoxicity by EGFR-mediated transporter regulation, opening possibilities for nephroprotective co-medication therapies.

In **chapter 6** we investigated the function and differentiation potential of human fetal kidney-specific progenitor cells (KSPC). Cells were tested for the expression of differentiation markers and function over passage numbers (P6-P9) in PTEC culture medium. In addition, matrigel cultures were used to determine 3-dimensional differentiation. Results showed that the cells developed a PTEC-like phenotype over passages, increasing the expression of MRP4 and P-gp and maintaining BCRP expression on the gene level. Functional assays revealed that MRP4 and P-gp activity increased over passage number, while BCRP was functionally inactive despite its gene expression. In matrigel the cells formed functional

tubule-like structures after two days in culture. These results indicated that KSPC can be functionally active *in vitro*, and although immature, KSPC can differentiate towards a PTEC phenotype over time and passage number.

Finally, **chapter 7** offers an in-depth discussion and summary of the results obtained. This thesis demonstrates that studying the activity of different xenobiotic transporters acting simultaneously can be achieved by using fluorescence-based tools. The role of EGFR as active component of a cell-signaling pathway was elucidated after investigating the pharmacological interaction between two anticancer drugs on the molecular level. Finally, we also showed that KSPCs can differentiate into functionally active PTEC. Overall this work pushes forward our understanding of renal xenobiotic transporters activity under physiological and pathological conditions, and provides a novel approach to explore further transporter regulation and renal drug interactions.

NEDERLANDSE SAMENVATTING

Dit proefschrift beschrijft het onderzoek naar de functie en de regulatie van transporteiwitten in gekweekte cellen van de menselijke nier (*in vitro*). De nieren zijn verantwoordelijk voor het handhaven van het interne milieu van het lichaam, o.a. door de verwijdering van afvalproducten uit de algemene circulatie. Hiervoor zijn een aantal energie-afhankelijke transporteiwitten verantwoordelijk, die aanwezig zijn in de proximale tubulus-epitheelcellen (PTEC). Aan de basolaterale zijde van de cellen, de kant die in nauw contact staat met de bloedbaan, zijn de organische aniontransporters 1 en 3 (OAT1 en 3) en organisch-kationtransporter 2 (OCT2) verantwoordelijk voor de opname van stoffen. Aan de andere kant, de apicale zijde, zijn het borstkankerresistentieeiwit (BCRP), P-glycoproteïne (P-gp), de multidrug-resistentieeiwitten 2 en 4 (MRP2 en MRP4) en de multidrug en toxine-extrusie-eiwitten 1 en 2 (MATE1 en MATE2) verantwoordelijk voor transport naar de voorurine. Gezamenlijk verwijderen de transporteiwitten veel verschillende stoffen, waaronder geneesmiddelen en lichaamseigen afbraakproducten, ook wel uremische toxines genoemd.

Verstoring van de nierfunctie kan leiden tot de ontwikkeling van chronische nierziekte (CKD), een inflammatoir proces dat gepaard gaat met een geleidelijk verder verlies van functie en uiteindelijk tot eindstadium-nierfalen waarvoor alleen nog dialyse en niertransplantatie als behandelingsmogelijkheden zijn. CKD is een belangrijk en duur gezondheidsprobleem. Om nieuwe therapieen te kunnen ontwikkelen die eerder ingrijpen in het ziekteproces, is het belangrijk om goed te begrijpen hoe de nieren functioneren. Er zijn slechts een beperkt aantal humane celmodellen beschikbaar om dit op cellulair niveau te kunnen doen. Een van de meest gekarakteriseerde celsystemen is het conditioneel-geimmortaliseerde humane PTEC (ciPTEC) model. In dit celmodel zijn een aantal karakteristieken behouden gebleven, waaronder de aanwezigheid van transporteiwitten, waardoor het geschikt is voor bestudering van nierfysiologische processen zoals beschreven in dit proefschrift. Hoofdstuk 1 geeft een overzicht van de rol van PTEC in algemene nierfysiologie en de motivatie voor het gebruik van ciPTEC als model voor het bestuderen van geneesmiddeltransporters in de nier.

Hoofdstuk 2 geeft een overzicht van het belang van BCRP in nierfunctie en in geneesmiddelresistentie bij kankertherapie. In de nier is BCRP minder aanwezig dan andere ATP-bindingscassette (ABC) transporters, zoals P-gp en MRP4, niettemin is de activiteit van belang voor de uitscheiding van een groot aantal lichaamseigen afbraakstoffen en veel geneesmiddelen, waaronder chemotherapeutica. BCRP is betrokken bij kankertherapieresistentie, waarbij de chemotherapeutica in staat zijn om de activiteit en de aanwezigheid van de transporter in tumorcellen te beinvloeden. Of ditzelfde proces ook in niertubuluscellen plaatsvindt is niet bekend. Wel is het zo dat chemotherapeutica niertoxisch kunnen zijn en nierfalen kunnen veroorzaken.

In hoofdstuk 3 staan de assays beschreven die zijn ontwikkeld voor het bestuderen van transportprocessen over de apicale celmembraan van PTEC. Hierbij is gebruik gemaakt

van fluorescerende modelstoffen, waarmee de functie van BCRP, Pgp en MRP4, alsmede receptor-gemedieerde eiwitendocytose konden worden geevalueerd. De substraten Hoechst33342, chloromethylfluorescein-diacetaat (CMFDA / GS-MF) en calceïne-AM zijn bestudeerd in aanwezigheid of afwezigheid van bekende remmers voor, respectievelijk, BCRP (KO143), MRP's (MK571) of P-gp (PSC833). Receptor-gemedieerde endocytose werd geanalyseerd aan de hand van de intracellulaire ophoping van GST-gelabeld receptor-geassocieerdeiwit (RAP-GST), wat aan het megaline-cubiline-receptorcomplex bindt. Uit het onderzoek is gebleken dat de getestte modelsubstraten en -remmers niet specifiek zijn voor de bestudeerde transporteiwitten. Desondanks kunnen condities zo gekozen worden dat wel een uitspraak over de activiteit van individuele transporteiwitten kan worden gedaan. De ontwikkelde assays zijn vervolgens in **hoofdstuk 4** toegepast, waarin de effecten van de uremische toxines p-cresylsulfaat (pCS) en p-cresylglucuronide (pCG) op niertubulusceltransport en progressie van nierfalen werd bestudeerd. Bij nierfalen hopen deze afbraakproducten zich op in het lichaam wat kan bijdragen aan een verder verlies van nierfunctie. Het onderzoek wees uit dat pCS en pCG substraten en remmers zijn voor de transporteiwitten BCRP en MRP4. Daarnaast bleek dat blootstelling aan pCS leidde tot een toename in de aanwezigheid en functie van BCRP in PTEC; pCG daarentegen had een negatieve invloed op PTEC-specifieke karakteristieken. Hieruit hebben we geconcludeerd dat de afbraakproducten kunnen bijdragen aan verergering van nierfalen door aantasting van de functie van PTEC.

In **hoofdstuk 5** is met de niercellijn de interactie onderzocht tussen twee geneesmiddelen tegen kanker, te weten cetuximab, een monoklonaal antilichaam gericht tegen de epidermale groeifactorreceptor (EGFR), en methotrexaat, een chemotherapeutisch en antiinflammatoir geneesmiddel. Eerst is het effect van methotrexaat op de activiteit van de niertransporters OAT1, BCRP, P-gp en MRP4 onderzocht met behulp van de eerder ontwikkelde fluorescentie-assays. Hieruit bleek dat het middel de OAT1-gemedieerde opname van fluoresceïne, en de BCRP- en MRP4-gemedieerde uitscheiding van, respectievelijk, Hoechst33342 en GS-MF verminderde. Een gelijktijdige toediening van cetuximab zorgde voor een vermindering in aanwezigheid en activiteit van OAT1 en BCRP, terwijl die van P-gp en MRP4 werden verhoogd. Met behulp van een Pamchip-kinasearray zijn de mechanismen die hierbij betrokken zijn verder onderzocht. Hieruit bleek dat cetuximab een intracellulaire signaleringsroute activeert die de veranderingen in de expressie en activiteit van de niertransporters veroorzaakt. Tenslotte kon worden aangetoond dat voorbehandeling van de cellen met cetuximab ervoor zorgt dat methotrexaat verminderd wordt opgenomen en hierdoor minder schadelijk is voor de niercellen. Deze interactie kan in gunstige zin gebruikt worden om de therapeutische effectiviteit van methotrexaat te verhogen en de nierbijwerkingen te verminderen.

Voor de behandeling van eindstadium-nierfalen wordt nog naar nieuwe mogelijkheden gezocht, waaronder ook op cellen-gebaseerde therapieen om het herstel van de beschadigde nieren te bevorderen. Stamcellen zijn hiervoor een goede mogelijkheid. In **hoodstuk 6** is onderzoek gedaan naar de mogelijkheid van menselijke,

foetale, nierstamcellen (KSPC) om zich te ontwikkelen to gedifferentieerde PTECs. Cellen werden getest op de aanwezigheid van differentiatiemarkers en vergeleken met ciPTECs in functieontwikkelen wanneer ze langere tijd in kweek werden gehouden. Daarnaast is onderzocht hoe de cellen zich 3-dimensionaal ontwikkelen in een hiervoor geschikt medium, zgn. Matrigel. De resultaten toonden aan dat wanneer KSPCs in PTEC-medium worden gekweekt ze ook PTEC karakteristieken gaan vertonen, zoals de aanwezigheid van MRP4, P-gp en BCRP op genniveau. Uit functionele testen is vervolgens gebleken dat ook de activiteit van MRP4 en P-gp kon worden waargenomen in kweek, terwijl BCRP niet actief aanwezig was. In Matrigel vormden de cellen functionele, buisachtige structuren. Uit de resultaten beschreven in dit hoofdstuk hebben we geconcludeerd dat de foetale KSPC zich kunnen ontwikkelen tot PTEC wanneer ze onder de juiste omstandigheden worden gekweekt.

Tot slot zijn de resultaten van dit proefschrift in **hoofdstuk 7** bediscussieerd, met bijzondere aandacht voor de toepassingen van de in dit proefschrift beschreven ontwikkelde methode om nierfunctie te kunnen bestuderen met behulp van fluorescentietechnologie en de rol van de EGFR in het aansturen van transporteiwitten in de nier. In het hoofdstuk staat verder beschreven hoe het niercelmodel, ciPTEC, een rol kan spelen bij de ontwikkeling van nieuwe geneesmiddelen door als menselijk-testsysteem te dienen waardoor mogelijk minder proefdieren nodig zijn voor het onderzoek naar de veiligheid van potentieel nieuwe middelen. Vervolgens zijn de toepassing van ciPTEC in een biologische kunstnier alsmede nieuwe ontwikkelingen binnen het nierregeneratie-onderzoek nog kort aangestipt. Het hoofdstuk wordt afgesloten met aanbevelingen voor implementatie van *in vitro*-testsystemen in nierfarmacologisch onderzoek.

RESUMO EM PORTUGUÊS

A presente tese descreve a investigação da função e regulação de transportadores renais em células de túbulos proximais *in vitro*, incluindo o desenvolvimento de métodos viáveis utilizando células humanas representativas da fisiologia renal. Os rins são responsáveis pela manutenção da homeostase corporal e pela remoção de resíduos da circulação sistémica. Um número de transportadores de xenobióticos, dependentes de energia, está presente nas células epiteliais dos túbulos proximais (CETP) e são responsáveis pela capacidade de excreção dos rins. Do lado basolateral, as CETP expressam os transportadores de absorção *organic anion transporter 1* e *3* (OAT1 e 3) e *organic cation transporter 2* (OCT2); do lado apical, entre outros, expressam os transportadores de efluxo *breast cancer resistance protein* (BCRP), *P-glycoprotein* (P-gp), *multidrug resistance proteins 2* e *4* (MRP2 e MRP4) e as *multidrug and toxin extrusion proteins 1* e *2* (MATE1 e MATE2). Juntos, estes transportadores removem uma variedade de solutos, incluindo fármacos e toxinas urémicas. A interrupção das funções normais dos rins conduz ao frequente desenvolvimento de doença renal crónica (DRC), caracterizada pela perda progressiva de função renal ao longo de um prolongado período de tempo. A DRC é uma das principais preocupações para a saúde pública e frequentemente culmina em doença renal terminal, para a qual os tratamentos disponíveis actualmente consistem em diálise e transplante renal. Uma melhor compreensão da função renal é importante para o desenvolvimento de novas estratégias no tratamento de doenças renais. Até á data vários modelos *in vitro* representativos da fisiologia de túbulos proximais estão disponíveis. No entanto apenas poucas linhas celulares retêm a actividade de transporte membranar nativa como as *conditionally immortalized human proximal tubule cell line* (ciPTEC), que expressam um importante número de transportadores presentes em CEPT, endogenamente. O capítulo 1 apresenta uma revisão geral do papel das CEPT na fisiologia renal e descreve os fundamentos para o uso de ciPTEC no estudo de transportadores de xenobióticos em detalhe.

No capítulo 2 é revista a importância do BCRP para a função renal. A expressão de BCRP é baixa quando comparada com outros transportadores ABC (*ATP binding cassette*) presentes em CEPT, no entanto a sua actividade permite a excreção de um elevado número de substancias endógenas e de fármacos amplamente utilizadas, inclusive em quimioterapia. Incidentalmente, BCRP está intrinsecamente associado a multi-resistência em quimioterapia e por outro lado os fármacos utilizados no tratamento de cancro influenciam a sua expressão e actividade.

O capítulo 3 descreve um conjunto de ensaios desenvolvidos para estudar os mecanismos de transporte apical em CETP. Recorrendo ao uso de métodos baseados em fluorescência, as funções de BCRP, P-gp e MRP4, tal como a absorção de proteínas por endocitose mediada por receptores, foi determinada. Os substratos *Hoechst33342*, *chloromethylfluorescein-diacetate* (CMFDA/GS-MF) e *calcein-AM* foram utilizados na presença ou ausência dos inibidores padrão para BCRP (KO143), MRP's (MK571) ou

P-gp (PSC833), respectivamente. A endocitose mediada por receptores foi determinada analisando a acumulação intracelular de *receptor associated protein*, marcada com fluorescência (RAP-GST), um ligando do complexo *megalin-cubilin receptor*. A retenção de Hoechst33342, GS-MF e calcein na presença de KO143, MK571 and PSC833 revelou a redundância de afinidades entre os transportadores. Enquanto KO143 e MK571 bloquearam BCRP, P-gp e MRP4, PSC833 bloqueou BCRP e P-gp, mas não MRP4. As células acumularam RAP-GST, em endossomas, de forma dependente da concentração e do tempo. No geral, métodos baseados em fluorescência surgem aqui como uma ferramenta viável e reprodutível para determinar a actividade dos mecanismos apicais de transporte, *in vitro.* Os substratos e inibidores padrão utilizados não aparentam ser específicos para os transportadores designados, reflectindo a complexidade das interacções que ocorrem, *in vivo*, em CETP.

No **capítulo 4** foram estudados os efeitos das toxinas urémicas *p-cresyl sulfate* (pCS) e *p-cresyl glucuronide* (pCG) no efluxo renal e progressão de DRC. Em pacientes com DRC, as toxinas urémicas acumulam-se devido a uma deficiente excreção renal e podem contribuir para a progressão da doença. Ao expor ciPTEC a pCS e pCG foi demonstrado que estas toxinas inibem competitivamente as actividades de BCRP e MRP4. Além disso, pCS teve um impacto positivo na expressão e função de BCRP. Por outro lado pCG promoveu alterações fenotípicas, aumentando a expressão de *vimentin* enquanto diminui os níveis de *E-cadherin*, indicando a indução de uma transição epitélio-mesenquimal. Estes resultados suportam a evidência prévia que aponta para o envolvimento de toxinas urémicas na progressão de DRC, afectando o fenótipo e a função dos túbulos renais.

A interacção de dois fármacos utilizados no tratamento de cancro – cetuximab, um anticorpo monoclonal recombinante *anti-epidermal growth factor receptor* (EGFR) e *methotrexate* uma agente quimioterapêutico e anti-inflamatório – foi investigada ao nivel das CEPT, no **capitulo 5**. A actividade de OAT1, BCRP, P-gp e MRP4 foi investigada recorrendo a ensaios de fluorescência em ciPTEC, modificadas para sobre-expressar OAT1. O *methotrexate* inibiu a absorção de *fluorescein* mediada por OAT1, e diminuiu o efluxo de *Hoechst33342* e GS-MF mediado por BCRP e MRP4. Após exposição a cetuximab as actividades de OAT1 e BCRP foram desreguladas negativamente, enquanto as actividades de P-gp e MRP4 sofreram um incremento positivo. Os mecanismos implicados na regulação da actividade dos transportadores foram subsequentemente estudados, analisando a actividade de cinases através do *Pamchip kinase microarrays* e previsões de sinalização celular. A análise da sinalização (*pathway analysis*) e ensaios farmacológicos confirmaram que a exposição a cetuximab activou a cascata EGFR-AKT-MEK, conduzindo a profundas alterações na expressão e actividade dos transportadores renais. Por ultimo, foi demonstrado que o pré-tratamento com cetuximab reduziu a absorção de methotrexate em ciPTEC e consequentemente o seu potencial tóxico. Em conjunto, cetuximab atenua a nefrotoxicidade induzida por methotrexate, regulando a actividade de transportadores renais via EGFR. Estes resultados abrem possibilidades para co-medicação com efeitos nefro-protectores.

O **capítulo 6** é dedicado ao estudo da função e potencial de diferenciação de células progenitoras renais fetais humanas (CPRFT). A expressão de marcadores de diferenciação e a função destas células foram testadas em meio de cultura específico para CETP, em passagens sequenciais (P6-P9). Adicionalmente, cultura em *matrigel* foi usada para determinar a sua diferenciação tridimensional. Os resultados mostram que as células adquiriram um fenótipo similar ao de CETP em sucessivas passagens, aumentando a expressão de MRP4 e P-gp, mantendo constante a expressão de BCRP ao nivel genético. Ensaios funcionais demonstraram que a actividade de MRP4 e P-gp aumentou em sucessivas passagens, enquanto BCRP se manteve inativo apesar da sua expressão. Em *matrigel* as CPRFT formaram estruturas funcionais, similares a túbulos após dois dias em cultura. Estes resultados indicam que CPRFT são funcionalmente activas *in vitro,* apesar de imaturas e que a sua diferenciação pode ser direccionada para um fenótipo de CETP em cultura, ao longo to tempo e passagens sucessivas.

Por último, o **capítulo 7** oferece uma discussão aprofundada e um resumo dos resultados obtidos. É aqui demonstrado que o estudo da actividade de diferentes transportadores renais de xenobióticos pode ser efectuado com recurso a métodos baseados em fluorescência. O papel de EGFR como um componente fundamental para sinalização celular e regulação de transportadores foi elucidado após a investigação da interacção entre dois fármacos anticancro, ao nível molecular. Finalmente foi demonstrado que é possível diferenciar CPRFT em CETP funcionalmente activas. No geral este trabalho contribui para melhorar a nossa compreensão sobre a actividade de transportadores renais em condições fisiológicas e oferece uma nova abordagem para explorar a regulação do transporte de xenobióticos e interacções farmacológicas nos rins.

LIST OF ABBREVIATIONS

3R's	Replacement, reduction and refinement
ABC	Adenosine tri-phosphate binding cassette
ADME	Absorption, distribution, metabolism and excretion
Ahr	Aryl hydrocarbon receptor
AKI	Acute kidney injury
Akt	Alpha serine-threonine protein kinase
AQP	Aquaporins
ASP^+	4-(4-(dimethylamino) styryl)-*N*-methylpyridinium
a.u.	Arbitrary units
BAK	Bio-artificial kidney
BCRP	Breast cancer resistance protein
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
cDNA	Complementary deoxyribonucleic acid
ciPTEC	Conditionally immortalized human proximal tubule epithelial cells
CKD	Chronic kidney disease
CM	Complete medium
CMFDA	Chloromethylfluorescein-diacetate
Creb1	cAMP responsive element binding protein 1
CRISPR	Clustered regularly interspaced short palindromic repeats
CTX	Cetuximab
CYP	Cytochrome enzymes
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DN	Double negative
DP	Double positive
E1S	Estrone-sulfate
E2	17-estradiol
EGFR	Epidermal growth factor receptor
EMT	Epithelial-to-mesenchymal transition
Erbb2	Receptor tyrosine-protein kinase
ESRD	End stage renal disease
eYFP	Yellow fluorescent protein
FCS	Fetal calf serum
FVB	Friend Leukemia virus B strain
GAP	GTPase-activation protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GFR	Glomerular filtration rate
GS-MF	Glutathione-methylfluorescein
GTP	Guanosine tri-phosphate
HBSS	Hank's balanced salt solution
HEK293	Human embryonic kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HK-2	Human kidney 2
HPLC	High-performance liquid chromatography
HPV	Human papilloma virus
hTERT	Human telomerase reserve transcriptase
HUGO	Human genome organization
IC50	Half maximal inhibitory concentration
IL-1β	Interleukin 1-β
IL-6	Interleukin 6
iPS	Induced pluripotent stem cells
ITC	International transporter consortium
K^+	Potassium
KDa	Kilo-dalton
KEAP1	Kelch-like ECH-associated protein 1
Klf4	Kruppel-like factor 4
Km	Michaelis-Menten constant
KSPC	Ketal kidney-specific progenitor cells
LC/MS-MS	Liquid chromatography/tandem mass spectrometry
MAPK	Mitogen activated protein kinase
MATE1	Multidrug and toxin extrusion 1
MATE2	Multidrug and toxin extrusion 2
MDCK	Madin-Darby canine kidney
MDR	Multi-drug resistance
Mg^{2+}	Magnesium
$MgCl_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid
MRP2	Multidrug resistance protein 2
MRP4	Multidrug resistance protein 4
mTOR	Mechanistic target of rapamycin
MTT	Dimethylthiazol bromide
MTX	Methotrexate
Na^{2+}	Sodium
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NBD	Nucleotide-binding domain

NF-kβ1	Nuclear factor kappa light polypeptide gene enhancer
Nrf2	Erythroid-derived 2
OAT1	Organic anion transporter 1
OAT3	Organic anion transporter 3
OAT4	Organic anion transporter 4
OATP4C1	Organic anion transporter polypeptide
OCT2	Organic cation transporter 2
Oct4	Octamer binding transpiration factors 4
OCTN1	Carnitine transporters 1
OCTN2	Carnitine transporters 2
PAH	Polycyclic aromatic hydrocarbons
PB	Presto blue
PBS	Phosphate buffer solution
pCG	pP-cresyl glucuronide
PCR	Polymerase chain reaction
pCS	pP-cresyl sulfate
PDK	Phosphoinositide dependent protein kinase
P-gp	P-glycoprotein
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
PLCγ	Phospholipase C-γ
PO_4^{3-}	Phosphate
PODXL	Podocalyxin
Pparα	Peroxisome proliferator-activated receptor α
PTEC	Proximal tubule epithelial cells
PTK	Protein tyrosine kinase
RAP-GST	Fluorescently labeled receptor associated protein
RPTEC	Renal proximal tubule epithelial cell line
SCCHN	Advanced squamous cell carcinoma of the head and neck
SD	Standard deviation
SEM	Standard error of the mean
SFM	Serum free medium
SGLT2	Sodium glucose co-transporter 2
SLC	Solute carrier family
SNP	Single nucleotide polymorphism
SOX2	Sex determining region Y-box2
STK	Serine/threonine kinase
SULT	Sulfotransferase
SV40T	Simian virus 40 large T antigen

TKI	Tyrosine kinase inhibitors
TMRF	Transcutaneous measurement of renal function
TNα	Tumor necrosis factor α
UGT	Glucuronosyltransferases
URAT	Urate transporter

CHAPTER 9

Acknowledgements

Curriculum vitae

List of publications

PhD Portfolio

ACKNOWLEDGEMENTS

As this thesis writing marathon is approaching the end, it is now time to look back into this journey of over four years and write a few acknowledgements and thoughts about the people that directly and indirectly, scientifically or not, have made this thesis possible and that, in a way, this work is also theirs.

After my time in the University of Coimbra, where I graduated in biology and grew a taste for research, I enrolled in the MIT-Portugal training program in bioengineering. Having worked in a diverse international environment, the goal was set to pursuit a doctorate abroad. When the opportunity came through my time in the Netherlands, a land of great flatness inhabited by welcoming, polite, highly educated, kind, milk drinking, taller than average people, where the sun barely shines, began.

To begin, I want to express my immense gratitude to **Roos**, my promotor and supervisor, that, for some mysterious reason, believed I was qualified enough to fill a PhD position in her group. From our initial meeting in Nijmegen, a place that until then I was oblivious of, you went to great lengths to ensure that my transition to a new lab, new group, new country and new city would go as smooth as possible. I felt welcomed from the first day. I cannot thank you enough for all your effort, guidance and time, from the initial planning of my research to the completion of my thesis. Thank you for sharing your extensive scientific insight and allow me to explore my own ideas, present my work in several occasions and get in contact with many experts in the fields of transporters and kidney research. Although four years went by fast, these were very busy, sometimes stressful, often fun years and it has been a privilege working with you. **Martijn**, my co-promoter and original 'daily-supervisor', that when I started my work at the (now) Radboud Institute for Molecular Life Sciences, took the time, effort and patience to introduce me to life and work in the 7th floor. I thank you very much for making my transition to this new environment easy. Your impact in my working habits will be everlasting, now I can say that I work in a more focused, less random and more efficient way. I much appreciated our planning meetings and brainstorming about the direction of my research, and your deep scientific and methodic knowledge were of great help for me over these years. Last I cannot thank you enough for introducing me to the local watering hole at the Radboudumc, where we and others spend very productive after work hours.

I cannot stress enough my gratitude to my colleagues and friends in the FarmTox department in Nijmegen for three years of a really great working experience. I would like to start by thanking **Rachel**, that left the lab shortly after I started, allowing me to take over her working space and enjoy a great view of the city. **Karl**, I much enjoyed our discussions about very import issues, like air-travel and crane rentals, thank you for keeping me up to date with what was going on in the Dutch society. **Janny P**, thanks

for keeping track of my orders, better than I did and all your immense helpfulness in the lab. I would like to thank **Lindsey**, **Maarten, Sanna** and **Esther** for always being in the frontline of office gossip and your positive attitude towards sometimes very crappy results and 'desperate' times. **Jeroen**, many thanks for all the help with vesicle-related work and for a good time in Baltimore. **Tom N,** tanks for explaining me tons of stuff about the Netherlands and Dutch culture and also for the countless hours interpreting fluorescent, and trying to figure out the best way to perform experiments and all the help with my work. **Jeanne** and **Vivienne**, many thanks for your patience, insights and good spirits during our experimental together, it was worth it despite ending up nowhere. **Julien**, many thanks for your support and ensuring me that the Netherlands are not that alien of a world to live in. **Tom S**, a common presence among those of us working late into the evening, thanks for your calm advice on how to deal with a lot of work. **Rick M,** thanks for your uncompromising views on work and help in my initial times. **Petra**, thanks for your patience and assistance with the LC-MS, and **Ab** many thanks for sharing your cinematic expertise and showing me a good time. **Rick G**, I much appreciated your comments to my work. **Elnaz**, thanks for introducing me to the world of DLC's and **Marieke** thanks for all the help with cell work. **Niels S**, though brief, the times spent in the lab were much fun and **Jelle**, thanks for helping in the transition out of the lab. Last, **Jan** and **Frans**, many thanks for your insights on my work and support during my time and Nijmegen. Again many thanks to you all, I certainly learned a lot from all of you.

The last year of my work, brought somewhat of a change, when I and a few colleagues moved to the University of Utrecht to start a new group and a new lab, as Roos (our boss) became the new Professor of experimental pharmacology at the Department of Pharmaceutical Sciences. Here I would very much like to acknowledge the small group of people that embarked in this new project. **Manoe,** many thanks for your support and help, for all the scientific discussions and the ones not so scientific and for facing many issues when we started up the experiments again. You are a very talented scientist with immense patience and a remarkable working capability and I am truly enjoyed working with you. **Michele,** many thanks for all your assistance and help in the lab, and for all the additional problem-solving that we had to do. Your very Italian way of doing things and facing issues was always refreshing and I much enjoyed our countless hours, counting weekends in the lab. **Milos**, many thanks for all your support and help and above all patience, that you kept in times when some of us were running out. Your very precise lab skill and detailed planning often reminded that trying to fit too much work in little time may not be the way to go. **Jitske**, although you joined us in Utrecht later, in a certain way you were always present before, especially when the cells behaved weird. Many thanks for your help, support, discussions, brainstorming, and lack of patience to put up with less important work-related issues. We starting working together in Nijmegen and you were always present throughout my work, and I cannot thank you enough for all your assistance.

Many thanks to **Paula** for showing me that life in Utrecht was more than just long hours in the lab and **Paul**, that was always fun to have around. Last, I would like to thank **Natalia** for helping me explore this new lab and start up my experiments during her initial visiting period. I also want to thank and acknowledge **Johan, Aletta, Frank R, Paul H, Lidja, Coen, Gemma, Mara, Susanne, Marlotte, Kirsten, Bart, Mojtaba, Aurora, Ying Xin, Mohamed, Anne-Metje, Sam** and **Nienke** for welcoming me in the making my brief (and busy) time in Utrecht easier and enjoyable, and also thanks for all the borrels. I am sure I will be forgetting someone here.

Now a word of appreciation for my collaborators in different projects. Many thanks to **Carla** and **Janneke** for their insights in oncology and cancer research and also for making sure that cetuximab never ran out in our lab. I would like to acknowledge the contributions of **Paul W** and **Chiara M** for their insights in development nephrology and providing the know-how for growing human fetal stem cells. **Norbert G** and **Jiaguo H,** for involving me in their extensive study of novel bio-tracers and for sharing their expertise with me. **Rob R** for his assistance and work with the kinomic assays and pathway analysis.

I would also like to thank **Patricia M**, coordinator of the Nephrotools project for having me for a short period in her group in Liverpool, and to **Ilaria, Sandra, Isabel, Agnes, Sofia** and **Andy** for a good and productive time in England. I also want to thank my other colleagues of the Nephrotools project (e.g. nephrotoolers), **Fanny, Stefania, Zeneida, Heather, Valentina, Shikar, Elli** and **Klaudyna** for their support, both moral and scientific and for all the fun times provided when we came together.

To my students, I sincerely hope you learned something from me. **Amer**, many thanks for your dedication and resilience. **Marieke**, **Anastasia** and **Janni** thank you all for your motivation, enthusiasm, effort and dedication and **Linda** thank you for all the hard work. Above all I thank you for extensively repeating many assays and not always doing what you were literally told to do.

Also, early in my Dutch enterprise I was fortunate enough to meet this remarkable group of people, beginning for me as the 'lunch-club', when a flood of emails would start shortly before midday and sometimes would be prolonged into the evening. From all the friendship, good and bad moments, wild and not so wild parties, advice, support and I could go on and on, thank you all, **Anchel, Tony, Davide, Nuria, Sip, Vicky, Benny, Niccolò, Angela, Rocío, Markus, Sip, Stefania, Marco, Simone, Philipp, Elígio, André** and **Katia**. To my house mates and also frequent visits to Derde Walstraat. Many thanks **Till** for keeping me and **Ganseh** from tearing the place down and for making us run a 'tight ship' (barely), and thank you both for the support and help and the food (often spicy) and all the amusing evenings and also the career and scientific advice, since we were all in the same boat, and also thanks to **Mario** that, although brief,

brought and Italian flavor to the household. Also to the small Portuguese community in Nijmegen, **Marta, Daniel, André, Patricia, Alan, Helena** and **Guilherme** , thank you for all the support, fun, friendship and the times we got together *para pôr a conversa em dia, dizer umas barbaridades e beber uns copos.*

And last, my dear **Cindy**, you have been an infinite source of support and motivation in this whole journey. I cannot thank enough for the long hours, advice and, off course, all the time spent in trains and planes for almost two years, commuting from the far end of Germany to the Netherlands. From when we met in Nijmegen, for lunch, to now, when we are about to write the next chapters of our lives together, I cannot thank you for everything, in particular the time I spent writing this thesis and you had to put up with all my frustrations and non-german working habits.

E por fim, umas palavras em português. Já lá vão uns bons anos que se tornou um hábito para mim mudar de sítio com alguma frequência e, aparentemente, cada vez para mais longe: Lisboa, Coimbra, Holanda, Inglaterra e agora Alemanha. Obviamante que nada disto seria possível sem o apoio e suporte dos meus pais, que sempre me apoiaram e acreditaram em mim. Desde cedo que me ensinaram e encorajaram a seguir o meu próprio caminho e a procurar por aquilo que eu mais gostasse de fazer na vida. É graças a vocês que sempre mantive uma atitude positiva de como encarar as coisas e todo este trabalho é dedicado a vocês. Á minha irmã e ao meu cunhado que também só me põem a vista em cima raras vez por ano, muito obrigado pelo apoio e paciência ao longo destes anos todos.

E para acabar, fica aqui um grande abraço á minha gente do académico e irmandade do Boda, que sempre foram uma fonte de apoio e suporte moral, motivação e por vezes inspiração desde os tempos de Coimbra até aos dias de hoje.

Muito obrigado a todos

CURRICULUM VITAE

Pedro Miguel Caetano Pinto was born in Vidigueira, on May 10, 1982. After graduating high-school in 2000 he enrolled in the Instituto Superior Técnico, in Lisbon and subsequently moved to the University of Coimbra were he obtained a bachelor degree in Biology in 2008. He pursued a master degree from the same University, having performed his training in the Institute of Pharmacology and Experimental Therapeutics, Coimbra Medical School, under the supervision of Prof. Luis Almeida and Dr. Nunes Gaspar. In 2010, Pedro presented the master thesis entitled: *The Effect of Endocannabinoid O2545 in Intra–ocular Pressure and Retinal Structure in New Zealand White Rabbits, obtaining his master's degree in Biology*. In the same year, he was admitted to the MIT-Portugal program in Bioengineering. During his track in the program he trained in both stem cell technologies and scientific management. His team was awarded the 1st prize in the 2011 bio-teams contest with the business plan: *The commercialization strategy for the Alumagna Technology*. In 2012, he was appointed as a PhD candidate at the Department of Pharmacology and Toxicology, Radboud Institute for Molecular Life Sciences in Nijmegen, The Netherlands. Within the Nephrotools consortium (EU-FP7) and, under the supervision of Prof. Dr. Roos Masereeuw and Dr. Martijn Wilmer, Pedro's research was focused on investigating renal function *in vitro*, developing assays to study the activity and regulation of specific membrane transporters. In 2013, he spent a visiting period in the group of Prof. Patricia Murray in the Institute of Translational Medicine, University of Liverpool, UK and in 2015 moved to the Utrecht Institute of Pharmaceutical Sciences, following the appointment of Prof. Dr. Roos Masereeuw as Professor of experimental pharmacology. Here, he completed his doctoral work, studying the function of human derived renal stem cells and the pharmacological interactions of cancer drugs in renal proximal tubules. As his doctoral studies concluded, in 2016, he received a presentation award from the American Association of Pharmaceutical Scientists for the work: *Cetuximab prevents methotrexate-induced nephrotoxicity in vitro through epidermal growth factor-dependent regulation of OAT1 and MRP4.*

LIST OF PUBLICATIONS

Proximal tubular efflux transporters involved in renal excretion of p-cresyl sulfate and p-cresyl glucuronide: Implications for chronic kidney disease pathophysiology
Mutsaers HA and **Caetano-Pinto P**, Seegers AE, Dankers AC, van den Broek PH, Wetzels JF, van den Brand JA, van den Heuvel LP, Hoenderop JG, Wilmer MJ, Masereeuw R. Toxicology In Vitro. 2015 Oct;29(7): p1868-77
DOI: 10.1016/j.tiv.2015.07.020

Fluorescence based transport assays revisited in a human proximal tubule cell line
Caetano-Pinto P., *Janssen MJ, Gijzen L, Verscheijden L, Wilmer MJ, Masereeuw R.*
Molecular Pharmaceutics. 2016 Mar 7;13(3): p933-44
DOI: 10.1021/acs.molpharmaceut.5b00821

Fluorescently Labeled Cyclodextrin Derivatives as Exogenous Markers for Real-Time Transcutaneous Measurement of Renal Function
Huang J, Weinfurter S, **Caetano-Pinto P**, Pretze M, Kränzlin B, Pill J, Federica R, Perciaccante R, Ciana LD, Masereeuw R, Gretz N.
Bioconjugate Chemistry. 2016 Oct 19;27(10): p2513-2526
DOI: 10.1021/acs.bioconjchem.6b00452

The Importance of Breast Cancer Resistance Protein to the Kidneys Excretory Function and Chemotherapeutic Resistance
Caetano-Pinto P, *Janssen J, and Masereeuw R.*
Drug Resistance Updates. 2017 Jan5(30): p15-27
DOI: http://dx.doi.org/10.1016/j.drup.2017.01.002

Cetuximab prevents methotrexate-induced nephrotoxicity in vitro through epidermal growth factor dependent regulation of renal drug transporters
Caetano-Pinto P, Jamalpoor A, Ham J, Goumenou A, Mommersteeg M, Pijnenburg D, Ruijtenbeek R, van Zelst B, Heil S, Jansen J, Wilmer MJ, van Herpen CML and Masereeuw R.
Submitted for publication

Biomedical membranes and bioartificial organs
Chapter 5: Membranes for bioartificial kidney.
Chevtchik N, **Caetano-Pinto P**, Masereeuw R and Stamatialis DF
Biomedical membranes and bioartificial organs, in preparation (2017)
Editor: Stamatialis DF
Publisher: World Scientific.

PHD PORTFOLIO

	Year
Courses and Workshops	
Radboud Institute for Molecular Life Sciences(RIMLS) graduate course	2012
16th European Nephrogenesis Workshop-Liverpool, UK	2012
Nephrotools Biomaterials Workshop-Dresden, Germany	2012
12th International *Workshop* on Developmental Nephrology-Edinburgh, UK	2013
17th European Nephrogenesis Workshop-Edinburgh, UK	2013
Public speaking workshop, Big Wheel Theatre-Liverpool, UK	2013
Nephrotools Stem Cell Workshop-Liverpool, UK	2013
Nephrotools & Bioart Renal Physiology Workshop-Nijmegen, The Netherlands	2014
Nephrology, Renal Injury Models & Methods Workshop**- Heidelberg, Germany	2014
Congress and symposium	
1st Nephrotools International Conference-Liverpool**, UK	2013
New Frontiers symposium in synthetic life-Nijmegen, The Netherlands	2013
RIMLS PhD retreat-Wageningen*, The Netherlands	2014
ESTIV: European Society of Toxicology In Vitro meeting*-Egmond aan Zee, The Netherlands	2014
2nd Nephrotools International Conference*-Turin, Italy	2014
New Frontiers symposium in regenerative medicine*-Nijmegen, The Netherlands	2014
AAPS/ITC Workshop on Drug Transporters-Baltimore*, USA	2015
Dutch pharmacology society spring meeting-Nijmegen*, The Netherlands	2015
RIMLS PhD retreat*-Veldhoven, The Netherlands	2015
3rd Nephrotools International Conference**-Liverpool, UK	2015
Figon-Dutch medicine days*- Ede, The Netherlands,	2015
10th Anniversary Strategic Alliance Nutricia Research & Utrecht University	2015
AAPS/ITC Workshop on Drug Transporters***-Baltimore, USA	2016
Göttinger transport meeting-Göttingen**, Germany	2016
Student supervision	
Linda Gijzen – RIMLS – bachelor internship – Biomedical sciences program	2014
Amer Jamalpoor – RIMLS – master internship – Biomedical sciences program	2015
Anastasia Goumenou – UPIS – master internship – Drug innovation program	2016
Marieke Wensveen – bachelor internship – College of pharmaceutical sciences	2016
Janni Mortensen – research internship – visiting student, Copenhagen University	2016
Other	
RIMLS Technical forum organizer – career development	2015
RIMLS PhD committee member/PhD retreat organization	2014-2015

* poster presentation **oral presentation***oral presentation awarded