Effect of selected ABC-drug transporters on anticancer drug disposition *in vitro* and *in vivo*

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ISBN: 978-94-6108-559-7

Lay-out and printed by: Gildeprint - Enschede

Cover design: S. Leijen & S. Marchetti.
Coverpicture: Serena's FIAT 500.

Effect of selected ABC-drug transporters on anticancer drug disposition *in vitro* and *in vivo*

Effect van specifieke ABC-geneesmiddeltransporters op de farmacokinetiek van antikankermiddelen *in vitro* en *in vivo*.
(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 donderdag 5 december 2013 des ochtends te 10.30 uur

door

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geboren op 9 juni 1974 te Pescia, Italië

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The research described in this thesis was performed at the department of Experimental Therapy of the Netherlands Cancer Institute, Amsterdam, the Netherlands.

Aan mijn ouders

CONTENTS

1

General introduction

Studies described in the thesis that is lying in front of you aim to address the possible implications of selected ABC-drug transporters on the disposition of a number of important anticancer drugs. Although variability in drug disposition has been known for as long as pharmacological studies supported drug development and clinical therapeutics general molecular pharmacological concepts explaining the given interpatient variation in drug disposition have been lacking for many decades. Firm expansion on the knowledge of drug disposition was ignited by the discovery of the first identified drug transporter P-glycoprotein (Pgp; ABCB1), or permeability glycoprotein, in the hallmark publication of Juliano & Ling in 1976 [1]. They identified that this is a 170 kD transmembrane protein that, when expressed, enables cells to become resistant to a range of well known anticancer drugs. Later it was discovered that Pgp extrudes drugs from the inside of the cell to the extracellular compartment at the cost of ATP. Further studies identified that efflux by Pgp could be inhibited by verapamil [2], which would form the basis for the understanding of the concept of drug-drug interactions (DDIs) *in vitro* as well as *in vivo* mediated by Pgp and other later identified drug transporter proteins. This knowledge has translated in a first clinical trial aiming to overcome tumor unresponsiveness by co-administration of a Pgp inhibitor and a Pgp substrate drug doxorubicin, as it had been identified that a range of solid tumors and hematological malignancies expressed Pgp a possible cause of so-called multidrug resistance [3]. Following this hallmark trial, improving this concept has been pursued in the laboratory and the clinic for a couple of decades. Despite the vast increase in knowledge of the family of drug transporters, the identification of a range of naturally occurring Pgp inhibiting substrate molecules and chemical synthesis of novel effective inhibitors clinical benefit of this concept turned out to be meager. Disappointingly, no combination of Pgp substrate anticancer drug plus Pgp inhibitor has shown a positive benefit/risk in pivotal studies and the concept has been abandoned almost completely for a number of reasons, of which discussion is considered beyond the scope of this introductory chapter as well as of this thesis as a whole. Knowledge of the field further deepened substantially by cloning and sequencing of the MDR (i.e. Multi Drug Resistance; ABCB family) gene encoding Pgp in 1986 [4]. Subsequently, identification of normal tissue expression of Pgp boosted research directed at unraveling the potential influence of Pgp on tissue distribution of affected substrate drugs [5]. Discovery of another family of drug transporters, the Multidrug Resistance (-associated) Protein (MRP) family in 1992 was another important event further shaping the landscape of drug transporters [6]. In the decade following this landmark discovery in the drug transporter field new families of drug transporters were identified and molecularly and pharmacologically characterized, including the Breast Cancer Resistance Protein (BCRP; ABCG2) [7]. The family members of the ABC-(i.e. ATP-Binding Cassette) drug transporters have now all been identified and for further details the reader is referred to recent reviews, including **chapters two and three** of this thesis [8,9], and websites, for example http://www.ncbi.nlm.nih.gov/books/NBK31/ & http://www.genenames.org/. Besides discovery of all colors of the pallet of ABC-drug transporters valuable novel tools were established to enable studying the *in vivo* pharmacological effects of ABC-drug transporters of which the first one was the *mdr1a* Pgp knockout mouse [10].

It is with this as a background that studies described in the individual chapters of this thesis should be read.

At the start of the research the literature was studied and reviewed in two publications printed in this thesis in **chapters two & three** [8,9]. The publication in **chapter three** has recently been updated and is now in press.

The tissue distribution of the important EGFR tyrosine kinase inhibitor erlotinib was studied in **chapter four** [11]. The pharmacokinetics of this orally applied anticancer drug are widely variable of which the underlying mechanism at the start of the studies described in this thesis was only partly understood. The preclinical studies employing cell models and novel genetically modified knockout mice as well as genetically unmodified wild-type mice were undertaken to explore the possible implications of tissue expression of the ABC-transporters Abcb1, Abcg2 and Abcc2. Animals used were female wild-type and Bcrp1/Mdr1a/1b$^{-/-}$ (Bcrp1/P-gp knockout) mice, obtained by cross breading Bcrp1$^{-/-}$ and Mdr1a/1b$^{-/-}$ mice, all with a > 99% FVB genetic background.

With the same purpose the affinity of the novel and potent camptothecin derivative anticancer drug gimatecan (7-t-butoxyiminomethylcamptothecin) for ABC-transporters ABCG2, Abcg2, ABCB1, and ABCC2 was explored in several *in vitro* models in **chapter five** [12].

The *in vivo* pharmacokinetics of gimatecan have been studied in **chapter six**. Animals used in this study were female Bcrp1$^{-/-}$/Mdr1a/1b $^{-/-}$ (Bcrp1/P-gp knockout) cross bread using Bcrp1$^{-/-}$ and Mdr1a/1b $^{-/-}$ mice, which were previously developed at our institute and wild-type mice of a comparable genetic background (FVB). Effect of co-administration of elacridar or pantoprazole, well-known Pgp and BCRP inhibitors, on the pharmacokinetics of gimatecan was also explored. [Manuscript in preparation]

The *in vivo* distribution of gimatecan and other camptothecin anticancer drugs has been further investigated in **chapter seven** [13]. The focus was to investigate in detail the pharmacological effect of the ABC-drug transporters Abcc4, Abcb1 and Abcg2 on the CNS penetration of topotecan, irinotecan, its active metabolite SN-38 and gimatecan, and the co-operative drug efflux system consisting of these drug transporters that could restrict the brain entry of camptothecin analogs [manuscript submitted]. Wild-type, Bcrp1$^{-/-}$, Mdr1a/1b$^{-/-}$, Mrp4$^{-/-}$, Mdr1a/1b/Bcrp1$^{-/-}$, Mrp2/Mrp4$^{-/-}$ and Mdr1a/b/Bcrp1/Mrp4$^{-/-}$ mice were used in these studies.

The effect of the drug transporters ABCG2, Abcg2, ABCB1 and ABCC2 on the disposition, brain accumulation and myelotoxicity of the aurora kinase B inhibitor AZD1152 (barasertib) and its active form AZD1152-hydroxy-QPA was investigated in **chapter eight** [14]. AZD1152 is being developed to treat acute myeloid leukemia, which tumor cells may express the indicated drug transporters. Hence, affinity of AZD1152 and its active form AZD1152-hydroxy-QPA were subjected to *in vivo* pharmacokinetic studies employing Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ (Bcrp1/Pgp knockout), cross bread using Bcrp1$^{-/-}$ and Mdr1a/1b$^{-/-}$ mice.

The effect of the drug transporters ABCB1, ABCC2, ABCG2 and Abcg2 on the disposition and brain accumulation of the taxane analog BMS- 275183 was studied in **chapter nine**. BMS- 275183 is an orally available C-4 methyl carbonate analogue of paclitaxel that has the same mechanism of action and stabilization of tubulin polymerization. The oral pharmacokinetics in man turned out to be highly variable. Moreover, a clinically relevant drug-drug interaction was observed in the clinic between BMS-275183 and benzimidazole proton-pump inhibitors. For these reasons investigation of the potential implications of affinity for ABCG2, ABCB1 and ABCC2, all highly expressed in the epithelial layer of the gastrointestinal tract, on the oral pharmacokinetics of BMS- 275183 was performed

in absence and in presence of the benzimidazole proton-pump inhibitor pantoprazole [manuscript in preparation].
It has been reported that the combination therapy of imatinib mesylate, a tyrosine kinase inhibitor, plus hydroxyurea, a ribonucleotide reductase inhibitor, is associated with remarkable antitumor activity in patients with recurrent glioblastoma multiforme. However, the mechanism of the added activity of hydroxyurea to imatinib is not known. Hence, studies were initiated to unravel whether the interaction could be explained by an effect of hydroxyurea on P-glycoprotein/BCRP-mediated transport and CYP3A metabolism of imatinib mesylate. The *in vitro* studies are described in **chapter ten** [15].
Conclusions & perspectives are described in **chapter eleven** of the thesis.

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2

Concise Review: Clinical relevance of drug-drug and herb-drug interactions mediated by the ABC transporter ABCB1 (MDR1, P-glycoprotein)

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Jan HM Schellens

The Oncologist 2007;12: 297-41.

SUMMARY

The importance of P-glycoprotein (P-gp) in drug-drug interactions is increasingly being identified. P-gp has been reported to affect the pharmacokinetics of numerous structurally and pharmacologically diverse substrate drugs. Furthermore, genetic variability in the multidrug resistance 1 gene influences absorption and tissue distribution of drugs transported. Inhibition or induction of P-gp by co-administered drugs or food as well as herbal constituents may result in pharmacokinetic interactions leading to unexpected toxicities or undertreatment. On the other hand, modulation of P-gp expression and/or activity may be a useful strategy to improve the pharmacological profile of anticancer P-gp substrate drugs.

In recent years, the use of complementary and alternative medicine (CAM), like herbs, food and vitamins, by cancer patients has increased significantly. CAM use substantially increases the risk of interactions with anticancer drugs, especially because of the narrow therapeutic window of these compounds. However, for most CAMs, it is unknown whether they affect metabolizing enzymes and/or drug transporter activity. Clinically relevant interactions are reported between St John's Worth or grapefruit juice and anticancer as well as non-anticancer drugs. CAM-drug interactions could explain, at least in part, the large interindividual variation in efficacy and toxicity associated with drug therapy in both cancer and non-cancer patients.

The study of drug-drug, food-drug and herb-drug interactions and of genetic factors affecting pharmacokinetics and pharmacodynamics is expected to improve drug safety and will enable individualized drug therapy.

INTRODUCTION

In patients, drug-drug interactions can result in unexpected life threatening and even lethal toxicities. Up to 10% of all admissions in general hospitals are caused by improper use of drugs and combinations of drugs, resulting in potentially severe drug-drug interactions [1, 2]. Adverse drug reactions can especially be severe when these interactions involve cytotoxic anticancer agents [3, 4]. Anticancer drugs are dosed close to the maximum-tolerated dose, and factors affecting the pharmacokinetics may therefore greatly increase the likelihood of development of life-threatening toxicities.

Thus far drug-drug interactions have been thought to result from inhibition of drug metabolism, inhibition of renal drug excretion, displacement out of the protein binding, or pharmaceutical interactions. However, interference at the level of ATP binding cassette (ABC) and other transporters is increasingly being identified as the mechanism behind clinically important drug-drug interactions. Drug-drug and herb-drug interactions at the level of ABCB1 (multidrug resistance 1 [MDR1], P-glycoprotein [P-gp]) is the subject of this paper.

Milestones, Position in ABC transporter family, Main molecular mechanism

P-gp was first identified by Juliano and Ling in 1976 as a surface glycoprotein in Chinese hamster ovary cells expressing the MDR phenotype [5]. Cloning of the encoding gene and structure analysis of the protein revealed that P-gp is a 160 kDa ATP-dependent efflux transporter, belonging to the ABC transporter superfamily [6, 7].

Tissue distribution and physiological function

The anatomical localization of P-gp in various tumors (where it confers the MDR phenotype) and at the apical/luminal membrane of polarized cells in several normal human tissues with excretory (liver, kidney, adrenal gland) and barrier (intestine, blood-brain barrier, placenta, blood-testis and blood-ovarian barriers) functions [8-11] suggests for P-gp a physiological role in detoxification and protection of the body against toxic xenobiotics and metabolites by secreting these compounds into bile, urine and intestinal lumen and by preventing their accumulation in brain, testis and fetus [12] [Fig. 1].

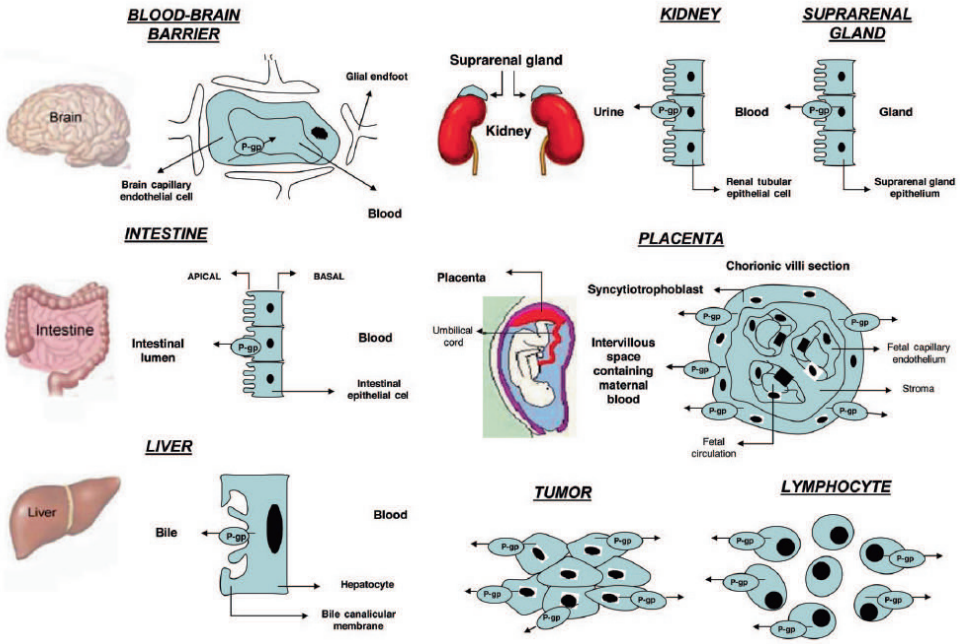

Figure 1 Schematic representation of the main sites of localization of P-glycoprotein in the body.

Impact of genetic polymorphism in the ABCB1 gene on function

Currently, at least 105 variants in the ABCB1 gene have been identified, with significant differences in their frequencies among different ethnic groups. The majority of these single nucleotide polymorphisms (SNPs) involve intronic or non-coding regions, thus not affecting P-gp amino acid sequence. However, several variants in the ABCB1 coding regions result in amino acid change and potentially affect P-gp expression and activity. Hoffmeyer et al. reported an association between a SNP in exon 26 (C3435T) of ABCB1, reduction of duodenal P-gp levels and higher peak plasma concentrations of the P-gp substrate digoxin in healthy volunteers [13]. Confirming and contradicting studies have subsequently been published about the influence of SNPs in ABCB1 on disposition of digoxin and also on other P-gp substrate drugs (such as fexofenadine, tacrolimus, irinotecan, SN-38, paclitaxel and cyclosporin A) and on P-gp expression and activity (see reviews [14-21].

Moreover, genetic variation in ABCB1, by potentially altering the physiologic protective role of P-gp, has recently been assessed in the etiology of several human pathophysiological conditions. An increasing number of studies have associated certain SNPs in ABCB1 with susceptibility to diseases such as pharmacoresistant epilepsy, Parkinson's disease, inflammatory bowel diseases (ulcerative colitis and Crohn's disease), colorectal cancer and renal carcinoma [22-27].

Recently ABCB1 SNP C3435T has been associated with the efficacy of anti-emetic treatment with 5-hydroxytryptamine type 3 receptor antagonists (such as granisetron, ondansetron, tropisetron) in patients with cancer [28], whereas in patients affected by depression, the same polymorphism has been linked to the development of postural hypotension induced by the antidepressant nortriptyline [29].

MDR1 gene polymorphism is also suggested to affect the therapy outcome of patients with several malignancies. Goreva et al. reported an association between C3435T and G3677T SNPs in ABCB1 and the risk of drug resistance in patients with lymphoproliferative diseases [30]. A correlation between several commonly occurring ABCB1 SNPs and overall survival and risk of relapse has been reported in patients affected by acute myeloid leukemia treated with etoposide, mitoxantrone or daunorubicin (well known P-gp substrates) [31]. Moreover, the ABCB1 SNP C3435T has been suggested as a significant predictor of treatment outcome in children affected by acute lymphoblastic leukemia, although these findings have not been confirmed in adults [32, 33]. Another study showed an increased response to preoperative chemotherapy in breast cancer patients homozygous for the C3435T genotype [34], whereas conflicting results have been reported about the impact of genetic variation of the MDR1 gene (in particular, G2677T/A) on the response to paclitaxel chemotherapy in patients with ovarian carcinoma [35].

Several factors may have contributed to the conflicting findings reported in the literature: demographic data from subjects slected for the various MDR1 SNPs and the methods used to measure P-gp expression differ from one study to another (i.e., protein detection by Western blot versus immunohistochemistry, various antibodies used, etc.). Moreover, discrepancies may be related to the route of drug administration and extent of metabolismrelative to P-gp-mediated transport. For instance, cyclosporine is a P-gp but also a cytochrome P450 3A4 enzyme (CYP3A4) substrate, therefore a potential P-gp effect may be hidden by CYP3A4 activity. In this regard, environmental factors, such as diet, that affect CYP enzyme activity could also influence transporter function. Differences in dietary constituents among different populations may have contributed to the conflicting results among studies. For examples, one of the possible reasons hypothesized for the reported divergent effects of MDR1 SNPs on fexofenadine disposition among whites living in U.S. and Germany was the difference in dietary salt intake between the two populations [14, 36, 37]. Furthermore, although well-known P-gp substrate drugs that are not metabolized to a relevant extent in humans (such as digoxin, fexofenadine, talinolol) have been used as probe drugs for P-gp function in humans, the involvement of other transporters and associated genetic variability could have influenced study results. Another possible reason for the contradictory reports associating ABCB1 variants with variation in drug response is that most of the studies have not considered aplotypes, whereas several recent studies suggested that the primary determinant of functional differences in P-gp resides not in SNP differences but in ABCB1 haplotypes [38]. Given the known interpopulation differences in drug response, it is especially important to consider variability among ethnic

groups and to characterize variability in haplotype structure and linkage disequilibrium and recombination within and among ethnic populations.
Additional studies involving larger sample sizes and stratification according to haplotype are required for a complete understanding of the contribution of genetic variability in ABCB1 and related proteins to drug disposition, therapeutic response, and toxicity [21, 39]. To reduce the risk of a spurious association between MDR1 genotypes and in vivo phenotypes, demographic data of subjects selected for the various MDR1 SNPs as well as sample size and environmental factors should also be considered carefully. Moreover, standardization of assays relating to P-gp protein and mRNA detection and quantification is desirable [14].

Main clinically applied substrates classes
P-gp presents high transport capacity and broad substrate specificity: a wide number of clinically relevant drugs with structurally different features and belonging to different classes (e.g., several anticancer drugs, some HIV protease inhibitors [HPIs], H2-receptor antagonists, antiarrythmics – cardiac glycosides and calcium channel blockers-, immunosuppressive agents, corticosteroids, anti-emetics and antidiarrheal agents, analgesics, antibiotics, anthelmintics, antiepileptics, sedatives, antidepressants) can be transported by P-gp (for review see [14, 40]); in general they are hydrophobic and amphipatic molecules in nature, uncharged or basic, although zwitterionic and negatively charged compounds can also be transported.

Inhibitors (competitive, non-competitive)
Some P-gp drug substrates are able to inhibit P-gp-mediated transport of others substrates. The discovery by Tsuruo and colleagues [41] that verapamil (weak P-gp substrate) could reverse P-gp-mediated MDR in leukemia cells was followed by the identification of several other P-gp inhibitors [42, 43] that can block P-gp activity by competition for drug-binding sites (competitive inhibitors) or by blockade of the ATP hydrolysis process (non-competitive inhibitors). The first agents identified as P-gp inhibitors were drugs (e.g., verapamil and cyclosporin A) already used in the clinic that were themselves transported by P-gp (so-called first generation inhibitors). Because of their low substrate selectivity and the concomitant inhibition of the drug-metabolizing CYP3A4, so-called second-generation (cyclosporin A analog PSC833) and third-generation (LY335979, VX710, GF120918, XR9576) P-gp inhibitors were developed. These and other selective P-gp inhibitors have been extensively tested preclinically and in patients to reverse MDR. Of interest is that GF120918 (elacridar), originally developed as a P-gp inhibitor, was also identified as an effective breast cancer resistance protein (BCRP; ABCG2) inhibitor [44].
Recently it has been reported that benzimidazole gastric H^+, K^+ -ATPase proton pump inhibitors (PPI - omeprazole, pantoprazole, lansoprazole and rabeprazole), which are used by up to 50% of patients with cancer, are effective inhibitors of P-gp in vitro [45], although their potency towards BCRP inhibition is even greater [46]. Drug interactions with benzimidazoles are increasingly reported [1, 2, 47-50]. However, it has been noted that the 50% inhibitory concentration (IC_{50}) values of PPIs in inhibiting P-gp observed in vitro are higher than their expected intraluminal (intestinal) and plasma concentrations obtained after oral dosing in humans, making a drug-drug interaction at these levels unlikely.

Considering that PPIs have also been shown to be CYP3A4 and CYP2C19 substrates, and that they are able to inhibit BCRP activity, under certain circumstances, for instance in poor metabolizers of CYP2C19, plasma levels of omeprazole and pantoprazole would reach the range of reported IC_{50} values, thus making a clinical drug-drug interaction possible with coadministered substrate drugs for P-gp and/or BCRP [45].
In addition, several widely used drugs have been described to inhibit P-gp function, thus potentially leading to relevant drug-drug interactions. They include various antimicrobial agents (e.g., ceftriaxone, cefoperazone, clarithromycin, erythromycin, itraconazole, ketoconazole), Ca^{2+} antagonists (verapamil, diltiazem, quinidine, quinine, nifedipine, nicardipine), HPIs (ritonavir, indinavir, saquinavir, nelfinavir), and other compounds such as amiodarone, propranolol, dipyridamole, tacrolimus, hydrocortisone, progesterone and tamoxifen, to name a few [51, 52].
Furthermore, many pure herbal constituents commonly used as complementary and alternative medicines (CAMs) by cancer patients and dietary phytochemicals have been reported to modulate P-gp expression and/or activity. Indeed, piperine [53], ginsenosides [54, 55], silymarin from milk thistle and other flavonoids [56], capsaicin [57], and resveratrol [57] were reported to inhibit P-gp activity *in vitro*, whereas curcumin [58, 59] and curcuminoids [60] and several catechins from green tea [61-63] were shown to reduce P-gp expression and activity in vitro. Importantly, some of these herbal constituents (such as piperine, silymarin) have been observed to interact with P-gp at dietary concentrations, thus making a drug-herbal interaction in vivo more likely [53, 56]. Similarly, constituents of grapefruit and orange juice were also found to block P-gp function and certain juice-drug interactions for commonly used drugs have been described too [64-70]. The modulation of P-gp as well as other transporters (i.e., organic anion transporting polypeptides [OATPs]) or drug-metabolizing enzymes (such as CYP3A4) may provide an explanation for many reported clinical herb/juice-drug interactions.

Inducers
Clinical and preclinical findings reveal that the expression of P-gp (like some of the CYP isoenzymes) is inducible. Expression levels of P-gp (as well as other transporters) and drug-metabolizing enzymes appear to be regulated by nuclear receptors like the pregnane X receptor (PXR), constitutive androstane receptor, and vitamin D binding receptor [71]. Some (active constituents of) herbs, like hyperforin from St John's wort (SJW), can activate one or more of these receptors, thereby increasing the expression of metabolizing enzymes and transporters [72, 73]. Several carotenoids and their metabolites (such as retinol and β-carotene) have been shown to activate the PXR in vitro [74]. Recent in vitro studies demonstrated that several drugs, including rifampicin, paclitaxel, and reserpine, can induce CYP3A4 and *MDR1* gene expression through a similar mechanism [75–77]. Other putative P-gp inducers are clotrimazole, phenobarbital, phenytoin, troglitazone, and the flavonoids kaempferol and quercetin [51]. However, thus far, only rifampin and SJW have been documented to significantly induce intestinal P-gp in humans: in duodenal biopsies performed in healthy volunteers after rifampin administration, P-gp was induced 3.5-fold [78]. Similarly, administration of SJW induced human intestinal P-gp 1.4 fold [79]. For the other inducers, only in vitro data are available, thus raising doubts whether results obtained in cell lines can be extrapolated to the human in vivo situation. Moreover, recent preclinical studies demonstrate a tissue specificity of P-gp induction with potential

differences among species [80]. Therefore, although interactions between P-gp substrates and commonly used drugs/CAMs that are reported to induce P-gp expression in vitro have been described in the literature (see below), clearly, preclinical animal models that behave similarly to humans in terms of transporter and metabolism induction as well as further studies in humans are needed to evaluate their clinical relevance.

Pharmacological and toxicological function

The pharmacological functions of P-gp have been extensively studied in in vitro and in vivo models: P-gp was first described as a plasma membrane protein that could cause MDR in tumor cells by actively extruding a wide range of structurally diverse compounds, thus contributing to the resistance against chemotherapy occurring in several cancers. In addition, the strategic physiological distribution of P-gp in organs that play key roles in processes of drug absorption, distribution, and elimination suggests that P-gp has a relevant impact on limiting cellular uptake of drugs out of the blood circulation into the brain, placenta, and testis and from the intestinal lumen into epithelial cells lining the gut. In addition, P-gp may also mediate excretion of drugs out of hepatocytes into the bile canaliculi and out of renal tubules into the urine.

The effect of P-gp on the pharmacokinetics of substrate drugs has been demonstrated in vivo using *mdr1a* and *mdr1a/1b* knockout mice. Mice lacking one or both *mdr* genes showed significant alterations in drug absorption, distribution, and elimination [81– 84]: compared with wildtype *mdr1a*$^{(-/-)}$ and *mdr1a/1b*$^{(-/-)}$ mice displayed greater sensitivity to the centrally neurotoxic anthelmintic ivermectin and other known P-gp substrates like vinblastine, digoxin, and cyclosporin A. Compared with wild-type, *mdr1a*$^{(-/-)}$ and *mdr1a/1b*$^{(-/-)}$ mice also presented higher concentrations of drugs in many tissues (especially in the brain) and a slower rate of drug elimination. Other in vitro and in vivo studies documented the effect of P-gp expression on the apparent oral bioavailability of substrate drugs. Hunter et al. [85] reported the apical efflux of vinblastine across intestinal Caco-2 cell layers and the efflux was inhibited in the presence of the P-gp inhibitor verapamil and other P-gp modulators. In in vivo experiments, the apparent bioavailability of the P-gp substrate paclitaxel after oral administration was 11% in wild-type and 35% in *mdr1a*$^{(-/-)}$ mice [86]. Bardelmeijer et al. [87] reported an apparent bioavailability after oral administration of docetaxel, another P-gp substrate, of 3.6% in wild-type and 22.7% in *mdr1a/1b*$^{(-/-)}$ mice. In other studies, oral administration of the P-gp inhibitors valspodar (PSC 833) or cyclosporin A or elacridar (GF120918) followed by oral paclitaxel [88 –90] or by oral docetaxel [87] resulted in significantly greater apparent oral bioavailability in wild-type mice compared with those treated without the P-gp inhibitor (Fig. 2A). These findings led to important potential clinical implications: drug– drug interactions between substrates and P-gp inhibitors can modify the drug's pharmacokinetics by increasing bioavailability and organ uptake, leading to more adverse drug reactions and toxicities. Possibly, coadministration of substrates for P-gp and P-gp–inducing agents may lead to a reduction in plasma drug levels and consequently undertreatment.

Furthermore, the localization of P-gp in the placenta has been shown to play a key role in preventing fetal exposure to various potentially harmful or therapeutic compounds. Inhibition of P-gp activity in the placenta can affect the distribution and consequently the fetal toxicity and/or efficacy of P-gp substrate drugs [91–94]. Drug–drug interactions should be considered very carefully in pregnant or lactating breast cancer patients who will be treated with anticancer drugs, substrates for P-gp, such as anthracyclines.

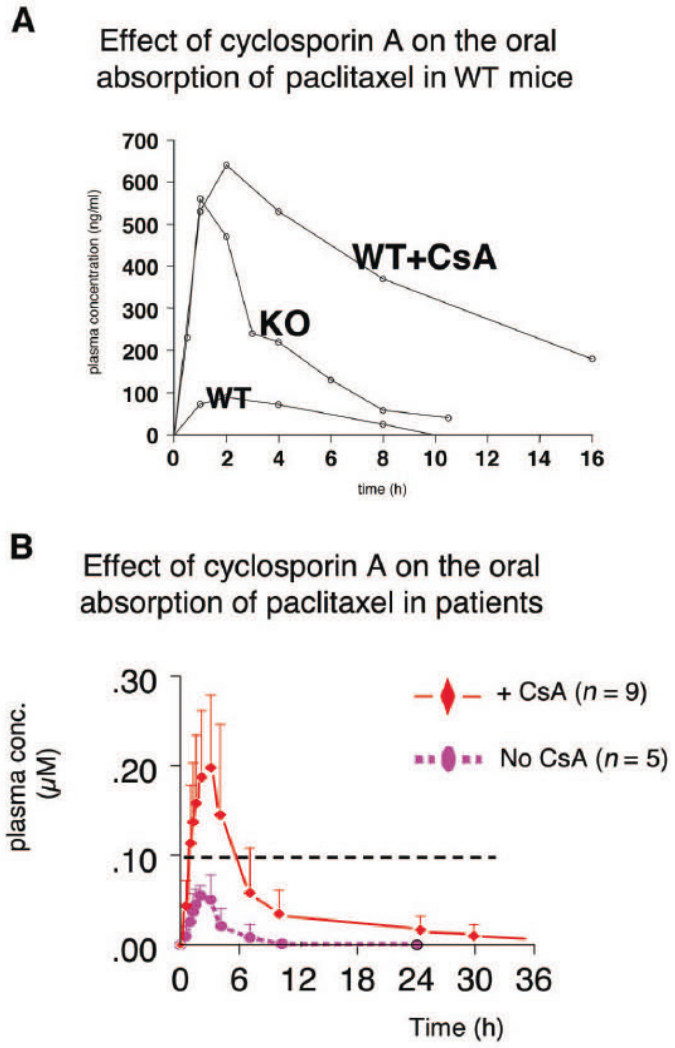

Figure 2 Effect of cyclosporine A on the oral absorption of paclitaxel.
(A) Co-administration of oral paclitaxel and cyclosporin A (CsA) in wild-type (WT) mice resulted in a significantly greater area under the curve (AUC) of paclitaxel in plasma. The effect was even greater than the AUC of paclitaxel when given to P-glycoprotein-deficient *mdr1a/b* knockout (KO) mice. The results indicate that P-glycoprotein effectively prevents oral uptake of paclitaxel from the gut. CsA may also have inhibited cytochrome P450 3A4 enzyme (CYP3A4) to explain the additional difference in the AUC compared with the experiment in KO mice. From Sparreboom A, van Asperen J, Mayer U et al. Limited oral bioavailability and active epithelial excretion of paclitaxel (Taxol) caused by P-glycoprotein in the intestine. Proc Natl Acad Sci U S A 1997;94:2031–2035, with permission.
(B) Co-administration of oral paclitaxel and cyclosporin A in patients with advanced cancer resulted in a significant increase in the AUC of paclitaxel in plasma. These results are the clinical proof of the concept that inhibition of P-glycoprotein (and possibly also CYP3A in the gut epithelium) results in a significant increase in the uptake of paclitaxel from the gut leading to in a significant increase in the systemic exposure to paclitaxel. Figure reproduced with permission from: Meerum Terwogt JM, Beijnen JH, Ten Bokkel Huinink WW, Rosing H and Schellens JHM. Co-administration of oral cyclosporin A enables oral therapy with paclitaxel. Lancet 325: 285, 1998 [ref 196].

Drug-drug interactions

In the literature several drug– drug interactions mediated by P-gp transporters have been described (Table 1). In general, the involvement of P-gp in drug– drug interactions is difficult to prove in humans because, as a result of the overlapping substrate specificity of inhibitors and inducersbetween CYP3A4 and P-gp, many drug interactions may involve both CYP3A4 enzymes and P-gp [95]. Moreover, P-gp and CYP3A4 may be functionally linked, and several potential mechanisms whereby the functions of P-gp and CYP3A4 could be complementary have been proposed [96]. Furthermore, drug– drug interactions may involve additional ABC transporters as well.

Clinical **drug–drug interactions** were reported in the literature between digoxin (a good P-gp substrate) and other drugs, such as quinidine [97–99], verapamil [100 –102], talinolol [103], clarithromycin [104], itraconazole [105], erythromycin [106], and propafenone [101, 107, 108].

Other clinically relevant drug interactions described in the literature involve the antimicrobial drug rifampicin. Rifampicin is a well-known inducer of intestinal CYP3A4. However, recent findings indicate that it can also induce P-gp expression. In a clinical study, the oral bioavailability of digoxin in eight healthy volunteers was decreased by 30% during rifampicin therapy. Intestinal biopsies obtained from the same patients before and after administration of rifampicin showed a significant increase in intestinal P-gp expression after administration of the antimicrobial drug, which correlated inversely with the oral area under the concentration–time curve (AUC) of digoxin. In addition, pretreatment with rifampicin had little effect on the renal clearance of digoxin. These results suggest that the digoxin–rifampicin interaction mainly occurs at the intestinal level and that chronic exposure to rifampicin can result in P-gp induction [78]. Similar interactions with rifampicin have been reported for talinolol [109], fexofenadine [110], and cyclosporin A [111].

Interactions mediated by P-gp that may have clinically relevant consequences have also been reported for some **excipients** used in pharmaceutical formulations. In in vitro experiments, polysorbate 80 was able to inhibit P-gp activity and to increase daunorubicin intracellular levels in cell cultures [112]. Polyoxyl castor oil and polysorbate 80 (substances used in drug formulations to dissolve some lipophylic and/or poorly soluble drugs, especially paclitaxel and docetaxel) were reported to increase the oral absorption of saquinavir and digoxin, respectively, through interaction with P-gp activity [113, 114].

In addition, **food and dietary constituents**, such as grapefruit, orange, apple, and pummelo juice, are also possible P-gp modulators. The in vivo effect of fruit juices, and in particular, grapefruit juice, on drug transport is still controversial, because some authors have predicted or reported greater whereas others have predicted or reported lower amounts of coadministered drugs reaching the systemic circulation. Indeed, several authors reported that flavonoids and furanocoumarins of grapefruit juice were able to inhibit P-gp and CYP3A4 activity, thus influencing accumulation and efflux of anticancer drugs (well-known P-gp substrates) in P-gp– overexpressing cell lines [66, 70, 115–117]. In contrast, the apparent bioavailability after oral administration and the plasma concentrations of etoposide were significantly lower in subjects taking grapefruit juice [118].

Table 1. Examples of the possible involvement of MDR1 in clinical drug-drug interactions

Drug	Inhibitor/inducer	Measured effect/toxicity	Putative mechanism	References
Digoxin	Quinidine	Greater plasma levels, lower renal clearance	Inhibition of MDR1	[97-99]
Digoxin	Verapamil	Greater plasma levels, lower renal clearance	Inhibition of MDR1	[100-102]
Digoxin	Talinolol	Greater plasma levels and AUC, lower renal clearance	Inhibition of MDR1	[103]
Digoxin	Clarithromycin	Greater plasma levels, lower renal clearance	Inhibition of MDR1	[104]
Digoxin	Erithromycin	Greater plasma levels, lower renal clearance	Inhibition of MDR1	[106, 177]
Digoxin	Itraconazole	Greater plasma levels, lower renal clearance	Inhibition of MDR1	[105, 178]
Digoxin	Ritonavir	Greater plasma AUC and terminal half-life and toxicity of digoxin	Inhibition of MDR1	[179, 180]
Paclitaxel	Cyclosporin A	Greater apparent bioavailability	Inhibition of MDR1, CYP3A	[157, 192]
Paclitaxel	Elacridar (GF120918)*	Greater bioavailability	Inhibition of MDR1, CYP3A	[181]
Docetaxel	Cyclosporin A	Greater bioavailability	Inhibition of MDR1, CYP3A	[158]
Saquinavir	Ritonavir	Greater apparent oral bioavailability	Inhibition of MDR1, CYP3A	[182, 183]
Tacrolimus	Verapamil	Greater plasma levels and toxicity of tacrolimus	Inhibition of MDR1, CYP3A	[184]
Talinolol	Erythromycin	Greater AUC	Inhibition of MDR1	[185]
Cyclosporin A	Erythromicin	Greater plasma AUC and peak plasma concentrations	Inhibition of MDR1, CYP3A	[186, 187]
Loperamide	Quinidine	Greater CNS adverse effects	Inhibition of MDR1	[175]
Digoxin	Rifampin	Lower plasma levels and AUC	Induction of MDR1, CYP3A	[78]
Talinolol	Rifampin	Lower AUC	Induction of MDR1	[109]
Tacrolimus	Rifampin	Lower apparent oral bioavailability, lower total clearance	Induction of MDR1, CYP3A	[188]
Cyclosporin A	Rifampin	Lower oral bioavailability	Induction of MDR1, CYP3A	[111]
Digoxin	St John's Worth	Lower AUC and peak plasma concentrations	Induction of MDR1	[79, 189]
Cyclosporin A	St John's Worth	Lower plasma levels	Induction of MDR1	[121, 122]
Indinavir	St John's Worth	Lower plasma levels	Induction of MDR1, CYP3A	[123]
Tacrolimus	St John's Worth	Lower plasma levels	Induction of MDR1, CYP3A	[190]
Topotecan	Elacridar (GF120918)*	Greater apparent oral availability	Inhibition of BCRP, MDR1	[159]
Methotrexate	Omeprazole/Pantoprazole	Greater AUC, lower clearance	Inhibition of BCRP, MDR1	[191]

* Experimental compound.
Abbreviations: AUC, area under the concentration-time curve; CNS, central nervous system; CYP3A4, cytochrome P450 3A4 enzyme; MDR, multidrug resistance

Similar results were reported in healthy volunteers taking grapefruit juice and the nonmetabolized and P-gp–transported drug talinolol [119], whereas in rats, administration of grapefruit juice resulted in higher plasma concentrations and lower apparent oral clearance of talinolol [115]. The reasons for these discrepancies are still unknown:
differences in the concentrations of druginteracting compounds in the juices (such as fouranocoumarins and flavonoids) have been proposed to contribute to the discrepancies in the results [120], as well as the modulation of other transporters (such as OATPs, multidrug resistance–associated proteins [MRPs]) and metabolizing enzymes by grapefruit juice constituents and other environmental factors (i.e., dietary constituents).

All these findings make it difficult to predict whether a grapefruit juice– drug interaction will occur and the magnitude of such interaction. Therefore, patients should be cautious with the consumption of grapefruit juice when treated with narrow-therapeutic-index drugs (especially with drugs whose absorption has been reported to be affected by P-gp, MRPs, OATPs).
Furthermore, many other **dietary food and pure herbal constituents** (see above) commonly used as CAMs directly inhibit CYP and P-gp activity in vitro, and some of them (like piperine and silymarin) were shown to act as P-gp inhibitors at dietary concentrations. P-gp expression is clearly induced by the over-the-counter antidepressant herbal SJW, and clinically relevant drug– drug interactions have been reported between SJW and a wide range of drugs. Chronic administration of SJW together with cyclosporin A has been associated with a significant reduction in cyclosporin plasma levels and a higher risk for acute organ rejection in transplanted patients [121, 122]. In healthy volunteers, administration of SJW together with the HPI indinavir produced an approximately 57% lower plasma AUC of indinavir [123]. Coadministration of SJW with digoxin produced an 18% lower plasma AUC of digoxin and a 40% higher expression level of intestinal P-gp [79]. Other clinical studies confirmed that coadministration of SJW significantly reduced plasma concentrations of drugs like oral contraceptives, tacrolimus, warfarin, verapamil, fexofenadine, and some others, leading to important clinical implications, that is undertreatment and failure of therapies. Similarly, in rats and in cancer patients, the plasma concentrations of SN-38 (the active metabolite of irinotecan) were significantly lower and hematological and gastrointestinal toxicities were less when SJW was coadministered [124, 125]. Furthermore, in healthy volunteers, administration of SJW together with the protein tyrosine kinase inhibitor imatinib resulted in a significantly greater oral clearance and lower AUC, maximum concentration, and half-life of imatinib [126, 127]. Induction of CYP3A4 and enhanced P-gp expression have been suggested to be responsible for these interactions (for review, see [128 – 135]).
However, for most CAMs, it is unknown whether they affect metabolizing enzymes and/or drug transporters, potentially leading to unwanted pharmacokinetic interactions with drug therapy. Altered expression or activity of several drug transporters and drug-metabolizing enzymes can lead to lower therapeutic efficacy or greater toxicity.
The risk for interactions is significantly high in cancer patients, considering that several anticancer drugs (such as vincristine, vinblastine, vinorelbine, irinotecan, etoposide, docetaxel, and paclitaxel) are P-gp and/or CYP3A4 substrates, as well as certain supportive care agents concomitantly and commonly used by cancer patients, such as ondansetron, fentanyl, morphine, loperamide, and domperidone [83, 136–138]. Clearly, the risk for

interaction is further increased by the intake of CAMs, products that are frequently used by people affected by cancer.

Possible clinical benefit of drug-drug interactions

On the other hand, the study of drug– drug interactions with P-gp modulators is an interesting research field, because P-gp was discovered and described for its ability to confer the MDR phenotype to cancer cells. The modulation of P-gp activity was at first seen as a useful strategy for increasing the penetration and retention of anticancer drugs in resistant tumor cells, thus overcoming the intrinsic or acquired resistance against chemotherapy occurring in several cancers. Since the mid-1980s, various clinical trials with anticancer drugs in combination with P-gp modulators (calcium channel blockers—nifedipine or verapamil—or cyclosporin A) have been performed [139–141]. Unfortunately, with only few exceptions [142–146], these studies did not show any survival benefit for the combination of an anticancer drug plus a P-gp inhibitor [147–151]. In addition, because the P-gp inhibitors used in those trials presented overlap in substrate specificity with CYP3A4 inhibitors, pharmacokinetic interactions occurred, resulting in greater toxicity. To date, some clinical trials using second- and third-generation P-gp inhibitors with the aim of reversing MDR in tumor cells have been performed and others are still ongoing [152–154]. In a recent pilot phase II trial, the combination of valspodar (PSC 833, a second generation P-gp inhibitor) plus paclitaxel (administered i.v. at a reduced dose because of the expected pharmacokinetic interaction [155]) in patients with metastatic breast cancer showed acceptable toxicity but the activity was not significantly increased compared with single-agent paclitaxel [156]. Additional trials will further explore the feasibility and efficacy of this strategy.

Modulation of P-gp activity with selective inhibitors could also be a useful strategy to increase the oral bioavailability of P-gp substrate drugs, in particular, to develop oral formulations of anticancer drugs transported by P-gp. Several preclinical animal studies (see above) and clinical trials in humans have been performed to evaluate the feasibility and the safety of this approach (coadministration of a substrate drug and a P-gp inhibitor). In a clinical study, cyclosporin A, an effective P-gp blocker, followed by oral paclitaxel (a well-known P-gp substrate) resulted in an eightfold higher systemic exposure to paclitaxel (Fig. 2B) [157]. Cyclosporin A also effectively resulted in a greater oral bioavailability of docetaxel, 91% versus 8% [158]. Elacridar, an effective inhibitor of BCRP as well as of P-gp produced a greater oral bioavailability of topotecan, 97% versus 40% [159]. Further studies in patients with advanced solid tumors confirmed that this strategy for oral treatment is at least as effective and safe as standard i.v. administration of these drugs, and clinical trials with third-generation modulators of P-gp (e.g., biricodar, zosuquidar, and laniquidar) specifically developed for MDR reversal are ongoing. The results will give insight into the possible clinical feasibility of this strategy [159 –163]. Indeed, an interesting clinical application of selective modulation of P-gp activity might lead to greater passage of certain drugs across the blood– brain barrier, which might profoundly extend the range of drugs available for treatment of brain disorders [164]. These include primary and metastatic tumors, microbial infections, HIV infections, mood disorders, and neurological treatment–resistant disease, for example, refractory epilepsy and schizophrenia. Furthermore, preclinical studies have shown that the brain penetration of anticancer drugs that are transported by P-gp, such as paclitaxel, docetaxel, and imatinib,

can be improved by concomitant use of P-gp inhibitors, such as cyclosporin A, valspodar, elacridar, and zosuquidar [165–169]. A clinical study determining the brain penetration of paclitaxel in combination with elacridar in patients with primary brain tumors is ongoing and the preliminary results are reported to be promising [170]. Similarly, clinical trials are exploring the activity of imatinib (Gleevec®; Novartis Pharmaceuticals Corporation, East Hanover, NJ) against the central nervous system (CNS) tumor glioblastoma [171] based on promising preclinical results. Taking into account that imatinib is a good P-gp and BCRP substrate drug with a limited distribution to the brain [172, 173] and that preclinical studies reported that the combination of imatinib with an effective P-gp inhibitor resulted in greater CNS accumulation [168, 174], modulation of P-gp as well as BCRP activity can be a useful strategy to improve CNS penetration of imatinib [170, 174].

However, the safety of this approach should be explored carefully as modulation of P-gp in the blood– brain barrier may lead to greater CNS accumulation of unwanted potentially toxic xenobiotics and endogenous compounds. Preclinical studies in wild-type and *mdr1a/b* knockout mice demonstrated that *mdr1a/1b* knockout mice are fertile and viable, but they are more sensitive to a range of drugs and toxins [81, 83, 84]. Moreover, absence or inhibition of P-gp activity can alter the specific pharmacodynamic activity of some P-gp substrate drugs, leading to CNS toxicity and adverse drug effects. For instance, the safe clinical use of the antidiarrheal drug loperamide may also be critically dependent on the presence of P-gp in the blood– brain barrier. Loperamide is a potent opiate, which demonstrates nearly exclusively peripheral opiate-like effects on the gastrointestinal tract and no central effects because it is a P-gp substrate. Thus, normally it cannot accumulate in the CNS. In *mdr1a* knockout mice, however, loperamide showed pronounced opiate-like effects and sometimes lethal effects at doses that are safe in wild-type mice [83]. In humans, coadministration of loperamide with the P-gp inhibitor quinidine produced opiate-induced respiratory depression, a clear central opiate effect that is normally not seen in humans [175].

On the same line, blocking of placental P-gp in HIVinfected pregnant women might be used to enhance HPI levels in the unborn child shortly before and during the delivery, thereby reducing the risk for HIV infection of the fetus. However, the safety of this approach needs to be studied in greater detail. Indeed, preclinical data using *mdr1a/1b* knockout mice demonstrated significantly greater fetal penetration of the HPIs indinavir and saquinavir, but also of other drugs and toxic compounds, indicating that placental P-gp might have a protective role for the fetus [92, 176].

SUMMARY, CONCLUSIONS AND PERSPECTIVES

The importance of ABC transporters in drug– drug interactions is increasingly being identified. P-gp is involved in the interactions between cyclosporin A or verapamil and oral digoxin. Azole antifungals, such as fluconazole and itraconazole, interact with P-gp, explaining drug interactions with digoxin and other drugs. Benzimidazoles are transported by and inhibit P-gp. P-gp regulates oral bioavailability and tissue distribution of the immunosuppressant tacrolimus. P-gp mediates drug interactions between antiretroviral drugs and comedications. Also, genetic variability in the *MDR1* gene affects absorption and tissue distribution of P-gp substrate drugs.

Furthermore, CAM use, like herbs, food, and vitamins, by patients has increased significantly in recent years. Surveys have shown that the prevalence of CAM use among cancer patients receiving conventional therapy is 54%–77%, and that about 72% of patients do not inform their treating physician.CAMuse significantly increases the risk for interactions with anticancer drugs, especially because of the small therapeutic range and steep dose–toxicity curve of these drugs. Clinically relevant problems are seen with SJW and grapefruit juice. SJW significantly decreases the plasma levels of SN-38, the active metabolite of irinotecan, and increases imatinib clearance. Grapefruit juice affects the oral bioavailability of etoposide. However, it is expected that CAM–drug interactions are responsible for more of the, so far unresolved, interindividual variation and clinical problems seen in cancer and noncancer patients.

The main causes of interactions are changes in the pharmacokinetics of drugs, although interactions at the pharmacodynamic level are also possible. Many drugs are cleared by biotransformation and subsequently transported by P-gp, BCRP, or other transporters. Altered expression or activity of these proteins can lead to lower therapeutic efficacy or greater toxicity.
Increased knowledge of drug-drug, food-drug, and herb-drug interactions and of genetic factors affecting pharmacokinetics and pharmacodynamics is expected to improve drug safety and will enable drug therapy tailored to the individual patients' needs.

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3

Clinical relevance: drug-drug interactions, pharmacokinetics, pharmacodynamics, and toxicity

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In: "Drug transporters: Molecular Characterization and Role in Drug Disposition", eds. Guofeng You & Marilyn E. Morris, John Wiley & Sons, Inc., Hoboken, NJ, USA, 2007; 747-880; update: 2013, in press.

HUMAN DRUG TRANSPORTERS

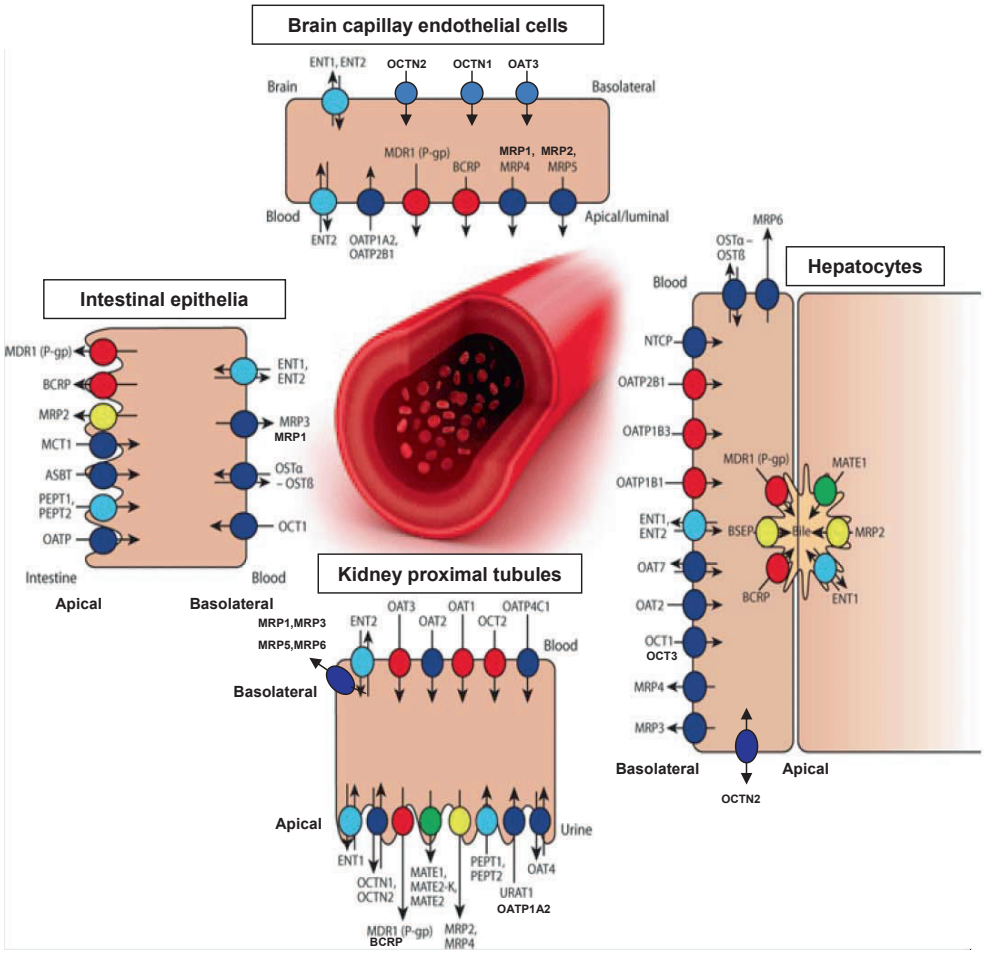

Fig. 1 Cellular localization and tissue distribution of human drug membrane transporters.
MDR1 (P-gp), Multidrug Resistance Protein 1 (P-glycoprotein); BCRP, Breast Cancer Resistance Protein; MRP, Multidrug Resistance –Associated Proteins; OAT, Organic Anion Transporter; OATP, Organic Anion-Transporting Polypeptides; OCT, Organic Cation Transporter; OCTN, Organic Cation Ergothioneine/Carnitine Transporter; MATE, Multidrug And Toxin Extrusion Transporter; PEPT, Peptide Transporter; MCT, Monocarboxylate Transporter; ENT, Equilibrative Nucleoside Transporter; URAT1, Urate Transporter; BSEP, Bile Salt Efflux Pomp. *(Adapted from Giacomini KM & Huang SM, Clin Pharmacol & Ther, 2013; 94: 3-10).*

1 - INTRODUCTION

Preclinical and clinical studies indicate that transporters are important determinants in mediating drug disposition, therapeutic efficacy and adverse drug reactions. In this chapter clinically relevant drug-drug, food-drug and herb-drug interactions mediated by drug transporters that have been described in the literature in the recent years will be

updated. A description of the hypothesized/demonstrated mechanism of pharmacokinetic and pharmacodynamic interactions will be provided employing pre-clinical and clinical data. The clinical consequences of such interactions for patients in terms of therapeutic efficacy and toxicity and for clinical practice will be presented. The role of genetically determined polymorphisms in drug-drug interactions mediated by transporters will be briefly discussed on the basis of recent insights.

2 - INTERACTIONS MEDIATED BY ABC DRUG TRANSPORTERS

2.1 ABCB1 (MDR1, P-glycoprotein, Pgp)

ABCB1 (MDR1, P-glycoprotein, Pgp) was first identified by Juliano and Ling in 1976 as a surface glycoprotein in Chinese hamster ovary cells expressing the multidrug resistance phenotype.[1] Cloning of the encoding gene and structure analysis of the protein revealed that P-gp is an ATP-dependent efflux transporter, belonging to the ABC (ATP binding cassette) transporter superfamily.[2,3]

The anatomical localization of P-gp in various tumors (where it confers the MDR phenotype) and at the apical/luminal membrane of polarized cells in several normal human tissues with excretory (liver, kidney, adrenal gland) and barrier functions (intestine, blood-brain-barrier, placenta, blood-testis and -ovarian barriers)[4-7] suggests for P-gp a physiological role in detoxification and protection of the body against toxic xenobiotics and metabolites by secreting these compounds into bile, urine and intestinal lumen and by preventing their accumulation in brain, testis and fetus.[8]

Main substrate classes, inhibitors (competitive, non-competitive), inducers

- *Substrate drugs.* P-gp presents high transport capacity and broad substrate specificity: a wide number of clinically relevant drugs with structurally different features and belonging to different classes (e.g. several anticancer drugs, some HIV protease inhibitors, H2-receptor antagonists, anti-arrythmics – cardiac glycosides and calcium channel blockers-, immunosuppressive agents, corticosteroids, anti-emetics and anti-diarrheal agents, analgesics, antibiotics, anthelmintics, anti-epileptics, sedatives, antidepressants) can be transported by P-gp [for review see Schinkel et al.][9]. In general they are hydrophobic and amphipatic molecules in nature, uncharged or basic, although zwitterionic and negatively charged compounds can also be transported.

- *Inhibitors (competitive, non-competitive).* Some P-gp drug substrates are able to inhibit P-gp mediated transport of others substrates. The discovery by Tsuruo and colleagues[10] that verapamil (weak P-gp substrate) could reverse P-gp mediated multidrug resistance in leukemia cells was followed by the identification of several other P-gp inhibitors[11] that can block P-gp activity by competition for drug-binding sites (competitive inhibitors) or by blockade of the ATP hydrolysis process (non-competitive inhibitors). The first agents identified as P-gp inhibitors were drugs (e.g. verapamil and cyclosporin A) already used in the clinic and which were themselves transported by P-gp (so-called first generation inhibitors). Because of their low substrate selectivity and the concomitant inhibition of the drug-metabolizing cytochrome P4503A4 enzyme (CYP3A4), so-called second (biricodar) and third (zosuquidar, valspodar, tariquidar, elacridar) generation of P-gp inhibitors were developed. These and other selective P-gp inhibitors have been extensively tested

preclinically and in patients to reverse multidrug resistance. Of interest is that elacridar (GF120918), originally developed as a P-gp inhibitor, was also identified as an effective BCRP (ABCG2) inhibitor.[12] Recently it has been reported that benzimidazole gastric H+, K+ -ATPase proton pump inhibitors (PPI, omeprazole, pantoprazole, lansoprazole and rabeprazole), which are used by up to 50% of patients with cancer, are effective inhibitors of P-gp[13], although their potency towards BCRP inhibition is even greater.[14] Other widely used drugs described to inhibit P-gp function, thus potentially leading to relevant drug-drug interactions, include various antimicrobial agents (e.g. ceftriaxone, cefoperazone, clarithromycin, erythromycin, itraconazole, ketoconazole), Ca^{2+} antagonists (verapamil, diltiazem, quinidine, quinine, nifedipine, nicardipine), HIV protease inhibitors (ritonavir, indinavir, saquinavir, nelfinavir, lopinavir), anticancer tyrosine kinase inhibitors (gefitinib, erlotinib, lapatinib, sunitinib, apatinib) and other compounds such as amiodarone, propanolol, dipyridamole, tacrolimus, hydrocortisone, progesterone and tamoxifen, to name a few.[15-19]

- *Inducers.* Clinical and preclinical findings reveal that the expression of P-gp (like some of the CYP isoenzymes) is inducible: recent *in vitro* studies demonstrated that several drugs, including rifampicin, reserpine and hyperforin (one of the components of St John's wort) can induce CYP3A4 and MDR1 gene expression through a similar mechanism.[20-22] Other P-gp inducers are clotrimazole, phenobarbital, phenytoin, troglitazone and the flavonoids kaempferol and quercetin.[15] Clinically relevant drug-drug interactions between P-gp substrates and inducers have been reported [see below].

Pharmacological and Toxicological Function

The strategic physiological expression in the apical membrane of enterocytes, hepatocytes, and kidney proximal tubule, as well as in the endothelial cells of the blood brain barrier and placenta, and its activity as ATP-dependent efflux transporter, suggests that Pgp may alter the pharmacokinetics of substrate drugs. Indeed, Pgp may limit uptake of drugs from the intestinal lumen, and from blood circulation into the brain, placenta and testis. Moreover, Pgp may facilitate excretion of drugs from the renal tubule in the urine and out of hepatocytes into the bile canaliculi. The effect of Pgp on the pharmacokinetics of substrate drugs has been demonstrated in vivo using Mdr1a/1b knockout mice. More recently, in view of the overlapping substrate specificity between Pgp, Breast Cancer Resistance Protein (BCRP, ABCG2) and cytochrome P450 3A4 (CYP3A4) metabolizing enzymes, combined Mdr1a/1b and Cyp3A knockout as well as combined Mdr1a/1b and Bcrp1 knockout have been generated.[23] Mice lacking one or both *Mdr* genes were viable, fertile and without marked spontaneous abnormalities, but displayed significant alterations in drug absorption, distribution and elimination.[24-27] When treated with the neurotoxic antihelmintic ivermectin or with other drugs (i.e., digoxin, vinblastin, cyclosporin A) Mdr1a(-/-) and Mdr1a/1b(-/-) mice displayed increased ivermectin-mediated neurotoxicity, higher plasma and tissue concentrations (especially in the brain) and reduced rate of drug elimination, when compared with wild-type mice. Moreover, genetic deletion of MDR1 resulted in increased bioavailability of orally administered drugs. Oral bioavailability of the anticancer agent and Pgp substrate paclitaxel increased from 11% in wild-type to 35% in Mdr1a(-/-) mice.[28] In other experiments the oral bioavailability of docetaxel, another Pgp substrate, improved from 3.6% to 22.7% in wild-type and Mdr1a/1b(-/-) mice, respectively. Interestingly, oral administration of the Pgp inhibitors

valspodar, cyclosporin A, or elacridar followed by oral paclitaxel or docetaxel resulted in a significant increase in the apparent oral taxane bioavailability in wild-type mice.[29-32] These findings have important clinical implications. Indeed, drug-drug interactions between Pgp substrates and Pgp inhibitors may increase oral bioavailability and organ drug uptake, leading to more drug-related toxicity. On the other hand, concomitant administration of Pgp substrates and Pgp inducers could potentially lead to reduction in plasma drug levels and, consequently under treatment.

The localization of Pgp in the placenta has been shown to protect the fetus by preventing exposure to various potentially harmful compounds. In preclinical studies treatment of pregnant Mdr1a(-/-) mice to an isomer of the pesticide ivermectin, known for teratogenic effects, resulted in increased fetal exposure and increased incidence of malformation (cleft palate) from 0% in wild-type, to 30% in Mdr1a(+/-), to 100% in Mdr1a(-/-) litters.[33] In other experiments fetal penetration of the Pgp substrates digoxin, saquinavir, and paclitaxel was significantly higher in Mdr1a(-/-) compared with wild type mice. Interestingly, administration of a Pgp inhibitor (valspodar or elacridar) to pregnant Mdr1a/1b(+/-) mice lead to an increase in fetal drug distribution similar to that observed in Mdr1a/1b(-/-) mice.[34] In humans, treatment with valspodar and elacridar increased fetal exposure to the Pgp substrate saquinavir.[35]

Very recently, specific PET (Positron emission tomography) and SPECT (single-photon emission computed tomography) radiotracers have been developed in order to measure in vivo the functionality and the expression of Pgp and other ABC transporters in animal models and in humans. The availability of a non-invasive imaging method to measure presence and activity of ABC transporters in patients could be useful when it would be able to identify patients that would benefit from treatment with ABC transporter modulating drugs. Pgp tracers, consisting in the form of substrates or modulators of the transporter are currently developed and tested. Preliminary application of such techniques appears promising, but further studies are necessary in order to evaluate whether their implementation will be beneficial and feasible, and whether it will translate in better tailored therapy for patients in clinical practice.[36]

Impact of Polymorphism on Function

Naturally occurring polymorphisms have been recognized in the *ABCB1* gene. However, the in vivo role of these polymorphisms has not been consistently demonstrated. To date, hundreds of studies in genotype defined population have been performed in order to evaluate the effects of genetic variants on disease, pharmacokinetics of substrate drugs, therapeutic efficacy and response, but results are often conflicting. Hoffmeyer et al. reported as first an association between an SNP in exon 26 (C3435T) of ABCB1 reduction in duodenal Pgp levels, and higher plasma concentrations of the Pgp substrate digoxin in healthy volunteers.[37] Confirming and contradicting studies have subsequently been published about the influence of SNPs in *ABCB1* on the disposition of digoxin and on other Pgp substrate drugs (such as fexofenadine, tacrolimus irinotecan, SN-38, paclitaxel, docetaxel, rosuvastatin and cyclosporin A) and on Pgp expression and activity.[38-46] Numerous studies have also investigated the influence of *ABCB1* SNPs on toxicity and therapeutic efficacy of clinically used Pgp substrate drugs, such as anticancer agents, antidepressant and immunosuppressant. Conflicting results have been shown regarding the association between 3435C>T or other SNPs and pharmaco-resistance to antiepileptic

drugs, response to nelfinavir or to other anti-HIV protease inhibitors[47-53], and treatment outcome in patients taking the antidepressant paroxetine.[59] *ABCB1* SNP C3435T has been associated with antiemetic treatment efficacy with 5-hydroxytryptamine type 3 receptor antagonists (e.g., granisetron, ondansetron, tropisetron) in patients with cancer,[54] whereas in patients affected by depression, the same polymorphism has been linked to the development of postural hypotension induced by the antidepressant nortriptyline.[55] The 2677T SNP has been reported as a positive predictor of tacrolimus-induced neurotoxicity,[56] whereas hypercholesterolemic patients carrying the 1236T variant allele showed higher lipid-lowering efficacy and reduced incidence of side effects during simvastatin treatment.[57] Recently, ABCB1 SNPs were also reported to predict central side effects in patients treated with the dopamine receptor agonist cabergoline.[58] Moreover, considering that Pgp transports various endogenous compounds and that non-functional genetic variants could potentially alter the physiologic protective role of Pgp, several studies have explored the association of *ABCB1* SNPs and several human pathophysiological conditions, such as Parkinson's disease, pharmaco-resistant epilepsy, inflammatory bowel diseases (e.g., ulcerative colitis, Crohn's disease), cancers (leukemia, colon cancer, renal carcinoma, glioma), liver and renal diseases (cirrhosis and nephritic syndrome, respectively), hypertension, rheumatoid arthritis and gingival hyperplasia. However, no definitive conclusion can be drawn from the data available to date.[59] Various studies have also tried to find a correlation between *ABCB1* SNPs and outcome of patients with several malignancies (in particular lymphoproliferative diseases), but definitive conclusions remain to be reached.[60-66] Despite the conflicting results, the data indicate that *ABCB1* SNPs resulting in variants with altered function may contribute to the interindividual variability in pharmacokinetics, efficacy and toxicity of administered drugs and to the susceptibility to certain diseases. However, additional studies are needed in order to clarify the clinical implications of *ABCB1* SNPs.

Drug-Drug Interactions

In the literature, several drug-drug interactions mediated by Pgp have been described. In general, involvement of Pgp in drug-drug interactions is difficult to prove in humans due to the overlapping substrate specificity between Pgp, other transporters and CYP3A4, and the possible involvement of multiple mechanisms.[67] The recent generation of mice knockout for multiple ABC transporter genes and metabolizing enzymes has improved the understanding of the overlapping functions of different transporters and the contributions of specific transporters to clinically observed relevant interactions. However, there are several limitations of these models, due to difference in species specificities and to compensatory up- or down-regulation of alternative pathways frequently observed in knockout models.[23] The clinical importance of Pgp as mechanism involved in drug-drug interactions is underlined by the inclusion in the recently published EMA and FDA guidelines of recommendations to study *in vitro* and *in vivo* the potential for Pgp-mediated interactions during the development of new molecular entities. In EMA guidelines digoxin, the oral anticoagulant dabigatran exetilate or the H1 antagonist fexofenadine are recommended as probe substrates to assess the potential for Pgp mediated drug-drug interactions in specific circumstances which have been described in the guidance documents.[68,69]

Drug-drug, drug-food and drug-herbal interactions mediated by Pgp can be divided in interactions between a substrate and an inhibitor of Pgp and interactions between a drug substrate and a Pgp inducer.

a) *Interactions between drug substrates and inhibitors of Pgp*

Clinically relevant drug-drug interactions have been reported in the literature between digoxin and other drugs, such as quinidine,[70-72] verapamil,[73] propafenone,[74,75] talinolol,[76] clarithromycin,[77] itraconazole,[78] erythromycin,[79] cyclosporin A and the specific Pgp inhibitor valspodar.[80] Co-administration of quinidine and digoxin increased digoxin plasma concentrations, resulting in clinical toxicity. This interaction could be explained by inhibition of renal secretion and intestinal elimination of digoxin mediated by quinidine due to interaction with Pgp. *In vitro* and *in vivo* experiments have shown that digoxin is a Pgp substrate[25] with only a minimal contribution of metabolism to its disposition.[81] *In vivo*, co-administration of quinidine with digoxin in wild-type mice increased plasma digoxin levels by more than 70%, whereas no effect was observed in Mdr1a/1b(-/-) mice.[82,83] Similar mechanisms involved in the digoxin-quinidine interaction can be postulated for the interactions described between digoxin and propafenone, clarithromycin, or erythromycin, as in vitro all these drugs could reduce the renal secretion of digoxin by inhibiting Pgp in the renal tubule.[84,85]

A drug-drug interaction between the HIV protease inhibitor ritonavir and digoxin has been described in a 61-year-old woman undergoing antiretroviral triple therapy combined with digoxin, resulting in development of digoxin-related toxicity.[86] In a clinical study in 12 healthy volunteers, repeated oral administration of ritonavir increased the area under the plasma concentration-time curve (AUC) of digoxin by 86% and its volume of distribution by 77%. Ritonavir decreased the non-renal and renal digoxin clearance by 48 and 35%, respectively, resulting in an increase in digoxin terminal half-life in plasma of 156%.[87] As the contribution of metabolism to digoxin elimination is negligible in humans, inhibition of CYP3A4 mediated by ritonavir as primary underlying mechanism for this interaction is rather unlikely. As ritonavir is a substrate and inhibitor of Pgp in vitro, inhibition of Pgp-mediated transport of digoxin exerted by ritonavir could explain such interaction.

A drug-drug interaction has been described between ritonavir and another HIV protease inhibitor, saquinavir, resulting in a dramatical increase in oral bioavailability of saquinavir. Although this interaction is probably explained by ritonavir-mediated inhibition of CYP3A4 mediated drug metabolism, a contribution of Pgp modulation cannot be excluded, as saquinavir and ritonavir are both substrates and inhibitors of Pgp.[16,88-91]

An important drug-drug interaction is also documented between the immunosuppressive tacrolimus and verapamil, resulting in dramatically increased tacrolimus plasma levels and toxicity. Since tacrolimus and verapamil are well-known substrates and/or inhibitors of Pgp and CYP3A, a contribution of Pgp modulation could be postulated in this interaction. *In vitro* studies showed Pgp-mediated efflux of tacrolimus from intestinal epithelial cells (Caco-2 cells),[92] and *in vivo* studies demonstrated a contribution of Pgp to the oral bioavailability of tacrolimus in rats.[93]

Several clinical reports regarding drug-drug interactions involving benzimidazoles proton pump inhibitors have been published. A serious case of rhabdomyolysis causing atrioventricular block in a patient taking atorvastatin, esomeprazole, and clarithromycin has been described, all well-known Pgp substrates.[94] In patients receiving high-dose methotrexate therapy, concurrent administration of benzimidazoles was associated with

a significant decrease in clearance and significantly higher plasma concentrations of methotrexate, resulting in severe toxicity.[95] Although proton pump inhibitors are known to interact with drug-metabolizing enzymes and to block BCRP *in vitro*, since a pre-clinical study has demonstrated that omeprazole, lansoprazole, and pantoprazole are substrates and inhibitors of Pgp,[13] hence a possible contribution of Pgp modulation to this interaction cannot be excluded.

Clinically relevant drug-drug interactions have also been reported between the β_1-adrenergic antagonist talinolol (a Pgp substrate) and erythromycin or verapamil in humans.[96-98]

Clinically relevant drug-drug interactions have also been described between fexofenadine and the antimicrobials ketoconazole or erythromycin. Although these antimicrobials have been reported to inhibit OATP activity, the interactions appear more likely to be mediated through inhibition of Pgp.[99]

A relatively new and important drug-drug interaction probably mediated by Pgp has been documented between the new anticoagulant dabigatran etexilate and verapamil.[100] Administration of dabigatran etexilate to 40 healthy volunteers in combination with verapamil resulted in increased dabigatran bioavailability, increase in plasma AUC (143%) and maximum plasma concentrations (179%) of dabigatran, probably due to Pgp inhibition mediated by verapamil.[100]

b) *Interactions between drug substrates and inducers of Pgp*

Several clinically relevant drug interactions described in the literature involve the antimicrobial drug rifampicin and several Pgp substrate drugs (e.g., digoxin, talinolol[101], fexofenadine[102], cyclosporin A[103], dabigatran etexilate)[104], and can be explained, at least partly, by induction of Pgp mediated by rifampicin. Rifampicin is a well-known inducer of Pgp and of CYP3A4. In a clinical study the oral bioavailability of digoxin in 8 healthy volunteers was decreased by 30% during rifampicin therapy.[105] Intestinal biopsies obtained from the same patients before and after administration of rifampicin showed a significant increase in intestinal Pgp expression after intake of the antimicrobial drug, which correlated inversely with the oral AUC of digoxin. Pretreatment with rifampicin had little effect on the AUC and renal clearance of digoxin. These results suggest that the digoxin-rifampicin interaction occurs primarily at the intestinal level.[105] Administration of rifampicin and talinolol to 8 healthy male volunteers resulted in a significant reduction in the AUC after intravenous and oral talinolol and substantially increased expression of duodenal Pgp.[101] In another study, concomitant administration of fexofenadine and rifampicin to 20 healthy volunteers resulted in reduction of peak plasma concentration and increased oral clearance of fexofenadine.[102] Although induction of intestinal Pgp could be the major mechanism contributing to this interaction, other factors can be involved as well, as fexofenadine is able to induce CYP3A4 and is a substrate of organic anion-transporting polypeptides (OATPs).[99] In another clinical trial, administration of oral rifampicin with cyclosporin A to 6 healthy volunteers lead to reduction in oral bioavailability of cyclosporin A from 27% to 10%. Since cyclosporin A is a substrate for both CYP3A4 and Pgp, and since rifampicin is an inducer of both CYP3A4 and Pgp, the interaction is probably due to a combination of CYP3A4 and Pgp induction.

Other clinically relevant drug-drug interactions mediated, at least in part, by Pgp induction have been reported between the over-the-counter antidepressant herbal St John's wort (SJW) and a wide range of drugs (i.e., cyclosporin A, digoxin, indinavir, tacrolimus, oral

contraceptives, fexofenadine, warfarin, verapamil, carvedilol).[106-109] Chronic administration of SJW together with cyclosporin A has been associated with a significant reduction of cyclosporin plasma levels and increased risk of acute organ rejection in transplanted patients.[106] Concomitant administration of SJW with digoxin produced a 18% reduction in the plasma AUC of digoxin and a 40% increase in the expression of intestinal Pgp.[108] In healthy volunteers administration of SJW together with the HIV protease inhibitor indinavir lead to 57% reduction of plasma AUC of indinavir and increased risk of treatment failure.[107] In all these studies administration of SJW resulted in significant reduction of plasma concentrations of co-administered drugs, leading to important clinical consequences (i.e., undertreatment and therapeutic failure). Induction of Pgp and of CYP3A4 metabolizing enzymes is responsible for these drug-drug interactions.[106-109]

c) *Interactions between Pgp substrate drugs and food*

As food and several dietary constituents appear to modulate Pgp activity in vitro, food-drug interactions may be speculated. However, the clinical evidence confirming such interactions is very limited and controversial. A possible interaction between grapefruit juice and Pgp has been postulated, but two clinical studies reported a very limited effect of grapefruit juice on the pharmacokinetics of digoxin[110-112], indicating that Pgp modulation by grapefruit juice is of limited clinical significance. Other in vitro studies have reported that ginger, tangerine, mango and guava could inhibit Pgp as well as OATPs.[113] However, further studies are needed in order to assess the in vivo relevance of such findings.

Several examples of drug-drug interactions possibly mediated by Pgp are reported in **table 1**.

Pharmacological modulation of Pgp

The modulation of Pgp activity represents a very active field of research. This concerns the following strategies: improvement of antitumor activity against cancers expressing the multidrug resistance (MDR) phenotype, and increase of oral bioavailability and of brain penetration of Pgp substrate drugs, which will be discussed in this order.

The classical approach consisting of administration of a compound able to inhibit Pgp was originally hypothesized as a strategy to increase penetration and accumulation of anticancer drugs in tumor cells expressing the multidrug resistance phenotype. Several trials have been performed to date exploring the concept of combining anticancer drugs with first, second and third generation Pgp inhibitors (e.g., verapamil, nifedipine, cyclosporin A, elacridar, valspodar, zosuquidar, tariquidar), but, with few exceptions, results have been disappointing. In general, no improved therapeutic efficacy has been observed with combination treatments, whereas in several cases the addition of the Pgp inhibitor resulted in significantly increased toxicity.[114-127] However, currently new strategies for Pgp modulation in tumor tissues are being explored.

Inhibition of Pgp activity by specific modulators has also been explored as a useful approach to improve the oral bioavailability of Pgp substrate drugs. This strategy is being evaluated in particular to develop oral formulations of anticancer drugs or to reduce the high interpatient variability observed with several orally administered anticancer compounds. In an original study concomitant administration of topotecan with the Pgp and BCRP inhibitor elacridar resulted in an increase of oral bioavailability of topotecan from 40% to 97% in patients.[128] In view of other preliminary preclinical and clinical results showing significantly improved apparent bioavailability after oral administration

of docetaxel or paclitaxel, two Pgp substrates, in combination with the Pgp inhibitor cyclosporin A or with the Pgp and CYP3A4 inhibitor ritonavir, several investigators are now exploring the feasibility of this strategy in the clinic.[129-131]

Modulation of Pgp activity has also been widely explored as a potentially beneficial approach in the field of HIV treatment, as almost all HIV protease inhibitors available have been demonstrated to be substrates and/or inhibitors of Pgp. Modulation of Pgp (and CYP3A4) achieved by combination treatment with different HIV protease inhibitors has become standard of practice to improve the oral bioavailability and delay drug elimination in order to reduce dosing frequency, thereby improving patient compliance and therapeutic efficacy. Enhanced drug penetration in lymphocytes expressing Pgp as well as in putative pharmacological sanctuary sites (e.g., CNS, testis) has also been hypothesized with this approach. Moreover, inhibition of placental Pgp in HIV infected pregnant women might be employed to increase the concentrations of HIV protease inhibitors in the fetus shortly before delivery, in order to reduce the risk of HIV infection of the child.[132,133]

Table 1. Examples of the possible involvement of MDR1 in clinical drug-drug interactions

Drug	*Inhibitor/inducer*	*Measured effect/ toxicity*	*Putative mechanism*	*References*
Digoxin	Quinidine	Increased plasma levels, decreased renal clearance	Inhibition of MDR1	70, 71
Digoxin	Verapamil	Increased plasma levels, decreased renal clearance	Inhibition of MDR1	73, 85
Digoxin	Talinolol	Increased plasma levels and AUC, decreased renal clearance	Inhibition of MDR1	76
Digoxin	Clarithromycin	Increased plasma levels, decreased renal clearance	Inhibition of MDR1	77
Digoxin	Erithromycin	Increased plasma levels, decreased renal clearance	Inhibition of MDR1	79, 956
Digoxin	Itraconazole	Increased plasma levels, decreased renal clearance	Inhibition of MDR1	78, 957
Digoxin	Statins (e.g. atorvastatin)	Increased plasma levels, decreased renal clearance	Inhibition of MDR1	958
Digoxin	Ritonavir	Increased plasma AUC and terminal half-life, toxicity effects	Inhibition of MDR1	86, 87
Paclitaxel	Cyclosporin A	Increased apparent bioavailability	Inhibition of MDR1, CYP3A	129
Paclitaxel	Elacridar	Increased bioavailability	Inhibition of MDR1, CYP3A	959
Docetaxel	Cyclosporin A	Increased bioavailability	Inhibition of MDR1, CYP3A	130
Saquinavir	Ritonavir	Increased apparent oral bioavailability	Inhibition of MDR1, CYP3A	88, 921
Tacrolimus	Verapamil	Increased plasma levels and toxicity	Inhibition of MDR1, CYP3A	960
Tacrolimus	Ketoconazole	Increased apparent oral bioavailability	Inhibition of MDR1, CYP3A	961
Talinolol	Verapamil	Increased plasma levels	Inhibition of MDR1	962, 963
Talinolol	Erythromycin	Increased AUC	Inhibition of MDR1	964

Table 1. ***(continued)***

Drug	*Inhibitor/inducer*	*Measured effect/ toxicity*	*Putative mechanism*	*References*
Cyclosporin A	Erythromicin	Increased plasma AUC and peak plasma concentrations	Inhibition of MDR1, CYP3A	9625 966
Loperamide	Quinidine	Increased CNS adverse effects	Inhibition of MDR1	146
Digoxin	Rifampin	Decreased plasma levels and AUC	Induction of MDR1, CYP3A	105
Talinolol	Rifampin	Decreased AUC	Induction of MDR1	101
Tacrolimus	Rifampin	Decreased apparent oral bioavailability, decreased total clearance	Induction of MDR1, CYP3A	967
Fexofenadine	Rifampin	Decreased AUC, peak plasma concentrations, increased oral clearance	Induction of MDR1, CYP3A	102
Cyclosporin A	Rifampin	Decreased oral bioavailability	Induction of MDR1, CYP3A	103
Digoxin	St John's Worth	Decreased AUC and peak plasma concentrations	Induction of MDR1	108, 968
Cyclosporin A	St John's Worth	Decreased plasma levels	Induction of MDR1	106
Indinavir	St John's Worth	Decreased plasma levels	Induction of MDR1, CYP3A	107
Tacrolimus	St John's Worth	Decreased plasma levels	Induction of MDR1, CYP3A	969
Saquinavir/Ritonavir	Garlic supplements	Decreased AUC	Induction of MDR1, CYP3A	970, 971
Topotecan	Elacridar (GF120918)	Increased apparent oral availability	Inhibition of BCRP, MDR1	128
Methotrexate	Omeprazole/ Pantoprazole	Increased AUC, decreased clearance	Inhibition of BCRP, MDR1	14, 95
Dabigatran etexilate	Verapamil	Increased AUC	Inhibition of MDR1	100

Finally, modulation of Pgp at the blood-brain barrier has been extensively studied in order to improve the brain penetration of Pgp substrate drugs, thereby improving treatment of brain disorders, including primary and metastatic tumors, microbial and HIV infections, mood disorders, degenerative CNS diseases and neurological conditions resistant to treatment such as refractory epilepsy and schizophrenia. The poor passage across the blood brain barrier for the most anticancer drugs employed in clinical practice due to affinity for ABC transporters represents one of the major limitations in cancer treatment.

This has been extensively documented in numerous preclinical studies employing mouse models where deletion of one or more ABC transporter genes was associated with dramatically enhanced CNS accumulation of clinically used substrate anticancer drugs like topotecan, etoposide, paclitaxel, docetaxel, endoxifen, lapatinib, imatinib, gefitinib, erlotinib, sorafenib, sunitinib, dasatinib, cediranib, vemurafenib, and dabrafenib.[17,18,134-138] Interestingly, preclinical studies performed in Mdr1a/1b(-/-) and wild type mice have demonstrated that the brain penetration of anticancer drugs transported by Pgp, such as paclitaxel and docetaxel, could be enhanced by concomitant administration of Pgp inhibitors like cyclosporin A, valspodar, zosuquidar, elacridar and tariquidar.[17,139-143] More recently, preclinical studies conducted with triple Mdr1a/1b/Bcrp1 knockout mice have shown that brain penetration of dual Pgp/BCRP substrate drugs (e.g., topotecan, gefitinib, erlotinib, sorafenib, sunitinib, vemurafenib, trametinib, etc) was dramatically increased when both BCRP and Pgp were absent (i.e., in Mdr1a/1b/Bcrp1(-/-) mice), compared with wild-type or Mdr1a/1b(-/-) or Bcrp1(-/-) mice (**table 2**). This suggests a cooperation between Pgp and BCRP at the blood-brain barrier and would indicate that combined blockade of both transporters might have a greater impact in enhancing the brain penetration of dual Pgp-BCRP substrate drugs. In effect, in most of the above mentioned preclinical studies where dual Pgp/BCRP substrates were employed, treatment of wild-type mice with the BCRP/Pgp inhibitor elacridar was usually able to increase the CNS accumulation of the anticancer drug up to the level observed in Mdr1a/1b/Bcrp1(-/-) mice.[17,19,135-138] Finally, as several of the anticancer tyrosine kinase inhibitors (e.g., gefitinib, erlotinib, lapatinib, sunitinib, apatinib) known as dual Pgp/BCRP substrates are also able to inhibit ABC transporters (mainly Pgp and BCRP), various strategies are being evaluated in order to use these agents as combination treatment to improve drug pharmacokinetics and pharmacodynamics effects. For instance, in preclinical models co-administration of gefitinib with topotecan resulted in enhanced penetration of topotecan in the CNS and in gliomas.[144,145] However, the safety of this strategy should be scrutinized carefully, as modulation of transporters at the blood-brain barrier may result in increased brain penetration of unwanted potentially toxic endogenous compounds and xenobiotics. The pharmacokinetics and pharmacodynamic activity of co-administered drugs could be altered too, with enhanced (CNS) toxicity. For instance, the lack of CNS side effects of the opiate anti-diarrheal loperamide and the anti-histamine fexofenadine, are due to their Pgp affinity, which limits their brain accumulation. In a clinical study, concomitant administration of loperamide with the Pgp inhibitor quinidine resulted in respiratory depression, a central opiate effect.[146] Similarly, administration of loperamide to Mdr1a(-/-) resulted in opiate-like, sometimes life-threatening, side effects.[26]

Moreover, along with the classical approach of direct transport inhibition, another strategy currently explored consists of targeting transport regulation in order to down-regulate transporter expression and functional activity. Two signaling pathways have been identified to date that could potentially be employed to down-regulate Pgp transport activity in particular at the blood-brain barrier. One pathway involves signaling of inflammatory mediators via PKC. In preclinical models targeting of PKC resulted in enhanced brain penetration of the Pgp substrate verapamil.[147] The other one includes VEGF signaling via flk-1 and src.[17] Small molecule inhibitors of MDR1 gene expression are currently under evaluation.[148] For the same purpose, recent studies have reported BCRP regulation via the PI3K/AKT signaling pathway.[17] However, whether targeting these

pathways will improve delivery of anticancer drugs across the blood-brain barrier and into brain tumors, thereby improving therapeutic efficacy, needs to be demonstrated.[17] Moreover, theoretically dual Pgp and BCRP down regulation would be expected to better enhance the brain penetration of dual Pgp/BCRP substrate drugs. However, a strategy to target both transporters simultaneously for therapeutic purposes has not been identified yet.

In order to explore the usefulness and feasibility of the above mentioned strategies targeted on modulation of Pgp and other ABC transporters expression/activity, new methodologies have been developed. Currently, PET and SPECT imaging using Pgp or other ABC transporter tracers are explored as potentially useful tools in order to characterize expression and activity of ABC transporters in vivo. These techniques are expected to facilitate dynamic evaluation of transporters in physiology and pharmacology, by following distribution and localization of tracer substrates for the specific transporter in normal tissues and tumors. Real time evaluation of the consequences of introduction/co-administration of a specific transporter modulator on the pharmacokinetics and activity of substrates would potentially help in the development of novel strategies and new compounds for the treatment of cancer and other diseases.[36,149,150] However, whether such approach could finally result in development of more specific diagnostic tools able to identify and predict which patients would benefit from a specific treatment is an open question.

Clearly, further studies are needed in order to demonstrate whether modulation of Pgp and or BCRP expression/activity through different strategies, will result in new effective and safe treatment modalities for patients.

Table 2 Brain distribution of dual Pgp and BCRP substrates.

Drug	Fold increase in Brain/Plasma ratios relative to WT mice			Reference
	Pgp KO	BCRP KO	Pgp/BCRP KO	
Topotecan	1.5	1.5	12	1021
Dasatinib	4	1	9	1022
Gefitinib	31	13.7	108	175
Sorafenib	1	4	10	18
Erlotinib	2.9	1.2	8.5	1023
Imatinib	1	1	12.6	1024
Tandutinib	2	1	13	1025
Lapatinib	4	1	42.5	1026
Vemurafenib	1.7	1	21.4	136
Flavoperidol	3.4	1.2	14.2	1023
Mitoxantrone	1.7	1.4	8	1023

WT: Wild-type (mice); KO: knockout (mice).

2.2 ABCG2 (Breast Cancer Resistance Protein, BCRP)

BCRP was cloned in 1998 based on its overexpression in MCF-7/AdrVp cells, a human breast cancer cell line displaying a typical multidrug resistance phenotype against doxorubicin with cross-resistance to daunorubicin and mitoxantrone and no expression of P-gp or MRP1.[151] Shortly afterwards Miyake et al discovered a transporter, called MXR, in a highly mitoxantrone resistant cell line (S1-M1-80) obtained upon stepwise selection in

mitoxantrone from S1 human colon carcinoma cells.[152] Structural and sequencing analysis of cloned BCRP-MXR DNA confirmed that there were almost identical sequences encoding for a new transporter belonging to the subfamily G of the ABC transporter superfamily, hence designated as ABCG2.[152] Almost simultaneously, Allikmets et al. cloned cDNA sequences that were essentially identical to BCRP and MXR cDNA and named it ABCP for its high expression in the human placenta.

Human BCRP is a "half transporter" composed of a single nucleotide binding domain followed by six putative transmembrane segments. BCRP is an ATP-drug efflux transporter that pumps a wide range of endogenous compounds, xenobiotics and substrate-drugs, out of cells.[153]

BCRP expression was found in several human tissues. The apical localization in the epithelium of the gut wall indicates a role of BCRP in the regulation of uptake of orally ingested BCRP substrates by back-transport of substrate drugs entering from the gut lumen.[154] Furthermore, BCRP is present in bile canaliculi of the liver and to a lesser extent in human kidney, suggesting a role of BCRP in urinary drug elimination. BCRP is also expressed in the endothelial cells at the blood-brain barrier and at the placental syncytiotrophoblasts suggesting a protective role for the brain and the fetus, respectively.[155-157] Recently BCRP has been identified in lactating mammary gland, where it participates to secretion of vitamin B12 and other substrates in human milk.[158-159] A role of BCRP in the differentiation of hematopoietic stem cells has also been hypothesized.[160-161]

Main substrate classes, inhibitors (competitive, non-competitive)

- *Substrate drugs.* Functional characterization studies have demonstrated that BCRP can transport a wide range of substrates, ranging from chemotherapeutic agents to conjugated and unconjugated organic anions and chemical toxicants.[162] Thus BCRP displays a broad spectrum of substrate specificity that is overlapping, but distinct from that of P-gp and MRP1. Clinical and preclinical findings suggest that mitoxantrone, epipodophyllotoxins (etoposide), methotrexate and methotrexate polyglutamates, and several camptothecin derivatives (like irinotecan and its active metabolite SN-38, topotecan, homocamptothecins) are substrates of BCRP.[14,162-168] BCRP also actively transports sulfated conjugates (like estrone-3-sulfate and dehydroepiandosterone) and, with less affinity, GSH and glucuronide conjugates.[169,170] Other BCRP substrates include some chemical toxicants and carcinogenic substances (like pheophorbide a and 2-amino-1-methyl-6-phenylimidazol[4,5-b]pyridine (PhIP)), several anticancer tyrosine kinase inhibitors (e.g., imatinib, vemurafenib, dabrafenib, sorafenib), PPIs (pantoprazole, omeprazole, lansoprazole), various HMG CoA reductase inhibitors (e.g., rosuvastatin, pitavastatin), HIV nucleoside reverse transcriptase inhibitors (lamivudine and zidovudine), the antibiotic nitrofurantoin, phytoestrogens/flavonoids, prazosin, flavopiridol, and several fluoroquinolone antibiotics.[17,18,136,137,171-184] In addition, fluorescent compounds, like BODIPY-prazosin and Hoechst 33342 are BCRP substrates.[182,185,186]

- *Inhibitors (competitive, non-competitive).* Recently, a large number of BCRP inhibitors with diverse chemical structures have been described of which some show overlap in substrate selectivity with other ABC transporters, whereas some others are substrates of BCRP, thus inhibiting BCRP in a competitive manner. The acridone carboxamide derivative GF120918 (elacridar), as well as benzimidazole PPIs (omeprazole, pantoprazole and lansoprazole) are effective P-gp and BCRP inhibitors.[12-14,128,187,188] The pipecolinate derivative VX-710

(biricodar) is reported to be a BCRP as well as a Pgp and a MRP1 inhibitor.[189] The HIV protease inhibitors ritonavir, saquinavir and nelfinavir, lopinavir, azatanavir are effective inhibitors (but not substrates) of BCRP.[87,89,190-192] Selective BCRP inhibitors are the natural product fumitremorgin C and its derivatives Ko132, Ko134 and K143.[193-195] The coumermycin antibiotic novobiocin, tryptostatin A, reserpine, tamoxifen, estrone, and several food dietary flavonoids (in particular biochanin A and chrysin) have been shown to block BCRP activity.[164,181,196,197] Recently, the tyrosine kinase inhibitors canertinib, imatinib, and gefitinib were reported to be potent BCRP inhibitors *in vitro* and *in vivo*.[198-202] Finally, in *in vitro* studies the immunosuppressants cyclosporin A, tacrolimus and sirolimus revealed to be effective inhibitors (but not substrates) of BCRP at clinically relevant concentrations.[203]

Pharmacological and Toxicological Function

The strategic localization of BCRP in placenta, blood-brain barrier, small intestine, colon, liver, testis and to a lesser extent in human kidney suggests that BCRP, as well as other ABC transporters, plays a crucial protective role for the fetus, for the brain and for the body as a whole. BCRP limits the oral absorption and facilitates biliary excretion of substrate xenobiotics, thus affecting the pharmacological behavior of these compounds. The role of BCRP in drug disposition and toxicity has been explored by generating Bcrp1 (the homolog of human BCRP) knockout mice, and more recently, mice with deletion of multiple ABC transporter genes (i.e., Bcrp1/Mrp2(-/-), Bcrp1/Mrp2/Mrp3(-/-), Mdr1a/1b/Bcrp1(-/-)).

In preclinical studies the oral bioavailability of topotecan, a BCRP and Pgp substrate, increased dramatically by oral co-administration of the BCRP/Pgp inhibitor elacridar in both wild-type and Pgp knockout mice.[188] In a subsequent clinical study, co-administration of elacridar increased the apparent oral bioavailability of topotecan in cancer patients significantly, from 40% to 97%. Elacridar reduced the plasma clearance of topotecan administered intravenously by only 10%, indicating that the increased systemic exposure after oral administration of topotecan is almost entirely due to increased intestinal drug uptake.[128]

In subsequent studies increase in plasma concentration due to improved intestinal absorption and reduced biliary excretion was observed when BCRP substrate drugs (i.e., ciprofloxacin, sulfasalazine, methotrexate, nitrofurantoine, vemurafenib, sorafenib, dabrafenib) as well as the dietary carcinogen PhIP (2-amino-1-methyl-6-phenylimidazol[4,5-b]pyridine) were administered to Bcrp1(-/-) compared to wild-type mice.[173,184,204] These findings clearly indicate that BCRP can substantially affect the pharmacokinetics of substrate drugs.

In view of the overlapping tissue distribution, substrate specificity and functional role of different ABC transporters, multiple ABC transporter gene knockout mice have been generated in order to evaluate the contribution of BCRP and of other transporters, to the disposition of endogenous compounds and xenobiotics. For instance, the pharmacokinetics of methotrexate (MTX) and of its toxic metabolite 7-hydroxymethotrexate (7OH-MTX) have been studied in single [Bcrp1(-/-), Mrp2(-/-)], double [Bcrp1/Mrp2(-/-)], and triple [Bcrp1/Mrp2/Mrp3(-/-)] knockout mice after intravenous (50 mg/kg) or oral (50 mg/kg) administration. Compared with wild-type, the plasma areas under the curve (AUC) for MTX after i.v. administration were 1.6-fold and 2.0-fold higher in Bcrp1(-/-) and Mrp2(-/-) mice, respectively, and 3.3-fold increased in Bcrp1/Mrp2(-/-) mice. The biliary excretion

of MTX was 23-fold reduced in Bcrp1/Mrp2(-/-) mice. Plasma levels of 7OH-MTX were not significantly altered in Bcrp1(-/-) mice, but the areas under the curve were 6.2-fold and even 12.4-fold increased in Mrp2(-/-) and Bcrp1/Mrp2(-/-) mice, respectively, indicating that, at least in this mouse model, Mrp2 is more able than Bcrp1 to compensate for the loss of the other transporter.[205] In triple knockout (Bcrp1/Mrp2/Mrp3) mice very high plasma concentrations and dramatic liver accumulation of MTX and 7OH-MTX were observed: 1 hour after administration 67% of the MTX dose was retained in livers of triple knockout mice as MTX or 7OH-MTX versus 7% in wild-type mice.[206] After oral administration MTX plasma AUCs in Bcrp1(-/-) and Bcrp1/Mrp2(-/-) mice were 1.7- and 3.0-fold higher than in wild-type mice, respectively, whereas in Bcrp1/Mrp2/Mrp3(-/-) mice MTX AUC was not different from wild-type mice, indicating that Mrp3 is necessary to obtain increased MTX plasma concentrations in the absence of Mrp2 and/or Bcrp1. MTX and 7OH-MTX in liver and kidney were significantly increased in Mrp2(-/-) and/or Bcrp1(-/-) mice too.[207-208] These findings suggest that BCRP and MRP2 are able to affect the oral bioavailability and tissue distribution of methotrexate. Therefore, impaired BCRP and/or MRP2 expression/activity due to co-administration of specific inhibitors, or due to non-functional SNPs, could result in increased oral bioavailability and drug-related toxicity in patients treated with oral MTX.
Recent findings also indicate that expression of BCRP at the blood brain barrier, together with other transporters (Pgp, MRP2, MRP4) play a crucial role in modulating brain penetration of substrate drugs. In vitro preclinical studies suggested that Bcrp1 reduced brain penetration of mitoxantrone and prazosin.[209] More recently, the use of Bcrp1(-/-), Mdr1a/1b(-/-) and Bcrp1/Mdr1a/1b(-/-) mice models has clarified that BCRP, together with Pgp, was able to significantly reduce the brain accumulation of the anticancer drug topotecan, various camptothecins, and of multiple tyrosine kinase inhibitors (TKIs) like sorafenib, sunitinib, imatinib, the EGFR TKIs gefitinib and erlotinib, and the selective BRAF V600 inhibitors vemurafenib and dabrafenib.[17,18,135-138,145,175,204] As in these animal models the use of Pgp/BCRP (e.g. elacridar) or of selective BCRP (e.g., Ko143) inhibitors was able to increase the brain penetration of BCRP substrates in wild-type mice to the levels observed in knockout mice, the use of a BCRP/Pgp inhibitor has been proposed as a useful strategy to improve brain penetration and therefore antitumor activity in the treatment or prevention of brain metastases in cancer patients.[144] In effect in mice co-administration of topotecan and gefitinib (a BCRP substrate and BCRP/Pgp inhibitor *in vitro*) has been reported to improve the accumulation of topotecan in brain and in glioma tumor tissue, potentially enhancing its antitumor activity.[145] Similarly, concomitant administration of elacridar with the selective BRAF V600 vemurafenib, currently used in the treatment of advanced V600 mutated melanoma, was reported to significantly improve the oral bioavailability and brain accumulation in wild-type mice, to levels observed in Bcrp1 and Bcrp1/Mdr1a/1b knockout mice.[136] Currently, several BCRP substrates (e.g., [(11)c]-topotecan, [(11)c]-sorafenib, etc) and BCRP/Pgp inhibitors (1-[(18F)]-fluoroelacridar) have been labeled in order to create positron emission tomography radiotracers to visualize Pgp/BCRP distribution in patients and to study the penetration of BCRP/Pgp substrates in brain and tumor tissue, potentially allowing better tailored drug therapy.[36,210-212]
Due to the potential impact on BCRP expression and function with relevant clinical implications, an increasing number of *in vitro* and *in vivo* studies have investigated the impact of genetic polymorphisms in ABCG2 on drug response and toxicity. Numerous genetic variants have been identified and characterized, several of which are associated

with impaired BCRP activity. The ABCG2 421C>A genotype leading to a non-functional BCRP variant has been reported to affect the pharmacokinetics and toxicity of topotecan, diflomotecan, 9-aminocamptothecin, gefitinib, erlotinib, rosuvastatin, simvastatin, sunitinib. Contrasting results have been published about the effect of the same ABCG2 genetic variant on the tissue distribution and efficacy of sulfasalazine, irinotecan and its active metabolite SN-38.[208,213-215]
The role of BCRP expression and its variability in cancer treatment efficacy and prognosis has been widely studied. In general, expression of BCRP in cancer cells is expected to be linked with poor prognosis, especially in patients with acute myeloid leukemia, but to date this association has not been demonstrated for all tumor types.[208]
Moreover, as expression of BCRP has been observed at the placenta where it plays a key role in transplacental pharmacokinetics, fetal protection and detoxification, a better understanding of genotype-phenotype relationships may allow personalized therapy in order to minimize fetal exposure to teratogens, or to maximize pharmacological therapy of the fetus.

Finally, as expression of BCRP is reported in lactating mammary glands where it contributes to excretion of riboflavin (vitamin B12) and xenobiotics in the milk, avoidance of BCRP substrate drugs/toxins should be advised in nursing women. Indeed, in milk of Bcrp1(-/-) mice treated with cimetidine, topotecan, nitrofurantoin or PhIP, the concentration of such compounds were significantly lower compared with wild-type mice.[208,216,217]

Drug-Drug Interactions
Clinically important drug-drug interactions have been described between the antifolate drug methotrexate (MTX), and the benzimidazoles omeprazole and pantoprazole, resulting in long-lasting extensive myelosuppression associated with systemic infections and severe mucositis.[218,219] In a clinical study, patients receiving benzimidazoles and high-dose MTX therapy displayed reduced MTX clearance and increased MTX plasma levels.[95] MTX and its active di- and triglutamylated forms are BCRP substrates[166-168], and in *in vitro* studies the proton pump inhibitors omeprazole and pantoprazole have been demonstrated to be BCRP substrates and effective BCRP and Pgp inhibitors.[13,14] In in vivo experiments, the plasma AUC of MTX after intravenous administration was increased 1.8-fold, and its clearance was reduced about 2-fold by co-administration of pantoprazole in wild-type mice, reaching similar levels as in Bcrp1 knockout mice, while plasma concentrations and clearance of MTX were not affected by pantoprazole in Bcrp1 knockout mice.[14] Although several transporters are involved in active transport of MTX (MRP1-4, RFC1, OAT1, OAT3, OAT4), these findings indicate that inhibition of BCRP may explain the clinical interaction observed between MTX and benzimidazoles. As a consequence, in clinical practice administration of proton pomp inhibitors should be avoided in patients treated with high dose MTX.
As several orally administered tyrosine kinase inhibitors employed for cancer treatment (e.g, sorafenib, sunitinib, imatinib, gefitinib, erlotinib, vemurafenib and dabrafenib) have been reported to be substrates of BCRP, co-administration of such drugs with BCRP inhibitors could result in increased oral bioavailability (due to increased intestinal uptake), tumor and brain penetration. This could result in increased antitumor efficacy by reversing tumor resistance mediated by BCRP, and open the possibility to treat/prevent primary or

metastatic brain tumors by increasing brain penetration of the drugs. Drug-related toxicity could also increase, due to enhanced plasma concentrations. Several preclinical and clinical findings support the above mentioned approach, but more studies are needed in order to evaluate the feasibility of such strategies in humans. For instance, in mice, gefitinib (a BCRP substrate and a Pgp/BCRP inhibitor) improved oral bioavailability of irinotecan (a BCRP substrate) in mice, and the concomitant administration of both compounds resulted in a greater than additive antitumor activity in tumor models, independent of tumor Erb-B1 status.[200] Similarly, co-administration of gefitinib significantly improved the oral bioavailability of topotecan and its penetration in brain and glioma tumor tissue expressing BCRP.[145] Furthermore, mice transplanted with BCRP-transfected lymphocytic leukemia cells treated with irinotecan and gefitinib in combination displayed significantly longer survival as compared to mice treated with one of the two drugs alone.[201]

Clinically relevant drug-drug interactions have been described between the HMG-CoA reductase inhibitor rosuvastatin and the HIV protease inhibitors ritonavir-boosted atazanavir or ritonavir-boosted lopinavir. In humans, rosuvastatin plasma AUC and maximum plasma concentrations were significantly increased (213% and 600%, respectively) when atazanavir was co-administered. As rosuvastatin is a substrate of BCRP (and OATPs) and several HIV protease inhibitors (e.g., ritonavir, saquinavir, nelfinavir, lopinavir, atazanavir) have recently been reported to be able to inhibit BCRP and OATP in vitro, a contribution of BCRP to this clinically relevant interaction is considered very likely.[220] Similarly, interactions between other clinically used BCRP substrates and HIV protease inhibitors can be predicted.

An important drug-drug interaction has been described between the anticancer drug irinotecan and the immunosuppressive tacrolimus in a patient with hepatocellular carcinoma after liver transplantation, resulting in increased plasma concentrations of SN-38 and severe diarrhea.[221] The mechanism of this interaction remains unknown, but as tacrolimus has recently been reported to inhibit BCRP efficiently in vitro[203], the inhibition of BCRP may explain, at least in part this clinically relevant feature.

Potentially clinically relevant BCRP-mediated drug-drug interactions can also be predicted between flavonoids or flavonoid-containing food or herbal products and BCRP-substrate drugs. Due to their claimed protective effect on cancer, cardiovascular diseases, and osteoporosis, flavonoid-containing dietary supplements and herbal products are widely used. Preclinical studies show that flavonoids are able to inhibit BCRP, Pgp, OATPs and CYP3A4 metabolizing enzymes at relatively low concentrations, which can be achieved in the gut after the ingestion of flavonoid-containing foods or dietary supplements.[181,222-226] 5,7-Dimethoxyflavone and other multiple flavonoids, described as able to inhibit BCRP in vitro, increased the plasma concentration and tissue distribution of mitoxantrone, a well-known BCRP substrate, in mice.[227,228] In another study, plasma concentrations of nitrofurantoine, a BCRP substrate, were 1.7-fold higher in wild-type mice after administration of the soy isoflavones genistein and daidzein. In particular, genistein and daidzein were reported to inhibit biliary and mammary excretion of nitrofurantoine, as demonstrated by the decreased milk/plasma and biliary/plasma ratio of the drug observed in mice treated with the isoflavones.[229]

In view of the wide substrate specificity and the pharmacological functions of BCRP, several drug-drug interactions can be speculated too. As various commercially available estrogen antagonists and agonists (e.g., diethylstilbestrol, tamoxifen, and tamoxifen derivatives) as

well as glucocorticoid drugs (e.g., prednisone, dexamethasone) have been reported to inhibit BCRP efficiently *in vitro*, drug-drug interactions can be predicted in patients taking these compounds and other BCRP-substrate drugs, especially when they display a narrow therapeutic window.[230,231] However, whether the modulation of BCRP activity by these and other clinically relevant drugs is clinically important is still an open question.

2.3 ABCC Family (Multidrug Resistance-Associated Proteins, MRP1 to MRP9)

The ABCC family includes 13 genes encoding the multidrug resistance proteins 1-9 (ABCC1-6, 10-12), the cystic fibrosis transmembrane conductance regulator (CFTR or ABCC7), two cell surface receptors that modulate insulin secretion (SUR1 and SUR2, ABCC8 and ABCC9 respectively), and a putative non-functional protein (ABCC13) [for review see Haimeur et al.].[232]

The multidrug resistance-associated protein (MRP) phenotype was described in 1987 as a different form of multidrug resistance in doxorubicin selected resistant human tumor cell lines and referred to as non-P-glycoprotein–mediated MDR.[233] A few years later the gene of the MRP1 transporter (ABCC1) was cloned from a multidrug-resistant human lung cancer cell line.[234] Subsequently, the other members of the subfamily were discovered, but to date some of them have not been fully characterized yet.

Structurally, the multidrug resistance associated proteins (MRP1-9) are fully ATP-drug efflux transporters and present two different structures: MRP4-5 and MRP8-9 proteins consist of two transmembrane domains, each containing 6 transmembrane segments, and two nucleotide binding domains. MRP1-3, MRP6 and MRP7 have the same basic structure as MRP4-5, however with an additional NH2-terminal extension consisting of 5 putative transmembrane segments.[235-239]

In general, MRPs are organic anion transporters with a broad substrate specificity that to a certain extent overlaps. MRPs can be distinguished also on their size and predicted membrane topology, cellular orientation (apical versus basolateral membrane) and tissue distribution.

Currently, the molecular properties, substrate specificity and physiological functions of MRP5 -9 have not been fully characterized yet. Therefore, their role in drug disposition and drug-drug interactions is unclear. In view of the lack of data, in the present chapter only MRP1-4 will be discussed and only drug-drug interactions mediated by MRP2 will be presented.

MRP1 (ABCC1)

MRP1 (ABCC1) is expressed in the basolateral membrane of polarized epithelial cells in most human tissues, with relatively higher levels found in lung, testis (Leydig and Sertoli cells), kidney, colon (with highest MRP1 levels in the Paneth cells of the crypts), peripheral blood mononuclear cells and in brain tissue (in particular in cells forming the choroid plexus).[234,240-242] The tissue distribution and the localization of MRP1 in the basolateral membrane of epithelial cells suggest for MRP1 a role in cellular defense rather than in body defense, as MRP1 secretes drugs into the body, rather than excrete them out of the body, as P-glycoprotein, BCRP and MRP2 do.[243]

Main substrate classes, inhibitors (competitive, non-competitive), inducers

- *Main substrate classes.* MRP1 is primarily an organic anion transporter. MRP1 can transport also compounds that are conjugated or complexed to glutathione (GSH), sulphate, glutamate or glucuronide.[244-252] In addition, MRP1 is able to co-transport neutral/basic drugs with reduced GSH: *in vitro*, transport of vincristine, daunorubicin and etoposide by MRP1 required reduced GSH, and GSH-depleting agents like buthionine sulfoximine (BSO) could sensitize MRP1-overexpressing cells to a number of anticancer agents.[248,253,255-259] Antimonial and arsenical oxianions are also co-transported by MRP1 with GSH or complexed to GSH.[259,260] Thus, MRP1 transports a broad range of physiological compounds, xenobiotics and drugs: LTC4 and its metabolites, D4 (LTD4) and E4 (LTE4) and other prostaglandin derivatives, the cholestatic 17ß-estradiol-glucuronide and the sulfated bile salt sulfatolithocholate.[246,248,251,261] A variety of chemical toxicants and their metabolites with potential carcinogenic activity are also transported by MRP1, including GSH conjugated stereoisomers of aflatoxin B1306, the glucuronide conjugate of some nicotine metabolites, the GSH conjugates of the herbicide metolachlor, and the toxicants 1-Cl-2,4-dinitrobenzene and 4-nitroquinoline 1-oxide.[252,262-266] Clinically relevant substrates of MRP1 are several classes of anticancer agents, such as anthracenedione (mitoxantrone), epipodophyllotoxins, vinca alkaloids, anthracyclines, camptothecins (topotecan, irinotecan and the unconjugated and conjugated forms of its active metabolite SN-38) and methotrexate, conjugates of alkylating agents (thiotepa, cyclophosphamide, chlorambucil and melphalan), and the antiandrogen flutamide.[244,245,267-272] Recently, the HIV protease inhibitors ritonavir and saquinavir were found to be transported by MRP1, as well as conjugates of the diuretic ethacrynic acid.[270-272]

- *Inhibitors (competitive, non-competitive).* Different classes of compounds with MRP-inhibiting activity have been described. Sulfinpyrazone, benzbromarone, probenecid and indomethacin are not specific MRP1 inhibitors, as they can modulate other transporters too.[273-276] Relatively specific modulators of MRP1 are MK571 (a LTD4 receptor antagonist), ONO-1078 (a peptide leukotriene receptor antagonist), glibenclamide (a sulfonylurea derivative that inhibits MRP1 as well as sulfonylurea receptor 1 (SUR1)) and several peptidomimetic GSH-conjugate analogs.[277-280] Several tricyclic isoxazole derivatives (e.g. LY475776, LY329146 and LY402913) are highly specific and potent MRP1 inhibitors.[250,281-283] Some P-gp inhibitors like VX-710 (biricodar), PSC833 (valspodar), verapamil, cyclosporin A, agosterol A, PAK-105P, S9788 as well as several bioflavonoids (e.g. genistein, quercetin), nonsteroidal anti-inflammatory drugs (NSAIDs), steroid derivatives (e.g. RU486, budesonide) and imidathiazole derivatives have shown to inhibit MRP1, but with low affinity and poor specificity.[252,284-291] Recently, MRP1-specific antisense oligonucleotides and cDNA, ribozymes and small interfering RNA molecules have been developed. For instance, ISIS 7597 (a MRP1-specific antisense oligonucleotide) has been shown to successfully downregulate MRP1 in a xenograft model of human neuroblastoma.[292]

- *Inducers.* In some cell systems MRP1 expression can be induced by pro-oxidant compounds such as quercetin, sulindac, menadione, pyrrolidinedithiocarbamate and tert-butylhydroquinone.[293-296] Several compounds that generate reactive oxygen species (e.g. TNF ***α***) and nitric oxide donors are also reported to induce MRP1 expression.[297,303]

Pharmacological and Toxicological Function, and Interactions

MRP1 is expressed in multiple tissues of the body, including lung, testis, kidney, skeletal and cardiac muscles, and macrophages. It is also localized at the apical syncytiotrophoblast membrane of the placenta and at the basolateral membrane of choroid plexus cells of the blood-cerebrospinal fluid barrier, suggesting a protective role for the body. At the cellular level, the endogenous expression of MRP1, together with Pgp, contributes to the multidrug resistance phenotype of several cancers, which lead to resistance to anticancer drugs like anthracyclines, topotecan, SN-38, and epipodophyllotoxins.[298-300] To date, MRP1 (*ABCC1*) SNPs have been described in various ethnic populations, but they are not associated with any known genetic disorder or phenotype, or with altered pharmacokinetics or toxicity of substrate drugs.

The pharmacological and toxicological functions of MRP1 have been studied by generating Mrp1(-/-) mice. Mrp1 knockout mice appeared to be healthy and fertile. However they displayed increased GSH levels, decreased response to inflammatory stimuli, and increased sensitivity to the anticancer drug etoposide, a well-known MRP1 substrate.[298,301-305] Moreover, toxicity of vincristine and etoposide was dramatically increased after intraperitoneal administration to Mrp1(-/-)/Mdr1a/1b(-/-) (triple knockout) mice compared with Mdr1a/1b(-/-) and wild-type mice.[301] In particular, the knockout mice showed increased toxicity to the bone marrow and to some epithelia containing high levels of Mrp1 (e.g., oropharyngeal mucosa, collecting tubules of the kidney, testis), as well as increased anticancer drug levels in the cerebrospinal fluid.[301-305] These findings suggest a protective role of MRP1 against drug induced toxicity, as well as a contribution of the transporter to the blood brain and blood cerebrospinal fluid barrier, in order to limit drug penetration and accumulation in the brain. In another preclinical study administration of saquinavir (a Pgp and MRP1 substrate) to mice in combination with the selective MRP inhibitor MK571 resulted in a more than 4-fold increased brain accumulation of saquinavir, indicating that MRP1, rather than Pgp, is the dominant transporter responsible for limiting the CNS penetration of saquinavir.[306] Taken together, these findings suggest a critical role of MRP1 in mediating drug disposition (in particular brain penetration) and toxicity, support a role of MRP1 in drug-drug interactions and as target of modulating compounds in order to improve the brain uptake of specific substrate drugs.

MRP2 (cMOAT, ABCC2)

MRP2 (cMOAT, ABCC2) MRP2 is expressed at the apical membrane of hepatocytes and of other polarized cells, e.g., in the epithelium of the small intestine, in the kidney tubular cells, gallbladder, bronchi, placenta and brain. The extensive overlap between the tissue distribution of MRP2 and P-gp together with the cellular apical location, suggests that these two transporters share physiological, pharmacological and toxicological functions, albeit with different (but partially overlapping) substrate specificities. The exclusive apical localization (different from selected other MRP transporters) underlines the important function of MRP2 in excretion and detoxification of endogenous compounds and xenobiotics.[307-312] MRP2 is involved in the hepatobiliary excretion of clinically important anionic drugs and intracellulary formed glucuronide and glutathione conjugates of many drugs and endogenous compounds, thus playing a key role in the phase III xenobiotic and endogenous detoxification system, i.e., the biliary excretion of conjugated metabolites produced by phase I and II hepatic enzymes.[313-321] MRP2 is considered the major

transporter of bilirubin glucuronides into the bile.[315,317] MRP2 probably plays a central role in the disposition and hepatobiliary elimination of substrate drugs.
Several mutations of the MRP2 (cMOAT, ABCC2) encoding gene have been described in humans and animals. In humans mutations in the *MRP2* gene resulting in the absence or functional inactivity of MRP2 in the bile canalicular membrane of hepatocytes have been linked with the Dubin-Johnson syndrome, an autosomal recessive inheritable disorder characterized by conjugated hyperbilirubinemia and increased urinary coproporphyria I fraction.[313,314,318-320] Although the hepatic function of subjects with Dubin-Johnson syndrome is reported to be normal (probably due to compensation by other transporters), it has been suggested that these individuals are at increased risk of drug-induced toxicity.[313,320]

Main substrate classes, inhibitors (competitive, non-competitive), inducers
-*Main substrate classes*. There are many similarities between compounds transported by MRP2 and MRP1, although there is not a complete overlap. Substrates of MRP2 include also many amphipathic anion drugs and endogenous compounds and GSH, glucuronide and sulfate conjugates. MRP2 is the principal transporter of bilirubin mono- and bis-glucuronides into the bile.[317,322] Other MRP2 endogenous substrates include LTC4, taurolithocholate sulfate, reduced and oxidized GSH or GSSG, LTD4, LTE4, estradiol-17β-glucuronide, L-thyronine and glucuronide conjugates of drugs like diclofenac and acetaminophen.[313,323,324,331] Anticancer drugs transported by MRP2 include anthracyclines (doxorubicin and epirubicin), camptothecin derivatives (irinotecan/SN-38), vinca alkaloids (vinblastin and vincristine), mitoxantrone, cisplatin, taxanes (paclitaxel, docetaxel), methotrexate, trabectedin and most likely also etoposide.[244,252,325-330] Other clinically important drugs transported by MRP2 are pravastatin, temocaprilat, ampicillin, ceftriaxone, grepafloxacin and its glucuronide conjugate, BQ-123, sulfinpyrazone, the HIV protease inhibitors saquinavir, ritonavir and indinavir, para-aminohippurate and possibly, arsenic trioxide.[252,331-335] Furthermore, MRP2 has been shown to mediate the transport of some carcinogens like the glucuronide conjugate of the nicotine metabolite NNAL307, the fungal toxin ochratoxin A, arsenite, cadmium and α-naphthylisothiocyanate.[334-338]
- *Inhibitors and Inducers*. Inhibitors of MRP2 have been described, but they are not highly specific. MK571 inhibits MRP2 but also MRP1 and MRP3 and cyclosporin A, PSC833, PAK-104P, sulfinpyrazone, benzbromarone, probenecid are reported to block MRP2 and Pgp. Interestingly, certain MRP2 modulators can inhibit MRP2-mediated transport of a number of substrates: for instance, probenecid inhibits methotrexate transport by MRP2 but stimulate transport of others compounds such as taxanes, etoposide, vinblastin and HPIs.[244,329,330,339,340]
Regulation of MRP2 activity can take place also at the cellular level: the dynamic endocytic retrieval and exocytic insertion of MRP2 between the canalicular membrane and an intracellular pool of vesicles are involved. Treatment of rats with lipopolysaccharide (which induces cholestasis) induces endocytic retrieval of MRP2.[341-343] *In vitro*, various MRP2 inducing agents have been found, including dexamethasone, rifampicin, tamoxifen, cisplatin, cycloheximide, phenobarbital, 2-acetaminofluorene, cholic acid, chenodeoxycholic acid, clotrimazole, pregnenolone, sodium arsenite, oltipraz.[313,343-353] Induction of liver Mrp2 expression has been described also in Wistar rats treated with St. John's Wort.[352]

Pharmacological and Toxicological Function

The cellular localization and tissue distribution of MRP2 (cMOAT, ABCC2), suggests a key role in mediating drug disposition and toxicity. The pharmacological and toxicological functions of MRP2 have been studied extensively using rat strains defective for Mrp2 (Groninger Yellow transporter [GY/TR$^-$] and Eisai hyperbilirubinemic [EHBR] rat) and cells from humans affected by the Dubin-Johnson syndrome.[313,314,354-356] In these models the pharmacokinetics of MRP2 substrate compounds are altered significantly, suggesting that MRP2 can affect the pharmacological behavior of these substrate drugs. Moreover, In Mrp2-deficient rats, the biliary excretion of cefodizime, ceftriaxone, and grepafloxacin, antimicrobials used clinically in the treatment of infections of the biliary tract, as well as of the HMG-CoA reductase inhibitor pravastatin was markedly reduced, indicating that efficient excretion of these drugs into the bile is probably mediated by MRP2.[332,357-361] MRP2, together with BCRP and MRP3 appears to significantly affect the pharmacokinetics of methotrexate and of its toxic metabolite 7-hydroxymethotrexate in vivo, as documented by experiments performed in Mrp2, Bcrp1, Mrp3 single and multiple knockout mice.[206,207] MRP2-mediated biliary excretion of drugs as part of multiple elimination pathways (such as biliary and urinary) has been proposed to confer pharmacokinetic advantage to substrate drugs (e.g., temocapril), thus avoiding potentially toxic effects, for example in patients with renal failure.[362-364]

Biliary excretion of drugs can result in drug-induced toxicity. Biliary excretion of methotrexate, a substrate of MRP2 and BCRP, has been proposed to be responsible for its intestinal toxicity,[14,365] whereas MRP2-mediated biliary secretion of the glucuronide derivative of diclofenac and other non-steroidal anti-inflammatory drugs (NSAIDs) is supposed to cause hepatic and intestinal toxicity.[324] MRP2 deficient rats (TR$^-$, EHBR) displayed lower excretion of indomethacin glucuronide (50%) and diclofenac glucuronide (98%) into bile and less intestinal ulceration compared with wild-type mice. Transfer of diclofenac glucuronide-containing bile into the intestine of TR$^-$ rats significantly increased intestinal damage.[213] Similarly, MRP2, together with BCRP and Pgp, may contribute to the gastrointestinal excretion of the anticancer drug irinotecan and its metabolites SN-38, and SN-38 glucuronide, which has been linked to the development of severe gastrointestinal diarrhea, a dose-limiting side effect.[266,366-374] The use of MRP2 inhibitors has been explored as a strategy to prevent this important toxicity. In rats, co-administration of the MRP2 inhibitor probenecid reduced SN-38 biliary excretion and decreased irinotecan-induced late-onset diarrhea.[374] Similarly, concomitant administration of irinotecan with cyclosporin A (a Pgp, BCRP, OATP and MRP2 inhibitor) resulted in significantly reduced excretion of irinotecan and SN-38 via the biliary and/or the intestinal route in rats.[202,375-377] In clinical studies conducted in cancer patients administration of intravenous irinotecan and oral cyclosporin A resulted in increased plasma AUC and reduced clearance of irinotecan, SN-38, and SN-38 glucuronide; frequency and severity of diarrhea were significantly reduced with preservation of antitumor activity.[361,378,379]

Intestinal MRP2 may limit the oral bioavailability of substrate compounds. In preclinical studies, absorption after oral administration and tissue distribution of the food-derived carcinogen PhIP, a proven MRP2 substrate, were approximately 2-fold higher in TR$^-$ than in Wistar rats.[335,338] Similarly, increased bioavailability of the HIV protease inhibitor saquinavir, a Pgp and MRP2 substrate, was observed in TR$^-$ compared with Wistar rats.[333]

In addition, recent research demonstrated that MRP2 modulates the brain penetration of

clinically important substrate drugs. In effect, it has been hypothesized that overexpression of Pgp and MRP2 may be involved in drug resistance in epileptic patients.[380] In TR$^-$ rats, brain extracellular levels and the anticonvulsant effect of the antiepileptic drug phenytoin were significantly increased compared with control rats. In another study increased anticonvulsant response to carbamazepine in the kindling model of temporal lobe epilepsy, but no difference in brain drug levels, were reported in TR$^-$ rats.[381-383] A significant increase of drug penetration into the brain by probenecid was reported previously for valproate[384] and carbamazepine[385] and has been attributed to inhibition of MRP2 at the blood-brain barrier. Therefore, the development of active and more specific MRPs and/or Pgp inhibitors might overcome drug resistance to antiepileptic drugs.[380]
Finally, genetic polymorphisms in *ABCC2* have been associated with altered pharmacokinetics and toxicity of several MRP2 substrates drugs, such as irinotecan and its active metabolite SN38, methotrexate, diclofenac, pitavastatin, and tenofovir-mediated nephrotoxicity.[213,214]

Drug-Drug interactions
Clinically relevant drug-drug interactions mediated by MRP2 have been described. Moreover, due to the broad substrate specificity, the pharmacological role and the wide range of clinically used drugs that can modulate MRP2 activity, several clinically relevant drug-drug interactions mediated by MRP2 could be anticipated.
A drug-drug interaction has been reported between phenobarbital and acetaminophen (i.e. paracetamol), resulting in reduced biliary excretion of acetaminophen-glucuronide in rats[386], possibly due to inhibition of Mrp2-mediated transport of acetaminophen-glucuronide by a metabolite of phenobarbital and/or by induction of hepatic MRP3 at the basolateral membrane of hepatocytes by phenobarbital.[387]
Another clinically relevant interaction has been described between ritonavir-boosted saquinavir (a MRP substrate) and probenecid, resulting in significantly decreased saquinavir plasma concentrations, potentially exposing HIV patients to suboptimal therapy and risk of treatment failure due to development of resistance.[379]
Clinically relevant drug-drug interactions have been reported between rifampicin and morphine, mycophenolate mofetil, ezetimibe, carvedilol or propafenone, resulting in reduced plasma concentrations and loss of therapeutic activity of the co-administered drug. Rifampicin-mediated induction of Pgp, drug metabolizing enzymes and MRP2 appear to be the mechanisms behind these clinically relevant interactions.[112,353,388] Similar interactions can be predicted by concomitant administration of MRP2 substrate drugs and compounds or herbal medicines that have been demonstrated to induce MRP2 in humans, rodents and primates (such as dexamethason, tamoxifen, St John's Wort). On the other hand, the use of such compounds able to induce intestinal and/or liver expression/activity of MRP2 could be a potentially useful strategy to stimulate the secretion of toxic metabolites, for instance to improve elimination of saquinavir in case of accidental overdose. However, careful interaction studies in humans are needed in order to evaluate the feasibility of such strategy.
Another drug-drug interaction has been documented by co-administration of the two antiretroviral drugs tenofovir and didanosine, leading to an increase in the plasma AUC of didanosine by 44 to 60%, due to tenofovir-induced inhibition of OAT1-mediated uptake of didanosine into the proximal tubule cells, and/or to inhibition of purine nucleoside

phosphorylase and/or to inhibition of MRP2-mediated efflux of didanosine.[112]
Finally, as MRP2 expression has been observed in several cancer cells where it contributes to the multidrug resistance phenotype, administration of a MRP2 inhibitor could be a useful strategy to overcome drug resistance. On the other hand, co-administration of a MRP2 inducer could be unwanted as it is expected to stimulate MRP2-mediated drug resistance and to reduce the oral bioavailability and consequently therapeutic efficacy of orally administered anticancer drugs that are substrates of MRP2.

MRP3 (ABCC3)

Human MRP3 (ABCC3) is localized in the basolateral membrane of polarized cells in the liver, gut, adrenal gland, and to a lower extent in the pancreas, kidney, prostate and placenta.[235,389-392] In normal liver MRP3 is found at low levels in the basolateral membrane of cholangiocytes and hepatocytes, however substantial variation between individuals in tissue expression has been observed.[391,393,394] MRP3 has been reported to be overexpressed in liver tissue of patients with Dubin-Johnson syndrome, in rats deficient for Mrp2 (TR-/EHBR) and in a cholestatic state.[391-397] These findings together with in vitro results showing that MRP3 transports a range of bile salts (glycocholate, taurocholate, and sulfo-conjugated bile acids) and bilirubin glucuronide suggest for MRP3 a role in the cholehepatic and enterohepatic circulation of bile salts and liver protection in cholestatic state.[396,396-402]

<u>*Main substrate classes, inhibitors (competitive, non-competitive), inducers*</u>

-Main substrate classes. MRP3 is an organic anion transporter with substrate specificity in part overlapping with MRP1 and MRP2. MRP3 transports several anticancer drugs such as the epipodophyllotoxin derivatives etoposide and teniposide, methotrexate and possibly vincristine, most likely in a non-GSH-dependent manner.[404] Substrates of MRP3 include E2βG, a variety of bile salts, LTC4, GS-DNP (but not GSH) and several glucuronide and sulfate conjugates of steroids, prostaglandins (in particular the glutathione conjugate of 15-d-PGJ2, an important product of PGD2 metabolism), bile acids and commonly used drugs (e.g. acetaminophen and morphine).[324,403-411]

- *Inhibitors (competitive, non-competitive)*. Several compounds like indomethacin, probenecid, benzbromarone and sulfinpyrazone have been identified as potential MRP3 inhibitors.[404]

- *Inducers*. Several groups have reported that liver MRP3 expression can be induced in response to a number of toxins, such as β-naphtoflavone and 2-acetylaminofluorene, oltipraz, diallyl sulfide, trans-stilbene oxide and drugs like phenobarbital, acetaminophen, the proton pump inhibitor omeprazole and several non-steroidal anti-inflammatory drugs.[296,324,412-417] Certain bile salts (e.g. chenodeoxycholic acid) can also act as inducers of MRP3 expression.[417] In humans, increased MRP3 expression has been detected in several hereditary and acquired liver diseases including Dubin-Johnson syndrome, primary biliary cirrhosis, type 3 progressive familial intrahepatic cholestasis and obstructive cholestasis.[397,417-422]

<u>*Pharmacological and Toxicological Function and Drug-Drug Interactions*</u>

ABCC3 is expressed at the basolateral membrane of hepatocytes and enterocytes, and it generally transports its substrates into the blood circulation, in contrast to MRP2 and BCRP that are localized at the apical membranes of cells transporting their substrates into

bile, feces and urine.[207] MRP3, MRP2 and BCRP have broad and substantially overlapping substrate specificities, but their affinity for substrates is considerably different. Elevated MRP3 expression has been reported in cholestatic human and rat liver, and in patients with Dubin-Johnson syndrome, characterized by lack of functional MRP2 in the liver canalicular membranes. This would indicate a compensatory activity of MRP3, allowing efflux of organic anions from the liver back into the blood compartment upon blockade of bile secretion due to cholestatic conditions. The role of MRP3 in drug disposition has been investigated in Mrp3(-/-) mice. Moreover, due to the overlapping substrate specificities with other transporters (BCRP, MRP2, Pgp), the interplay between transporters and their effect on the pharmacokinetics of substrate drugs has been studied by generation of multiple knockout models (Mrp2/Mrp3(-/-), Mrp2/Mrp3/Bcrp1(-/-), Mrp3/Bcrp1(-/-), Mrp2/Bcrp1(-/-)). In Mrp3 knockout mice, administration of the commonly used analgesic and antipyretic acetaminophen resulted in dramatically increased acetaminophen-glucuronide levels in the liver and bile and in a reduction of plasma acetaminophen-glucuronide levels compared with Mrp3(+/+) mice, suggesting that in the absence of Mrp3, acetaminophen- glucuronide accumulates in the liver and redirect a fraction of its excretion through a low-affinity canalicular transport involving Mrp2. This hypothesis is supported by previous preclinical studies in Mrp2 (TR-)-deficient rats, where liver Mrp3 up-regulation was observed, and where administration of acetaminophen resulted in a reduction in the biliary elimination of acetaminophen- glucuronide without retention of this metabolite in hepatocytes, and increased secretion of acetaminophen- glucuronide into the urine.[422,426] These findings suggest also a role of Mrp3 in modulating the (hepato) toxicity of substrate drugs by minimizing liver exposure, thus reducing acetaminophen activation to its toxic reactive metabolite. In effect it has been demonstrated in preclinical models that increased urinary excretion of the acetaminophen- glucuronide metabolite (due to a shift from canalicular to basolateral efflux of acetaminophen- glucuronide and a decrease in its enterohepatic recirculation) is obtained by significant Mrp3 induction, achieved through repeated acetaminophen dosages or by administration of Mrp3 inducers (e.g., phenobarbital).[322,413,416,424-425] This mechanism could explain, at least partly, clinical cases of patients who took high-doses of acetaminophen without developing liver damage.[423] Another mechanism could be related to acetaminophen-mediated induction of Mrp2, as in rats and mice, treatment with an acute toxic dose of acetaminophen increased the hepatic expression of Mrp2 and increased the biliary excretion of its model substrates dinitrophenyl-S-GSH and GSSG. In any case, induction of Mrp3 expression/activity by administration of Mrp3 inducers could be a useful strategy to reduce hepatotoxicity of clinically applied Mrp3 substrate drugs.

The role of Mrp3 in affecting drug pharmacokinetics and pharmacodynamics and the potential implication for drug-drug interactions have also recently been studied by evaluating the disposition and pharmacological effect of other clinically relevant Mrp3 substrate drugs in Mrp3 knockout models. Genetic deletion of Mrp3 in mice resulted in pharmacokinetic alterations and reduction in anti-nociceptive potency of injected morphine and morphine glucuronide, suggesting that MRP3 at the basolateral membrane of hepatocytes is responsible of transport of morphine glucuronide conjugates into the systemic circulation.[213,408-410] Therefore genetic variants resulting in impaired Mrp3 functional activity and/or co-administration of morphine with MRP3 inhibitors are predicted to reduce the analgesic effect of morphine.

Administration of the anticancer drug etoposide to Mrp3/Mrp2(-/-) mice resulted in significant accumulation of etoposide-glucuronide in the liver, whereas no accumulation was observed when etoposide was administered to single Mrp2(-/-) or Mrp3(-/-) mice, indicating alternative pathways provided by Mrp2 and Mrp3 for the hepatic elimination of etoposide glucuronide.[427,428]
Recently, oral administration of low dose methotrexate (1 mg/kg) in Mrp3(-/-) mice resulted in 3.4-fold lower plasma AUC compared with wild type mice, partly due to reduced basolateral efflux of methotrexate from the liver (and hence increased biliary clearance) and partly due to reduced intestinal uptake.[207,429] However, subsequent experiments conducted with a higher dose of methotrexate administered orally (50 mg/kg) in Mrp3(-/-) mice showed only a small effect on the pharmacokinetics of methotrexate, probably due to saturation of intestinal uptake at higher drug dose-levels.[207] Finally, after intravenous administration (50 mg/kg), plasma and liver concentrations of methotrexate were not significantly different between Mrp3(-/-) and wild-type mice, whereas when Mrp2 and/or Bcrp1 were additionally deleted a clear effect of Mrp3 expression was found, resulting in increased methotrexate plasma concentrations and toxicity (see section about BCRP).[207]

MRP4 (ABCC4)
The subcellular localization of MRP4 in humans is cell type dependent. In hepatocytes, choroid plexus epithelium, and prostate tubuloacinar cells MRP4 is localized on the basolateral membrane, whereas in renal proximal tubule cells and brain capillary endothelium, ABCC4 resides at the apical membrane. This suggests a contribution of MRP4 to the maintenance of body homeostasis by protecting crucial tissues against xenobiotics and by facilitating renal and biliary excretion of substrate compounds. Preclinical experiments conducted in Mrp4(-/-) and wild-type mice after common bile duct ligation suggest an essential protective role of Mrp4 in the adaptive response to obstructive cholestatic liver injury.[430]

Main substrate classes, inhibitors (competitive, non-competitive)
-*Main substrate classes.* MRP4 substrates include cyclic nucleotides and nucleotide analogs widely used in antiviral and anticancer therapy (e.g. 9-2-phosphonylmethoxyethyladenine (PMEA), azydothymidine (AZT) lamivudine, 2',3'-dideoxy-3'-thiacytidine, ganciclovir, 6-mercaptopurine, thioguanine, cladribine)[431-436], cyclic GMP and AMP, methotrexate, leucovorin[432,437], cyclophosphamide, several camptothecins (topotecan, irinotecan, its active metabolite SN-38, gimatecan and rubitecan)[438-440], GSH and folate.[437,438,441-443]
-*Inhibitors*. Several drugs have been reported to efficiently inhibit MRP4 activity in vitro, but their specificity and clinical applicability are still unclear. They include buthionine-sulfoximine (BSO), MK571, celecoxib and diclofenac as well as dipyridamole, dilazep, nitrobenzyl, mercaptopurine ribozide, probenecid and sulfinpyrazone.[435,438]

Pharmacological and Toxicological Function
The pharmacological role of MRP4 is not yet completely known. However, due to the broad tissue distribution of MRP4, it is likely that its modulation by co-administered drugs (inhibitors or inducers) may have important pharmacokinetic implications for substrate drugs. Mrp4(-/-) mice have recently been employed to explore the potential role of MRP4 on drug disposition. Intravenous administration of the anticancer drug topotecan (besides

a BCRP and Pgp also a MRP4 substrate) to Mrp4 knockout mice resulted in enhanced drug accumulation in brain tissue and cerebrospinal fluid compared with wild-type mice.[444] In a recent study, MRP4, together with BCRP and Pgp, appears to significantly reduce the brain accumulation of several camptothecin analogues.[445] The polarized expression of Mrp4 in the choroid plexus and brain capillary endothelial cells indicates that Mrp4 has a dual role in protecting the brain from xenobiotics and suggests that modulation of Mrp4 activity by development of specific inhibitors could be a useful strategy to increase the therapeutic efficacy of central nervous system-directed drugs that are Mrp4 substrates. Of interest, upregulation of MRP4 has been reported in patients with neuroblastoma and MRP4 expression levels were associated with poor prognosis.[440] In other studies conducted in single Mrp3 and Mrp4 and double Mrp3/Mrp4 knockout mice, MRP3, together with MRP4, appears to influence the oral bioavailability of the cephalosporin cefadroxil, and to be involved in the tubular secretion of ceftizoxime and cefazolin, in concert with basolateral uptake transporters.[446,447] A potentially beneficial drug-drug interaction between the anticancer drug irinotecan and the analgesic celecoxib mediated, at least in part, by MRP4, and resulting in enhanced therapeutic efficacy and reduced gastrointestinal toxicity of irinotecan, has been documented in preclinical models. However, clinical studies conducted in cancer patients in order to explore this approach display inconclusive results.[448]

MRP5-9 (ABCC5-9)

The physiological and pharmacological roles of other members of the MRP family (**MRP5-MRP9**, ABCC5-9) have not been (fully) characterized yet. Therefore, their possible involvement in drug disposition and in drug-drug interactions is unclear and every speculation appears premature at this stage.

3 - INTERACTIONS MEDIATED BY ORGANIC ANION AND CATION TRANSPORTERS (SOLUTE CARRIER FAMILY, SLC22)

3.1 Organic Anion Transporters (OATs)

The organic anion transporters (OATs) represent a group of transporters belonging to the Solute Carrier 22 family. Actually the SLC22 (organic ion transporter) family consist of the organic anion transporters (OATs), the organic cation transporters (OCTs) and the organic cation/carnitine/ergothioneine transporters (OCTNs). The OATs subgroup comprises the organic anion transporters OAT1-5 and the human urate transporter URAT1.[449] Novel OAT isoforms identified recently are the human OAT7 and the mouse Oat5, Oat6 and Oat8.[450] The first organic anion transporter, OAT1/SLC22A6 (also named p-aminohippurate (PAH) transporter), was independently cloned in 1997 from rat, mouse, and winter flounders.[450-454] One year later the human ortholog was cloned by cloning-expression systems.[455] Subsequently the other OATs and URAT1 were identified.

OATs mediate the transmembrane transport of endogenous and exogenous organic anions. OAT1, OAT3 and URAT1 have been reported to mediate exchange with divalent organic anions, whereas OAT2, OAT4 and OAT5 have been described as facilitated transporters or as exchangers. Structurally, all OATs have been predicted to be proteins with 12 putative transmembrane domains (TMDs) with a large glycosylated extracellular loop between the

first and the second TMDs and another extracellular hydrophilic loop between the sixth and the seventh TMDs with consensus sequences for phosphorylation.[456]
In humans, OAT1, OAT3 and to a lesser extent OAT2 are located in the basolateral membrane of renal proximal tubule cells and are responsible for uptake of substrate compounds from the blood compartment into the cells, which is the first step of renal organic anion secretion. OAT4, OAT10 and URAT1 are localized to the apical membrane of renal proximal tubule cells and essentially contribute to proximal tubular urate absorption (especially URAT1), whereas OAT4 is also involved in excretion of substrate drugs into the tubular lumen. Extrarenal locations of OATs, the function of which is less characterized at this time, include the basolateral membrane of hepatocytes in the liver for OAT2 and OAT7, the blood-brain barrier for OAT3 and OAT1, the basolateral membrane of syncythiotrophoblasts in the placenta for OAT4 and the nasal epithelium for OAT6.[456,457]

Main substrate classes, Inhibitors and Inducers
-Main substrate classes and Inhibitors. Generally, OATs mediate the transport of structurally diverse organic anions with broad overlapping substrate specificities. Interspecies differences in OATs substrates have been described too.

OAT1 is an organic anion/dicarboxylate exchanger and has been reported to transport a wide range of organic anions but also uncharged molecules such as steroid hormones. Typically, OAT1 substrates are compounds with a hydrophobic domain of 4-10 Å. In addition, functional studies suggested that substrates with an increase in the negative charge and/or with electron-attracting side groups (like Cl, Br, NO2) interact with more affinity with OAT1. OAT1 substrates include the model organic anion p-aminohippurate (PAH) and endogenous anionic compounds such as the cyclic nucleotides cAMP (adenosine 3',5'-cyclic monophosphate) and cGMP (guanosine 3',5'-cyclic monophosphate), folate, α-ketoglutarate, prostaglandin E2, urate, indole acetate and indoxyl sulfate. In addition, several classes of widely used drugs are transported by OAT1. Antimicrobic agents (e.g. β-lactam antibiotics-penicillins and cephalosporines, tetracyclines and quinolone gyrase inhibitors) and diuretics (e.g. furosemide, acetazolamide, bumetanide, hydrochlorothiazide, ethacrynate, tienilate) are transported by OAT1. Moreover OAT1 substrates comprise several antiviral drugs (e.g. acyclovir, ganciclovir, lamivudine, zidovudine, stavudine, trifluridine adefovir, cidofovir, tenofovir, zalcitabine), non-steroidal anti-inflammatory drugs (NSAIDs: indomethacin, acetylsalicylate, ketoprofen, salicylate), cimetidine, methotrexate, neurotransmitter metabolites (e.g. vanilmandelic acid), heavy metal chelators (2,3-dimercaptopropane sulfonate: DMPS), test agents (fluorescein and 6-carboxyfluorescein) and toxins (in particular ochratoxin A) [for review see ref. 457-461]. In addition to the human substrates, rat OAT1 has also been shown to interact with several ACE inhibitors (captopril, enalapril, delapril, quinapril, ramipril), angiotensin II antagonists (telmisartan, candesartan, valsartan, losartan), the anti-epileptic valproate and several neurotransmitter metabolites (e.g. 5-methoxytryptophol, 5-hydroxyindole-3-acetic acid, D,L-4-hydroxyl-3-methoxymandelic acid).[458-460] Inhibitors of human OAT1 have been reported too. Probenecid, the classic inhibitor of the renal organic anion secretion system, has been shown to block OAT1 activity with high affinity but low specificity, as it also inhibited sat-1, OAT2 and OAT3. Furthermore, in *in vitro* studies PAH cellular uptake OAT1-mediated was inhibited by benzylpenicillin, cephaloridine, cephradine, doxycycline,

minocycline, oxytetracycline and tetracycline. Human OAT1 inhibitors include also some antiviral drugs (e.g. acyclovir, adefovir, cidofovir and its prodrug, zidovudine and ganciclovir) as well as certain diuretics (furosemide, bumetanide) and NSAIDs (diclofenac, ibuprofen, flurbiprofen, indomethacin, ketoprofen, naproxen, etodolac, diflunisal, phenacetin, piroxicam and salicylate). Cimetidine was shown to inhibit OAT1 and OAT2 in a noncompetitive manner and OAT3 and OAT4 in a competitive way. Pravastatin was able to block OAT1 and OAT3 activity *in vitro*.[455,462-465]

Substrate specificity of human OAT2 has not been completely characterized yet, although based on *in vitro* studies it is currently accepted that OAT2 transports PAH, α-ketoglutarate, cAMP and prostaglandins E2 and F2α.[450,466] Clinically applied drugs substrates of OAT2 are for example the antiviral zidovudine, the cytostatic methotrexate and the antimicrobial tetracyclines and cephalosporines. Due to the low transport rates found in in vitro experiments for ochratoxin A, valproate, methotrexate and tetracyclines, it has been suggested that OAT2 does not significantly contribute to proximal tubular transport of these compounds. Probenecid was shown to inhibit human OAT2 in vitro but with low affinity.

Human OAT3 was demonstrated to translocate PAH, cAMP, glutarate, sulfate or glucuronide conjugates of steroid hormones (e.g. dehydroepiandrosterone sulfate, estrone sulfate, estradiol glucuronide), prostaglandins E2 and F2α, taurocholate and urate, as well as clinically relevant drugs such us tetracycline, methotrexate, salicylate, cimetidine, zidovudine, valacyclovir and ochratoxin A. Several cephalosporins (cefadroxil, cefamandole, cefazolin, cefoperazone, cefotaxime, ceftriaxone, cephaloridine and cephalothin) have also been reported to interact with human OAT3.[450,456,467-470] Besides probenecid, benzylpenicillin, quinidine, the loop diuretics furosemide and bumetanide, and the NSAIDs diclofenac, ibuprofen and indomethacin, have been reported to inhibit the cellular uptake of estrone-3-sulfate by human OAT3 in vitro. Furthermore, piroxicam was observed to inhibit the ochratoxin A transport OAT3-mediated in a competitive manner.[464]

Less is known regarding the substrate specificity and inhibitors of the other OAT members, with the exception of human URAT1. URAT1 is reported to act as an antiporter exchanging urate against certain organic anions (such as lactate, nicotinate, acetoacetate, hydroxybutirate) and chloride. Orotate, pyrazinecarboxylic acid and nicotinate have been reported to inhibit URAT1 as well as several uricosuric compounds that have been shown to reduce hyperuricemia in experimental and clinical conditions (e.g. benzbromarone, fenylbutazone, sulfinpyrazone, probenecid, NSAIDs and diuretics).

-*Inducers.* Little is reported about induction of OAT expression and activity. Steroid hormones, and in particular androgens, have been suggested to affect the OAT expression thus leading to the sexual dimorphism in OATs reported in rodents and supposed in humans. Indeed in rats, testosterone has been shown to increase OAT3 and to decrease OAT2 messenger RNA levels, respectively.[471-474] Moreover, phosphorylation has been hypothesized to post-translationally affect OAT activity: the epidermal growth factor (EGF) has been reported to induce OAT activity whereas protein kinase C and certain protein kinase C activators (e.g. phorbol esters) led to an inhibition/internalization of human OAT1.[474-476]

Pharmacological and Toxicological Function

The physiological activity, cellular location, tissue distribution and the wide substrate specificity of OATs suggest that they could affect the pharmacokinetics, efficacy and toxicity of substrate drugs.

To study the physiological, pharmacological and toxicological roles of OATs *in vivo*, single OAT gene knockout mice have been generated. However, only Oat1(-/-), Oat3(-/-), and URAT1(-/-) mice are available to date, and such models present several limitations in extrapolating to the human situation essentially related to species differences in OAT substrate specificity and tissue distribution. Moreover, due to the functional redundancy of OATs, generation of knockout models of multiple OAT genes (e.g., OAT1-/-/OAT3-/- or OAT4-/-/URAT1-/-) is expected to help further in the elucidation of the *in vivo* function of these clinically relevant transporters.

Both Oat1 and Oat3 knockout mice appeared viable, fertile and did not exhibit any obvious phenotype neither gross morphological tissue abnormalities. However, Oat1 knockout mice showed reduced clearance of the model substrate para-aminohippurate (PAH), and decreased renal excretion with increased plasma levels of a wide range of endogenous (e.g., benzoate, N-acetylaspartate, 4-hydroxyphenyllactate, 4-hydroxyphenylpyruvate, 4-hydroxyphenylacetate) and specific exogenous compounds, some of which have been demonstrated to interact with OAT1 *in vitro*. For instance, Oat1 knockout mice displayed a significant reduction in the tubular secretion of the loop diuretic furosemide (a well-known OAT1 substrate), resulting in altered diuretic effect: a rightward shift in a natriuresis dose-response curve was observed in Oat1(-/-) compared with wild-type mice. Similar findings were reported in Oat1 knockout mice with the thiazide diuretic bendroflumethiazide.[476] Therefore, it can be predicted that OAT1 affects the pharmacokinetics of other clinically used substrate drugs. Renal toxicity of several clinically used drugs (e.g. cephaloridine, adefovir, cidofovir) as well as nephrotoxins (ochratoxin A, citrinin, mercuric conjugates) and uremic toxins (indoxyl sulfate, indoleacetic acid, p- and o-hydroxyhippuric acids) has been suggested to be caused by their accumulation in the renal proximal tubular cells via OATs, in particular OAT1.[477,478] This hypothesis is further supported by the protective role observed for OAT inhibitors or competitive substrates, such as probenecid and several NSAIDs, against nephrotoxicity induced by ochratoxin A, adefovir, cidofovir, cephaloridine, and mercury. In this view, drug-drug interactions due to competition for OAT transport is expected to result in a decrease in the renal secretion of OAT substrates, thus leading to decreased nephrotoxicity, increased half-life, and potentially enhanced extrarenal toxicity of the affected compounds.

Oat3 knockout mice displayed an altered organic anion transport physiological phenotype in renal and choroid plexus epithelia. In *ex vivo* experiments, uptake of Oat3 substrates, in particular taurocholate, but also estrone sulfate and to a lesser extent PAH, was reduced significantly in renal slices prepared from Oat3 knockout mice compared with slices from wild-type mice. Administration of penicillin G to Oat3(-/-) mice resulted in a 2-fold increase in the half-life of the compound and a significant reduction in its volume of distribution, suggesting that OAT3 plays a crucial role in the clearance and efficacy of this drug.[479] Similarly, administration of ciprofloxacin to Oat3(-/-) mice resulted in 35% reduction in total clearance, significantly elevated plasma concentrations and reduced volume of distribution compared with wild-type mice.[480] Moreover, using intact choroid plexus slices, uptake of the Oat substrate fluorescein was markedly (75%) reduced in

Oat3 knockout mice compared with wild-type mice, suggesting that, at least in mice, OAT3 contributes to brain protection by regulating the distribution of endogenous organic anions and other substrates and xenobiotics within the cerebrospinal fluid.[476,481] In contrast, no differences were observed in hepatic activities of Oat3(-/-) compared with wild-type mice despite expression of OAT3 in the liver of wild-type animals, suggesting no major contribution of OAT3 to hepatic uptake of organic anions. A lower (15%) blood pressure and slightly increased plasma concentrations of several endogenous compounds (i.e., thymidine, ACTH, renin, aldosterone) was observed in Oat3(-/-) compared with wild-type mice, suggesting OAT as a potential new target for the treatment of hypertension. In effect treatment of wild-type mice with the potent OAT inhibitors probenecid and eosin-Y resulted in lowering of blood pressure similar to the effect observed in knockout mice.[483]
URAT1(-/-) mice were generated in order explore the contribution of URAT1 to the renal excretion of urate. Although viable, fertile and without any obvious abnormalities, URAT(-/-) mice displayed increased urinary urate-creatinine concentration ratio, indicating the involvement of URAT1 in tubular reabsorption of urate. Of note, urate reabsorption was still present in knockout mice, suggesting presence of overlapping activities of transporters or other mechanisms. This is in contrast with observations in humans, where genetic mutations of URAT1 resulting in loss of function have been associated with development of familial idiopathic hypouricemia, a syndrome commonly detected in Japanese and Korean populations and characterized by hypouricemia related to impaired renal reabsorption of urate. Species differences may explain such discrepancy observed between URAT1(-/-) mice and humans.[476,484] Pharmacological modulation of URAT1 expression and/or activity is predicted to affect urate plasma levels and several URAT1 inhibitors have been used for the treatment of hyperuricemia and gout. Probenecid is a uricosuric drug that reduces urate plasma levels by increasing urine urate excretion through inhibition of URAT1 and other OATs involved in renal tubular reabsorption of urate. Similarly, uricosuric activity by blocking URAT1 has been observed with several anti-inflammatory drugs (indomethacin and salicylate) and benzbromarone.[485] The angiotensin II receptor antagonist losartan has been described to determine a transient uricosuria with reduction in blood urate levels in healthy volunteers, probably due to inhibition of urate reabsorption in the proximal renal tubule.[486] In contrast, hyperuricemia with increased risk of gout has been described as an adverse event associated with treatment with the antitubercolous drug pyrazinamide, essentially due to activation of URAT1 mediated by the antimicrobial compound.[487]
Several genetic variants of OAT have been described and characterized, but their importance in affecting human variation in organic anion handling, drug disposition and response remain to be established. In effect, in view of the broad substrate specificity of OATs it could be expected that single nucleotide polymorphisms leading to variants with altered function would result in altered pharmacokinetics and toxicity of affected substrates. However, most studies performed to date failed to show an association between specific genetic variants and altered pharmacokinetics of substrate drugs (e.g. adefovir, pravastatine).[488-490] As an exception, a single nucleotide polymorphism in OAT4 gene (rs11231809) has been recently associated with altered renal clearance of the loop diuretic torsemide.[476,491]

Drug-Drug Interactions

A number of clinically relevant drug-drug interactions mediated by OAT family members have been described and others can be predicted, considering the wide number of clinically used drugs that have been reported to interact with OATs as substrates or (competitive) inhibitors (e.g., several β-lactam antibiotics, cephalosporins, quinolones, aminoglycosides and macrolides, the histamine receptor 2 antagonists cimetidine and ranitidine, several nonsteroidal anti-inflammatory drugs (NSAIDS), several antiviral nucleoside analogs, etc). Concomitant administration of OAT substrates and inhibitors may lead to a reduction in renal clearance and to an increase in plasma concentration and half-live of substrate compounds. This strategy has been used in the past to prolong the half-life of antibiotics and increase their plasma concentrations in case of limited antibiotic supplies. For instance during the World War II probenecid (a strong OAT inhibitor) was frequently administered with benzylpenicillin for this purpose.[112] However, the reduction of renal clearance of a drug can potentially increase the risk of its (extrarenal) toxicity.[482] In effect drug-drug interactions have been reported between methotrexate and probenecid, several NSAIDs, and penicillin G, resulting in severe and even life-threatening toxicity, including bone marrow suppression, hepatitis, and acute renal failure. These interactions may be explained at least in part by decreased renal secretion with subsequent increase in plasma concentrations of methotrexate due to (competitive) inhibition of renal OATs by co-administered drugs. Indeed, methotrexate has been reported to be transported by OAT1, OAT3, and OAT4. Probenecid, penicillin G and several NSAIDs (like indomethacin, salicylate, phenylbutazone, loxoprofen) have been reported to inhibit OATs *in vitro* at clinically relevant concentrations.[492-503]

Drug-drug interactions have also been described between cephalosporin antibiotics and the OAT inhibitor probenecid. In clinical studies probenecid has been shown to alter the pharmacokinetics of co-administered cefadroxil, cefamandole, and ceftriaxone (all recognized as OAT substrates *in vitro*) resulting in increased peak plasma concentration and half-life of these antibiotics.[504-506]

Administration of an OAT inhibitor could be a useful strategy to reduce the nephrotoxicity of OAT substrates, by lowering the tubular secretion of nephrotoxic substrates. Administration of the OAT inhibitors probenecid, piroxicam or octanoate has been reported to reduce the renal clearance and to prevent nephrotoxicity induced by ochratoxin A, a mycotoxin identified as an etiological factor of the endemic Balkan nephropathy and well-known substrate of OAT1, OAT2, and OAT4.[507-509] Moreover, probenecid has been reported to reduce the nephrotoxicity of cephaloridine, adefovir, cidofovir, and of mercury.[510] In effect, high doses of probenecid are co-administered with cidofovir in clinical practice to improve the renal tolerability of the antiviral drug. Similarly, betamipron and cilastatin have been suggested to prevent nephrotoxicity of the antimicrobials panipenem and imipenem, respectively, via inhibition of OATs.[112] Involvement of OATs has been proposed also in the chelation therapy of the environmental neurotoxicant methylmercury. N-acetylcysteine and dimercaptopropanesulfonate, used for mercury chelation, have been reported to increase dramatically urinary excretions of methylmercury in animals and humans, probably by forming mercaptide complexes, which are high-affinity substrates for OAT1. Therefore, organic anion transporters may facilitate the urinary methylmercury excretion.[512]

On the other hand, the concomitant administration of OAT substrate drugs and inhibitors may result in the impaired efficacy of drugs that exert their pharmacological activity in the kidney. Indeed, most loop and thiazide diuretics are actively secreted by renal OATs, reaching in this way their pharmacological target in the kidney. Co-administration of these diuretics with OAT1-3 inhibitors is predicted to reduce their diuretic effect. In Oat1 knockout mice a reduction in tubular secretion and a decrease in diuretic effect of the loop diuretic furosemide was demonstrated. In humans, after treatment with probenecid, the renal clearance of intravenously administered furosemide was decreased significantly, whereas elimination half-time and furosemide plasma concentrations were increased.[513] Diuresis and natriuresis were reduced during the first 90 minutes after furosemide administration compared with controls. Similarly, a drug-drug interaction probably mediated by OATs has been described between the antiviral zidovudine and the H2 receptor inhibitor cimetidine. In humans, cimetidine has been shown to inhibit the renal clearance of zidovudine. *In vitro* studies have shown that zidovudine is an OAT1-4 but not an OCTs substrate; cimetidine has been identified as a potent inhibitor of OAT3 (as well as OCTl and OCT2).[468,514-516] A drug-drug interaction probably mediated by OAT3 has also been described in humans between the H2 antagonist famotidine and probenecid. Co-administration of famotidine and probenecid resulted in increased plasma concentrations and decreased renal clearance of famotidine, probably due to inhibition of the OAT3-mediated transport of famotidine by probenecid.[517]

A clinically relevant interaction has been reported between the OAT3 inhibitor gemfibrozil and pravastatin. In 10 healthy volunteers gemfibrozil decreased the renal clearance of pravastatin by 43%, probably due to OAT3 inhibition, and increased pravastatin plasma concentrations, probably by inhibiting hepatic uptake transporters.[112] Similarly, gemfibrozil increased the plasma concentrations of the antidiabetic sitagliptin, a dipeptidyl peptidase IV inhibitor and substrate of OAT3 and P-gp in healthy volunteers.[112]

Finally, as OAT3 together with several MRPs (especially MRP4 and MRP5) have been suggested to reduce the brain penetration of the thiopurine nucleobase analogs 6-mercaptopurine and 6-thioguanine, used for the treatment of acute lymphoblastic leukemia, co-administration of 6-mercaptopurine together with OAT3-specific inhibitors has been proposed as a useful strategy to increase thiopurine concentrations in the brain interstitial and cerebrospinal fluid, thus potentially enhancing their pharmacological effect.[518] In animal models efflux of 6-mercaptopurine from the brain was inhibited by OATs and/or MRPs inhibitors, such as benzylpenicillin, cimetidine, and sulfinpyrazone.[518] Additional studies are needed to evaluate the feasibility of such approach in humans.

3.2 Organic Anion-Transporting Polypeptides (OATPs)

The OATP/SLCO superfamily of transporters has been divided in families (identified by Arabic numbering, e.g. OATP1, OATP2, etc), subfamilies (designated by capital letters for human members and small letters for rodent ones, e.g. OATP1A, OATP1B, etc) and single proteins (genes) (named by continuous Arabic numbering according to the chronology of protein (gene) identification (e.g. OATP1A2, OATP1B1, OATP1B3, etc). Thus far more than 35 Oatps/OATPs have been identified in humans, rats and mice. Not all of the known OATPs have been fully characterized and although some of them share tissue distribution and substrate specificity, differences between individual OATPs in substrate affinity and physiologic function have been reported.

Structurally OATPs are membrane proteins composed of 12 transmembrane spanning domains with a large extracellular loop with many conserved cysteine residues and N-glycosylation sites and a superfamily sequence (D-X-RW-(I,V)-GAWW-X-G-(F,L)-L) between the extracellular loop and the sixth transmembrane domain.[519] OATPs mediate the transport of a wide range of amphipatic endogenous and exogenous organic compounds including bile salts, steroid conjugates, thyroid hormones, anionic oligopeptides, several drugs and xenobiotics.

Main substrate classes, Inhibitors and Inducers

-*Main substrate classes.* In general OATPs mediate the transport of structurally diverse compounds such as organic anions, cations, neutral or zwitterionic substances and certain peptidomimetic agents. The main OATPs physiological substrates are bile acids, bilirubin, steroids, thyroid hormones, prostaglandins and cholecystokinine. An increasing number of clinically used drugs has been recognized to be transported by OATPs. They include several HMG-CoA reductase inhibitors (e.g., pravastatin, simvastatin, atorvastatin, cerivastatin, pitavastatin, rosuvastatin), anticancer drugs (the active metabolite of CPT-11 SN-38, gimatecan, methotrexate, imatinib, pazopanib, docetaxel, paclitaxel), endothelin receptor antagonists (atrasentan, bosentan), anti-hypertensive drugs (olmesartan, valsartan, temocapril, enalapril, aliskiren), anti-infective agents (benzylpenicillin, rifampicin, erythromycin, cefoperazone, cefazolin, caspofungin), HIV protease inhibitors (lopinavir, darunavir, saquinavir), anti-diabetics (repaglinide, troglitazone, glibenclamide), the cardiac glycoside digoxin, and the anti-histaminergic compound fexofenadine.[112,520]

-*Inhibitors (competitive, non-competitive).* Several compounds have been reported to inhibit OATP activity. The antimicrobial rifamycin SV was able to strongly block OATP1B1, OATP1B3, OATP2B1 and OATP1A2 mediated transport of bromosulfophthalein (BSP) *in vitro* and *in vivo* whereas rifampicin, a drug structurally related to rifamycin SV, was shown to inhibit in a competitive manner primarily OATP1B3 and, to a lesser extent, OATP1B1 in preclinical and clinical studies.[521-523] Inhibition of OATPs transport activity by rifamycin antibiotics has been suggested to determine the reduced hepatic bilirubin/organic anion elimination observed during initial treatment with these antimicrobial agents.[521-524] Analogously, the antimicrobials ketoconazole and erythromycin are described as OATP inhibitors.[99] Troglitazone sulfate, the metabolite of troglitazone (an insulin sensitizing drug developed for the treatment of type 2-diabetes mellitus) as well as other thiazolidinediones (e.g. pioglitazone and rosiglitazone) have been demonstrated to inhibit OATP1B1 and/or OATP1B3 *in vitro*, thus suggesting a possible involvement of troglitazone sulfate in troglitazone hepatotoxicity, the rare side effect that caused the withdrawal of the drug from the market.[525]

Grapefruit, orange and apple juices as well as several of their furanocoumarin, bioflavonoid and bergamottin constituents (such as 6′,7′-dihydroxybergamottin, bergamottin, naringin, hesperidin, methoxypsoralen) have been reported to reduce human OATP and rat Oatp activity *in vitro* and *in vivo* thus leading to clinically relevant drug-food interactions.[526,527] Finally, several P-gp substrates and inhibitors (e.g., lovastatin, saquinavir, nelfinavir, quinidine, ketoconazole, and verapamil) were shown to inhibit OATP transport activity to various extent *in vitro*.[99]

-*Inducers.* Several studies have demonstrated that OATP expression can be induced by various compounds that are well-known ligands and activators of the nuclear receptors

pregnane X receptor (PXR) and constitutive androstane receptor (CAR). Furthermore, the expression of certain OATPs (such as OATP1B1 and OATP1B3) has been suggested to be under transcriptional control by hepatocyte nuclear factor 1α (NF1α). In pre-clinical studies phenobarbital, spironolactone, and dexamethasone were shown to enhance rat Oatp1b1 expression, whereas testosterone has been reported to induce renal rat Oatp1a2. Of note, as the hepatic expression of OATP1B3 has been reported to depend on HNF-1a and on the bile acid nuclear receptor FXR/BAR, it has been suggested that the induction of SLCO1B3 gene expression by bile acids could maintain the hepatic elimination of xenobiotics and peptides under cholestatic conditions.[528-532]

Pharmacological and Toxicological function

The importance of Organic Anion Transporter Polypeptides (OATPs) in drug pharmacokinetics has been increasingly recognized during the last decade. The cellular localization and tissue distribution of several OATPs suggested a significant role in drug disposition, response and toxicity. Indeed, OATP1B1 and OATP1B3 are liver specific members of the OATP family of transporters and are expressed at the sinusoidal membrane of hepatocytes, modulating the hepatic uptake of drugs from the portal vein and thereby influencing hepatic and systemic exposure of substrates. Moreover, they may play a significant role in detoxification reactions, as they prevent accumulation of xenobiotics and toxic endogenous compounds in certain tissues. OATP2B1 and OATP1A2 are located at the apical membrane of intestinal enterocytes and therefore are expected to affect the absorption of substrates. Impaired OATP function may potentially reduce activity and increase the risk of toxicity of substrate drugs. In addition, the expression of OATP1A2 in the capillary endothelial cells of brain supports the hypothesis that they may modulate the brain penetration of substrate drugs. The study of the role of OATPs on pharmacokinetics and pharmacodynamics of substrate drugs is severely hampered by the lack of reliable animal models: mice with targeted disruption of OATP/Oatp encoding genes (Slco1b2(-/-), Slco1a/1b(-/-)) have been generated and they appear to support a crucial role of OATP in hepatic uptake of bilirubine and other endogenous compounds, as well as in distribution and toxicity of xenobiotics (e.g., pravastatin and rifampicin).[533] However, the lack of orthology between rodents and humans jeopardizes the translation of the findings observed in mice models to the human situation. SLCO1B1 transgenic mice have been generated too, but additional studies are needed to confirm the utility of rodent models to predict the function of human OATP1B1.[520] Therefore the pharmacological role of OATPs and the identification of substrates and inducers is essentially based on in vitro and ex-vivo models and on the identification of naturally occurring polymorphisms resulting in altered transporters expression and/or activity and their association with particular phenotypes or with specific impairment in the disposition of substrate drugs. Several pathophysiologic conditions associated to mutations of OATP genes have been described too. Mutations of SLCO1B1 and SLCO1B3 genes resulting in non-functional OATPs have been linked to the Rotor syndrome, an autosomal recessive syndrome characterized by conjugated and unconjugated hyperbilirubinemia, coproporphyrinuria and altered bromosulfophthalein.[534] In effect there is genetic evidence for a role of OATP1B1 and OATP1B3 in the hepatocellular uptake of conjugated and unconjugated bilirubin. Mice with genetic deletion of Slco1a/1b locus displayed conjugated and unconjugated hyperbilirubinemia (40% higher levels compared with wild-type mice).

Similarly, subjects with specific SNPs of OATP1B1 resulting in a non-functional protein displayed hyperbilirubinemia. Specific SNPs in SLCO1A2 have been associated with progressive supranuclear palsy or with high serum levels of reverse triiodothyronine.[534] Currently, OATP Activity has been reported to affect the disposition of several clinically used drugs, such as the majority of statins (e.g., pravastatin, simvastatin, atorvastatin, cerivastatin, pitavastatin, rosuvastatin), anticancer drugs (the active metabolite of CPT-11 SN-38, gimatecan, methotrexate, imatinib, pazopanib, docetaxel, paclitaxel), endothelin receptor antagonists (atrasentan, bosentan), anti-hypertensive drugs (olmesartan, valsartan, temocrapril, enalapril, aliskiren), anti-infective agents (benzylpenicillin, rifampicin, erythromycin, cefoperazone, cefazolin, caspofungin), HIV protease inhibitors (lopinavir, darunavir, saquinavir), anti-diabetics (repaglinide, troglitazone, glibenclamide), digoxin, and fexofenadine. Numerous genetic variants of OATPs, in particular of OATP1B1, OATP2B1 and OATP1A2, have been described, several of which were linked to impaired transport activity *in vitro* and *in vivo*. The body of evidence regarding the association between specific single nucleotide polymorphisms and altered disposition and response of substrate drugs is rapidly increasing, as demonstrated by the numerous articles and reviews recently published about this issue.[520,534-537] SNPs in OATP1B1 appear to contribute to the interindividual variability in the pharmacokinetics and pharmacodynamic profile of OATP substrate drugs that have the liver as one of their pharmacological targets (e.g., HMG-CoA reductase inhibitors) and/or have a plasma clearance affected by OATP1B 1-mediated hepatic uptake. Several SNPs have been associated with altered systemic exposure to different statins, substrates of OATP. In particular, it has been hypothesized that the 2-fold increase AUC of rosuvastatin, an OATP1B1-substrate in Asians than in Caucasians, responsible for different starting doses of the drugs in the two ethnic groups (5 mg and 10 mg, respectively) could be related to genetic variation o OATP1B1.[112] Similarly, Preclinical and clinical studies have shown a link between the SLCO1B1*5 (T521C, Val174Ala) polymorphism in the OATP1B1 gene and increased systemic exposure to pravastatin, atorvastatin and simvastatin in several ethnic populations. The increased plasma concentrations to these statins due to a decreased hepatocellular uptake from blood in subjects carrying these loss-of-function haplotypes has been suggested to enhance the risk of myotoxic effects, accompanied by reduced cholesterol-lowering efficacy due to insufficient hepatic concentration needed to inhibit HMG-CoA reductase.[538] In effect, SLCO1B1*5 allele was recently reported to predispose to statin intolerance and development of myopathy, whereas other studies appear to suggest that SLCO1B1 SNP does not have clinically meaningful effect on the cholesterol-lowering effect of statins, probably because the total hepatic exposure to statins is unlikely markedly affected by reduced OATP1B1 activity. Therefore, genotyping for OATP1B1 polymorphisms could be a strategy for tailored statin dosing in order to obtain optimal cholesterol lowering effects while minimizing the risk of side effects. In contrast, the haplotype 130AspVal174 was reported to increase the hepatic OATP1B1-dependent uptake of pravastatin, and possibly of valsartan and temocapril. Indeed, aside from statins, SLCO1B1 SNPs have been reported to impair the pharmacokinetics of several widely used drugs, such as ezetimibe, repaglinide, methotrexate, rifampicin, fexofenadine, tacrolimus, irinotecan, lopinavir, olmesartan, torsemide, and enalapril.[520,537]

Specific genetic variants of OATP2B1 have been linked to altered disposition of fexofenadine, celiprolol, and montelukast. As OATP1A2 is localized predominantly in the capillary

endothelial cells of the brain, allelic variants of OATP1A2 could affect the brain distribution and toxicity of several substrate drugs.[539,540] Similarly, few SPNs in OATP1B3 appear to be associated with increased clearance of imatinib, whereas contrasting results are available over the effect of other SNP variants on mycophenolic acid pharmacokinetics.[537]
In view of the clear influence of specific OATP SNPs on drug disposition or toxicity, genotyping for selective SLCO1B1 variants (e.g. c.521T>C) could be used to improve tolerability and efficacy of, for example statin therapy.

Drug-Drug interactions
An increasing number of clinically relevant drug-drug and food-drug interactions mediated by OATPs are reported. Moreover, in view of the wide spectrum of substrate specificity, other drug-drug interactions can be predicted by the concomitant use of OATP substrates and inhibitors/inducers. The majority of interactions described to date concerns OATP1B1 and OATP1B3, the best characterized family members. Their relevance in mediating clinically relevant drug interactions is underlined by their inclusion in the EMA and FDA guidance documents for the investigation of drug interactions during the clinical development of new drugs.[68,69] During drug development, studies should be undertaken to explore the possible role of OATPs, and in particular OATP1B1, in disposition, efficacy and toxicity of a new drug candidate. However, the study of drug-drug interactions mediated by OATPs is hampered by the lack of specific probe inhibitors, as cyclosporin A, gemfibrozil and rifampicin, drugs usually employed for this purpose, can also inhibit other transporters or metabolizing enzymes. Cyclosporin A is a potent inhibitor of OATP1B1, OATP1B3, OATP2B1, Pgp, and CYP3A4; gemfibrozil is reported to block also BCRP, MRP2, OAT3 and CYP2C8; rifampicin inhibits also OATP1B3, CYP2C8, and CYP3A4.[112,520]
Clinically relevant drug-drug interactions have been described between several HMG-CoA reductase inhibitors (simvastatin, rosuvastatin, cerivastatin, pravastatin, atorvastatin and pitavastatin) and cyclosporin A. Heart transplant patients receiving cyclosporin A for anti-rejection presented a significant increase in rosuvastatin AUC compared with historical controls.[543] Similarly, co-administration of cerivastatin and cyclosporin A resulted in a 4- and 5-fold increase in plasma AUC and maximum plasma concentrations, respectively.[544] As cerivastatin, as well as other statins, are metabolized by cytochrome P450 enzymes, and several OATPs inhibitors (like cyclosporin A) are also blockers of metabolizing enzymes and other transporters (MRP2, Pgp), interpretation of these interactions may be difficult. However, a similar effect has been observed with pravastatin in children taking immunosuppressive therapy with cyclosporin A after cardiac transplantation, where systemic exposure to pravastatin was nearly 10-fold higher compared to children with hypercholesterolemia treated with pravastatin and not with cyclosporin A. As pravastatin is not metabolized, the observed pharmacokinetic interaction is likely due to inhibition of OATP mediated transport, and suggests that the same mechanism contributes, at least partly, to the interactions observed with other statins.[112,520]
Clinically relevant drug-drug interactions have been reported between the anti-hypertriglyceridemic drug gemfibrozil and several statins, such as cerivastatin, pravastatin, simvastatin, lovastatin, pitavastatin and rosuvastatin, resulting in markedly increased statin plasma concentrations.[520] In particular, the interaction with cerivastatin has been considered responsible for the side effect of myotoxicity, including life-threatening rabdomyolysis, leading to the withdrawal from the market of cerivastatin. In a drug-

drug interaction study conducted in 10 healthy volunteers, administration of pravastatin during treatment with gemfibrozil resulted in 2-fold increase in mean AUC of pravastatin compared to patients taking placebo.[112] However, as gemfibrozil is also able to inhibit other transporters (i.e. BCRP, MRP2, OAT3) and metabolic enzymes (e.g. CYP2C8, CYP2C9), these data need to be interpreted with caution.

Similarly, concomitant administration of atorvastatin and rifampicin (an OATP1B1 and OATP1B3 inhibitor, and an inducer of drug-metabolizing enzymes) in healthy volunteers resulted in more than 6-fold increase in atorvastatin AUC, probably due to inhibition of hepatic uptake of the drug.[112] However, in clinical practice co-administration of rifampicin and statins is expected to decrease the plasma concentration of the co-administered drugs, as repeated dosing of rifampicin causes strong induction of several metabolizing enzymes (CYP3A4 and CYP2C8) and transporters (Pgp, MRP2, OATP1B1).

Recently oral administration of eltrombopag, an oral nonpeptide thrombopoietin receptor agonist reported to inhibit OATP1B1 and BCRP *in vitro*, to 42 healthy volunteers resulted in increased AUC (55%) and maximum plasma concentrations (103%) of co-administered rosuvastatin.[545]

Clinically relevant interactions have been described between macrolide antibiotics (in particular clarithromycin and roxithromycin) and simvastatin, atorvastatin. In humans clarithromycin increased the maximum plasma concentrations of simvastatin and atorvastatin, two substrates of OATP1B1, by more than 6- and 4-fold, respectively. These interactions were attributed to inhibition of metabolizing enzymes, as both statins are metabolized by CYP3A4 enzymes and clarithromycin is an efficient CYP3A4 inhibitor at clinically relevant concentrations. However, 50% increase in plasma AUC of pravastatin, a statin not substrate for metabolic enzymes, was observed also when clarithromycin was co-administered, suggesting that the above mentioned interactions could be explained, at least in part, by inhibition of OATP-mediated uptake of the statins into hepatocytes, resulting in increased plasma concentrations.[112]

A clinically relevant drug-drug interaction has been documented between the anti-diabetic repaglinide and cyclosporin A. A significant (2.4-fold) increase in the AUC and maximal plasma concentration of 244% and 175%, respectively, of repaglinide was reported in twelve healthy volunteers receiving cyclosporin A.[546] Cyclosporin A has also been reported to increase the plasma AUC of caspofungin (1.4-fold), of the active metabolite SN-38 of the anticancer drug irinotecan (1.2- to 7.3-fold), and of methotrexate (1.3-fold). Cyclosporin A- mediated inhibition of OATP1B1 can explain, at least partly, such interactions, as inhibition of other transporters and metabolizing enzymes can be involved too.

Drug-drug interactions between the endothelin receptor antagonist bosentan, a substrate for OATP1B1, OATP1B3, CYP3A4 and CYP2C9, and sildenafil, cyclosporin A, ketoconazole and rifampicin have been reported in humans, resulting in significantly increased bosentan plasma concentrations. As sildenafil is not a modulator of CYPs involved in the metabolism of bosentan but has been reported to inhibit OATP1B1 and OATP1B3 in vitro, inhibition of OATPs may be the major determinant for these clinically relevant interactions.[112]

Similarly, recent preclinical findings suggest that the drug-drug interaction described between digoxin and amiodarone, resulting in in increased digoxin plasma levels and toxicity and classically attributed to modulation of Pgp, could be explained, at least partly, by the inhibitory effect of amiodarone on OATPs.[547,548]

Clinically relevant drug-drug interactions have been described between the HIV protease inhibitors lopinavir and atazanavir, well-known OATP1B1 and OATP1B3 inhibitors, when administered with rosuvastatin. In humans, use of ritonavir-boosted lopinavir or ritonavir-boosted atazanavir increased the AUC of rosuvastatin by 2- to 4.7-fold, and by 2.1- to 7-fold, respectively.[520]

A significant food-drug interaction has been reported between several fruit juices (including grapefruit, orange, and apple juices) and the anti-histamine fexofenadine, as well as other OATP substrates such as aliskiren, celiprolol, montelukast. In different studies, pre-treatment of subjects with the above mentioned juices resulted in significant reduction of the plasma concentrations and/or oral bioavailability of the co-administered drug.[112,549] As these juices are enriched with different bioflavonoids and furanocoumarins, which are reported to interact with OATP1A2, but also with CYP metabolic enzymes and Pgp, several mechanisms are involved in these interactions. However, there is increasing evidence that for some of these interactions inhibition of OATP1A2-mediated intestinal uptake of the co-administered OATP substrate drugs represents the main underlying mechanism.

Finally, several genotype-dependent drug-drug interactions have been reported. Indeed, specific genetic variants may encode for variants of specific transporters with higher activity, which are expected to be more sensitive to inhibition compared with subjects with low activity variants. For instance, in subjects with a specific variant (TT genotype) of a clinically relevant polymorphism in the SLCO1B1 gene encoding for OATP1B1 (c.521T>C, OATP1B1*5) the increase in AUC of repaglinide observed by co-administration of cyclosporin A was significantly higher compared with patients with the TC genotype.[546] Similarly, increase of atorvastatine plasma concentrations after administration of a single dose of rifampin were genotype dependent, i.e., 8-, 5- and 3-fold increased in patients with genotype TT, TC, and CC, respectively. Other SLCO1B1 dependent drug interactions were observed between gemfibrozil and repaglinide, as well as between atorvastatine and repaglinide.[112]

The study of OATP inhibitors and/or inducers as well as of the impact of genetic variability in OATP on drug disposition is important to explain, predict, and thus avoid clinically disadvantageous drug-drug or drug-food interactions. On the other hand, the development and the clinical use of selective OATP inhibitors may have relevant implications for drug therapy. The oral bioavailability of an OATP substrate drug with significant OATP-mediated hepatic first-pass elimination could be improved by co-administration of an OATP-specific inhibitor. Administration of a selective OATP inhibitor could also be useful in reducing liver damage in patients with an OATP substrate intoxication (e.g., microcystin LR, phalloidin), by inhibiting the hepatic toxin uptake and the enterohepatic circulation of toxins. In effect Oatp1b2-null mice were resistant to the hepatotoxicity induced by phalloidin and microcystin-LR.[550]

3.3 Organic Cation Transporters (OCTs)

The organic cation transporters (OCT1/SLC22A1, OCT2/SLC22A2, OCT3/SLC22A3/EMT) are polyspecific and membrane potential-dependent but pH-independent transport proteins belonging to the Solute Carrier 22 subfamily.[449,551,552] The first organic cation transporter rOCT1 was identified by Grundemann et al. by expression cloning from rat kidney.[553] Structurally, they are characterized by 12 transmembrane domains (TMD) with

a large extracellular hydrophilic loop (between TMD1 and TMD2).[553] In general the OCTs are expressed in plasma membrane of epithelial cells where they mediate the transport of a wide range of relatively small hydrophilic organic cations.[554]
Human OCT1 is mainly expressed in the liver at the basolateral (sinusoidal) membrane of hepatocytes, whereas in rodents (rats) rOCT1 is highly expressed in liver, kidney and small intestine.[552-556] Human and rat OCT2 are expressed predominantly in the kidney and in various regions of the brain.[552,557-559] In the kidney both rOCT1 and rOCT2 are localized at the basolateral membrane of epithelial cells of the proximal tubules, and expression of rOCT2 is reported to be gender- and age-dependent.[560-563] In contrast to OCT1 and OCT2, which are mainly expressed in the major excretory organs, OCT3 shows a broad tissue distribution with high levels in the brain, heart, liver, aorta, prostate, skeletal muscle, adrenal gland, salivary glands and term placenta.[564-567] The strategic localization of OCT1 and OCT2 in excretory organs suggests that they play a key role in the elimination of cationic endogenous compounds and xenobiotics from the body, by mediating the up-take of these compounds from the blood into the excretory epithelial cells. The brain localization, together with the ability of OCTs to transport catecholamines and other biogenic amines, support the hypothesis that OCTs may be responsible of the extraneuronal removal of monoamine neurotransmitters that have escaped from re-uptake by high-affinity transporters.[559,568-571]

Main substrate classes and Inhibitors

In general the OCTs mediate the transport of structurally diverse small hydrophilic organic cations and they display a broadly but not completely overlapping substrate specificity. Interspecies differences in the function of the OCTs have been also described. OCT substrates include the model substrate tetraethylammonium (TEA), the neurotoxin 1-methyl-4-phenylpyridinium (MPP+) and clinically applied drugs such as antidiabetics (biguanides, in particular metformin), antiparkinson drugs (amantadine and memantine), β-blockers (acebutolol), antiarrhythmics (i.e., procainamide, pilsicainide), the H2-receptor antagonist cimetidine, the antidote pralidoxime, skeletal muscle relaxants (e.g. vecuronium), paraquat, and several other endogenous compounds such as dopamine, noradrenalin, serotonin, histamine, creatinine and choline.[572-582] Moreover, OCT1 and OCT2 have been demonstrated to transport some anionic prostanoids (i.e. prostaglandins and their derivatives) indicating that a positive charge is not an absolute prerequisite for being an OCT substrate.[583] The anticancer tyrosine kinase inhibitor imatinib is a substrate for OCT1, whereas the anticancer drug oxaliplatin (but carboplatin) appears to be transported by OCT1 and OCT2.[584-586]
Several compounds have been shown to inhibit the uptake of the prototypes TEA or MPP+ in a competitive or not competitive manner, suggesting that they can interact with OCTs. Cimetidine appears to produce a non-competitive inhibition of OCTs, whereas cisplatin has been reported to competitively inhibit TEA uptake by mouse Oct2.[586] The antiarrhytmic procainamide is reported to efficiently inhibit OCT1 as well as OCT2 and OCT3; quinine and quinidine appear to alter the activity of OCT1 and OCT2.[574,569,582,587]
In a mammalian expression system for murine OCT2 and OCT3 several steroids (in particular β estradiol, corticosterone, deoxycorticosterone, papaverine, testosterone and progesterone) were found to inhibit TEA uptake.[588] In addition, the HIV protease inhibitors ritonavir, saquinavir, indinavir and nelfinavir have been reported to be potent inhibitors

but poor substrates for human OCT1.[580,589] Finally, several flavonoids (such as quercetin, kaempferol, naringenin, isoquercitrin, spiraeoside and others) have been shown to inhibit OCT2 mediated transport *in vitro*.[590]

Pharmacological and Toxicological Function

The pharmacological and toxicological roles of OCT1-3 have been investigated in vivo using knockout mice generated for all three organic cation transporters.[590-592] Oct1(-/-), Oct2(-/-), Oct3(-/-) and Oct1/2(-/-) mice were viable, fertile and did not display any obvious physiological defects. However, the disposition of organic cation substrates was altered.

In Oct 1(-/-) mice accumulation of the model OCT1 substrate TEA (tetraethylammonium) in the liver was dramatically reduced, as well as biliary intestinal excretion of TEA was 50% lower compared with wild-type mice after intravenous administration.[590] Similarly, decreased liver accumulation but no difference in intestinal excretion was found in Oct1(-/-) mice for the neurotoxin l-methyl-4-phenylpyridium (MPP^+) and [^{131}I] metaiodobenzylguanidine (MIBG), a compound clinically used for detection and treatment of tumors of neuroadrenergic origin, such as neuroblastoma, pheochromocytoma, and carcinoid.[593] These findings indicate a major role for OCT1 in liver distribution of substrate drugs. Extrapolating to humans, modulation of OCT1 expression and/or activity might have clinically important consequences. Reduced liver uptake could be beneficial for hepatotoxic drugs, whereas it could lead to reduced therapeutic efficacy for drugs that undergo hepatic metabolic activation or exert their therapeutic activity in the liver. In effect, studies performed in Oct1 knockout mice with metformin, a biguanide used for the treatment of diabetes that acts through hepatic inhibition of gluconeogenesis, showed a significant reduction of hepatic exposure to metformin associated with reduced pharmacodynamic effects of the drug (i.e., lack of fasting glucose-lowering effects and reduced lactic acidosis, a life-threatening adverse effect of biguanides).[594,595]

Of note, systemic pharmacokinetics and renal clearance of metformin, but also of TEA and MIBG, were not altered in Oct1(-/-) mice, due to the functional redundancy of OCT1 and OCT2 in mice and to a shift in clearance of transported drugs from hepatobiliary towards renal elimination.[595] Therefore, Oct2 single and Oct1/2 double knockout mice were generated in order to explore the impact of OCTs on renal distribution of substrates. Although the absence of Oct2 had little effect on the renal distribution of substrates (such as TEA), the concomitant deficiency of Oct1 and Oct2 in mice resulted in a complete abolishment of the renal secretion of TEA.[596]

Similarly, administration of metformin to Oct1/2(-/-) mice resulted in a 4.5-fold decrease in systemic clearance, a 3.5 decrease in the volume of distribution, a 4.2- and 2.5-fold reduction of hepatic and renal tissue partitioning of metformin, respectively, leading to significantly increased metformin systemic concentrations.[597] These results are in line with observations in humans where a 2.6-fold reduction in secretory clearance of metformin was reported in subjects with a functional OCT2 genetic variant.[597-599] However, tissue (liver, kidney) drug exposure and pharmacodynamic effect (glucose lowering effect) of metformin were significantly reduced in Oct1/2(-/-) compared with wild-type mice, suggesting involvement of other transporters (MATEs, OCT3, etc).[597] By extrapolating from rodents to humans (where only OCT2 and not OCT1 are expressed in the kidney), it can be expected that OCT2 deficiency in humans may result in altered renal elimination and consequently, increased exposure to some drugs. Recently, expression of OCT2 in the

limbic system of mice was reported, and experiments performed in Oct2(-/-) mice showed altered anxiety and depression behavior, suggesting OCT2 as a potential pharmacological target for mood disorder therapy.[600]

The pharmacological and physiological functions of OCT3 were partly elucidated by generation of Oct3 knockout mice.[592] Oct3(-/-) mice do not present an obvious physiological phenotype; however aberrations were reported by specific or extreme conditions. Oct3(-/-) mice showed an increase in the level of ingestion of hypertonic saline under thirst and salt appetite conditions, as well as alterations of the neural response in the subfornical organ after sodium deprivation. Significantly less anxiety and less fear were reported by evaluation of cognitive functioning of Oct3(-/-) mice, probably related to OCT3 modulation of the serotonergic tone. Induction of transient focal cerebral ischemia in Oct3(-/-) mice resulted in increased histamine concentration in the ischemic cortex and significantly reduced infarct volume compared with wild-type mice, possibly due to involvement of OCT3 in clearance of ischemia-induced histamine and subsequent regulation of T cells. When treated with lipopolysaccharide, survival of Oct3(-/-) mice was significantly shorter compared with wild-type mice, possibly due to functional changes of immunological cells related to altered homeostasis of histamine, an OCT3 substrate. Impaired activity of the Uptake2 system (an extraneuronal monoamine clearance system) has been reported in Oct3(-/-) mice. Compared with wild-type mice, in Oct3(-/-) mice a significant (72%) reduction in heart accumulation of MPP+, a neurotoxin involved in the etiology of Parkinson's disease, was observed, further suggesting the major role of OCT3 in vivo in the transport activity of the Uptake-2 system.[592] The placenta was also identified as an additional Uptake-2 site, as in Oct2(-/-) heterozygous pregnant females a 3-fold reduced MPP+ accumulation was found in Oct3(-/-) compared with wild-type fetuses after intravenous administration.[592] No difference in MPP+ concentration was found in other Oct3-expressing organs, probably due to the functional redundancy within the different OCTs and other transporters.

Several genetic variants in OCTs have recently been identified, some of which with significantly altered (decreased, abolished or increased) transport activity or substrate selectivity. In effect, there is increasing evidence indicating a role of genetic polymorphism in OCT (and other) transporters in interindividual variability in disposition, response and toxicity of cationic substrate drugs like oral anti-diabetics (especially metformin), imatinib and cisplatin.[601-604] In general, OCT3 appears to be less polymorphic than OCT1 and OCT2 and more evolutionary conserved. However, several OCT3 variants have been recently described, three of which with altered substrate specificity with potential clinically relevant consequences. Indeed, as OCT3 appears to transport metformin efficiently and it has been reported as one of the determinants of metformin activity in skeletal muscles, variation in OCT3 expression/activity could modulate the pharmacological response to metformin.[605] Moreover, a non-synonymous variant of OCT3 (M370I), associated with a 40% reduction of norepinephrine transport capacity and a genetic variant in the promoter of OCT3 (g.-81G>delGA), which showed increased luciferase activity, were associated with obsessive-compulsive disorders in Caucasian children and adolescent.[605] The recognition of a role for OCT3 in the regulation of neurotransmission and maintenance of homeostasis within the CNS could lead to strategies targeting OCT3 for the treatment of several neurological and psychiatric diseases. Preclinical studies indicate OCT3 inhibition as one of the pharmacological effects exerted by antidepressant. In effect, OCT3 activity was inhibited

by several clinically used antidepressants (e.g., desipramine, sertraline, paroxetine, amitriptyline, imipramine, fluoxetine) in a concentration dependent manner.[606]

Drug-Drug Interactions

A number of potentially relevant drug-drug interactions probably mediated by OCTs have been described. However, due to increasing evidence of overlapping activity and substrate specificity between OCTs and other transporters, often more transporters are considered involved in these interactions. A clinically relevant interaction has been reported between the antiarrythmic drug pilsicainide and the H1-receptor antagonist cetirizine in a Japanese patient with moderate renal insufficiency.[608] In a subsequent study in healthy volunteers co-administration of the two drugs resulted in mutual inhibition of renal clearance and reduction of elimination constant. Considering that both cetirizine and pilsicainide were able to inhibit the MDR1- and OCT2-mediated transport of substrates, and pilsicainide was reported to be excreted into urine probably via both MDR1 and OCT2, both transporters have been implicated in such interaction. Similarly, several clinically observed drug-drug interactions involving the H2-receptor inhibitor cimetidine (an OCT blocker) and other OCT substrate drugs (metformin, procainamide, pilsicainide) have been considered as OCT2 mediated. Co-administration of cimetidine with metformin increased the plasma levels and reduced the renal excretion of metformin, thus leading to clinically relevant increased exposure in diabetic patients.[607] In healthy volunteers, co-administration of pilsicainide and cimetidine resulted in an increased AUC (by on average 33%), a prolonged elimination half-life, and a reduced apparent renal clearance of pilsicainide.[609] Similarly, treatment with cimetidine significantly increased the AUC and decreased the renal clearance of the co-administered antiarrythmic drug procainamide.[610] However, considering that recent pre-clinical studies have shown that cimetidine is a stronger inhibitor of Multidrug And Toxin Extrusion receptors (MATEs) than of OCT2 at clinically relevant plasma concentrations and that several compounds (e.g. metformin) are efficiently transported by MATEs, inhibition of MATEs, rather than OCT2, could be responsible for the drug-drug interaction with cimetidine at the level of renal elimination.[611]

As OCTs have been shown to influence cisplatin renal distribution and toxicity, co-administration of cisplatin with an OCT2 inhibitor could be a strategy in order to reduce cisplatin induced side effects. In pre-clinical studies Oct1/2(-/-) mice treated with cisplatin displayed significantly impaired plasma urinary excretion of cisplatin, and appeared to be protected against severe cisplatin-induced tubular damage and ototoxicity. Indeed no sign of ototoxicity and only mild nephrotoxicity was reported.[612] The hypothesis of a role of Oct2 in cisplatin-induced nephrotoxicity is further supported by the observation that a nonsynonimous single-nucleotide polymorphism in the OCT2 gene SLC22A2 (rs316019) was associated with reduced cisplatin-induced nephrotoxicity in patients.[613] Considering that co-administration of cisplatin with cimetidine in wild-type mice or with imatinib in rats (two OCT2 inhibitors) resulted in significant reduction in platinum accumulation and nephrotoxicity, administration of an OCT2 inhibitor has been proposed as a useful strategy in patients treated with cisplatin in order to reduce toxicity.[614] However, further studies are considered necessary before testing such hypothesis in patients, as several OCT2 inhibitors (e.g., cimetidine, imatinib) have been reported to inhibit MATEs even more efficiently and genetic deletion of MATE in mice resulted in increased cisplatin induced nephrotoxicity (see section about MATE transporters)[611,615]. Similarly, OCT2 has recently

been proposed as one of the major mechanisms for nephrotoxicity and renal Fanconi syndrome induced by ifosfamide, a widely used anticancer drug.[616] Additional studies are warranted in order to explore the potential protective effect of an OCT2 inhibitor during ifosfamide therapy, in order to prevent nephrotoxicity. Analogously, as paraquat (N,N-dimethyl-4-4′-bipiridinium), a commonly used herbicide causing life-threatening lung, liver and renal toxicity by (accidental) ingestion, is primary eliminated by the kidney by a mechanism partly mediated by OCT2 and MATE1, target modulation of such transporters could be useful for prevention and treatment of paraquat-induced toxicity.[579]
Furthermore, administration of an OCT2 inhibitor during treatment with the anticancer drug oxaliplatin (an OCT2 substrate) has recently been proposed to prevent/mitigate the development of dose-limiting neurotoxicity. Genetic deletion of OCT2 in mice resulted in reduction of hypersensitivity to cold and of mechanical-induced allodynia, tests employed to assess oxaliplatin induced neurotoxicity. Moreover, OCT2 appears to be expressed on dorsal root ganglia cells, where oxaliplatin is known to accumulate and cause neurotoxicity. However, further studies are needed to evaluate the feasibility of such strategy, as other groups have reported that uptake of oxaliplatin in dorsal root ganglia cells is essentially mediated by Organic cation/ergothionine/carnitine transporters (OCTNs), with a very limited contribution of OCTs.[617] Moreover, as expression of OCT1 and OCT2 has been reported in samples from colorectal cancer patients (the target population for oxaliplatin administration) reassurance should be given that co-administration of an OCT inhibitor might even reduce the therapeutic efficacy of oxaliplatin.
Finally, modulation of OCT activity has been proposed as a potential strategy to improve activity of pralidoxime, an antidote given together with atropine in case of organophosphate poisoning. Treatment of Oct1/2(-/-) mice with pralidoxime resulted in a significant increase in drug plasma concentrations and improved antidotal activity of the drug, with sustained return within the normal range of respiratory variables in a paraoxon-poisoned rat model.[578]

3.4 Organic Cation/Ergothioneine/Carnitine Transporters (OCTNs)

The organic cation/ergothioneine/carnitine transporters (OCTNs) is a group of transporter proteins belonging to the Solute Carrier 22A subfamily. Actually it consists of OCTN1 (SLC22A4), OCTN2 (SLC22A5) and OCTN3 (Slc22a9), three transporters that share high homology, but each has unique transport characteristics for carnitine and organic cations. OCTN1 was first cloned from human fetal liver in 1997 by Tamai et al.[618] Subsequently, OCTN2 was cloned from a human placental trophoblast cell line using homology screening.[567] To date, only chicken and rodent OCTN3 has been discovered and no human counterpart has been found.[619,620]
Like the other OCTs, structurally OCTNs are membrane proteins composed of 11 (OCTN1) or 12 (OCTN2 and OCTN3) transmembrane domains (TMDs) with a large hydrophilic loop between TMD1 and TMD2, which carries potential N-glycosylation sites. Unlike OCTs, OCTNs contains a nucleotide binding site sequence motif.[567,618,621] OCTN1 shares around 70% identity with OCTN2 in amino acid sequence and OCTN1 and OCTN2 share about 30% and 35% identity with members of the OCT family, respectively.

Organic cation/ergothioneine/carnitine transporters are physiologically important in mediating the transport of ergothionine (OCTN1), carnitine (OCTN2), carnitinine ester

derivatives and organic cations. In mouse OCTN3 have also been described, but no human ortholog has been identified.
OCTNs, and in particular OCTN2 are responsible for the cellular transport as well as the intestinal uptake and renal reabsorption of carnitine and other organic cations. L-carnitine (3-hydroxy-4-N-trimethylaminobutyric acid) is essential for β-oxidation of long-chain fatty acids into the mitochondria and for regenerating the cytosolic coenzyme A pools. Although several cells synthesize L-carnitine, a number of tissues, in particular skeletal and heart muscles, are not able to produce L-carnitine and therefore they depend on the transcellular uptake of L-carnitine mediated by carnitine transporters.
OCTN1 and OCTN2 display a broad tissue distribution. OCTN1 is strongly expressed in kidney, bone marrow, trachea, fetal liver and certain cancer cell lines whereas it is weakly expressed in several other tissues.[618] High levels of OCTN2 have been found in the kidney, skeletal muscle, placenta, heart, prostate and thyroid, whereas low levels are detected in several tissues including brain, small intestine, liver, pancreas and lung.[619-625] OCTN3 has only been detected in mouse testis and, to a lower extent, in the kidney.[619]

Main substrate classes, Inhibitors and Inducers
Ergothioneine, carnitine and carnitine-esters (acetyl- and propionyl-carnitine), together with other important organic cations, are the main physiological substrates of OCTN1 and OCTN2. OCTN1 and OCTN2 substrates include TEA (tetraethylammonium) and the Ca^{2+}-antagonists verapamil and quinidine. OCTN2 has been reported to transport betaine, choline, lysine and methionine.[626] In *in vitro* models several clinically applied drugs, such as the β-lactam antibiotics cephaloridine, cefepime, cefoselis and ceftuprenam were reported to inhibit OCTN2-mediated carnitine transport in a competitive manner, suggesting being themselves substrates of OCTN2.[627] Analogously, widely used compounds like quinidine, procainamide, desipramine, cimetidine, clonidine, emetine and the hormones aldosterone and corticosterone compete with carnitine and/or acetyl-carnitine for OCTN2 transport. OCTN1 is inhibited by structurally diverse compounds including cephaloridine, quinine, cimetidine, procainamide and pyrilamine. Valproic acid seems to interfere with the regulation or synthesis of carnitine transporters. Emetine (an anti-amoebic and emetic compound) and pivalic acid (a substance contained in several antibiotics used in the treatment of respiratory and urinary tract infections) have been reported to inhibit OCTN2 activity.[626-635]

Pharmacological and Toxicological Function and Interactions
The physiological and pharmacological functions of OCTNs have been explored by generation of Octn1 and Octn2 knockout mice, respectively.
Octn1(-/-) mice appear to be fertile and do not present any phenotypic abnormalities. Metabolome analysis of blood and several organs indicated complete deficiency of ergothioneine, a potent antioxidant, in Octn1 knockout mice.[636] The pharmacokinetics of ergothioneine after oral administration were altered in Octn1(-/-) compared with wild-type mice. In particular intestinal absorption and renal reabsorption of ergothioneine were significantly reduced in knockout mice. Despite the lower absorption, plasma concentrations of ergothioneine after oral administration were higher in Octn1(-/-) mice than in wild-type mice, essentially due to significantly reduced hepatic uptake. These findings suggest a role for OCTN1 in the intestinal and hepatic uptake, and renal

distribution of substrate compounds. However, as a substantial amount of ergothioneine was orally absorbed even in Oct1 knockout mice, a contribution of other mechanisms/transporters is hypothesized.[637] Of note, Octn1(-/-) mice exhibited greater susceptibility to intestinal inflammation in an ischemia and reperfusion model, suggesting a role of OCTN1 for maintenance of systemic and intestinal exposure of ergothioneine, which could be important for protective effects against intestinal tissue injuries. In effect several single nucleotide polymorphisms of OCTN1 and OCTN2 genes (in particular a missense substitution in OCTN1 gene and a G→C transversion in the OCTN2 promoter) in the IBD5 locus (chromosome 5q31) have been associated with increased risk of Crohn's disease.[638] In a study conducted in Japanese subjects plasma ergothionine concentrations were significantly reduced in patients with Crohn's disease compared with healthy volunteers, whereas in another study no difference in ileal carnitine transport was observed between intestinal tissues from Crohn's patients and controls.[639] The potential pharmacological role of OCTN1 has recently been explored by administration of the OCTN1 substrate metformin to Octn1(-/-) and wild-type mice. As expected, the maximum plasma concentration after oral administration of metformin in Octn1 knockout mice was significantly higher compared with control mice, suggesting a role of OCTN1 in the oral absorption of metformin in the small intestine.[640]

The crucial physiological role of OCTN2 in maintaining homeostasis of carnitine, an essential cofactor in mitochondrial fatty acid oxidation, is proven by the severe pathological consequences associated with absence or dysfunction of OCTN2. Defects in OCTN2 result in systemic carnitine deficiency, a rare autosomal recessive disease characterized by several symptoms ranging from fatigability, elevated transaminases and creatine kinase to cardiac and skeletal myopathy, hypoketotic hypoglycemia, hyperammonemia, and encephalopathy. Over 100 mutations have been reported in the SLC22A5 gene, encoding OCTN2, responsible for systemic primary carnitine deficiency, with the c136C>T (p.P46S) mutation being the most frequent mutation identified. As in humans and in mice heterozygosity for OCTN2 mutations has been shown to produce a moderate carnitine deficiency phenotype, it has been hypothesized that moderate defects in OCTN2 activity caused by genetic polymorphisms could result in variability in the disposition of carnitine and other OCTN2 substrates.[641] The functional consequences of Octn2 gene mutation have been studied in the juvenile visceral steatosis (jvs) mouse model, which has a mutation in the mouse ortholog of OCTN2 and exhibits a phenotype very similar to systemic carnitine deficiency. In jvs mice significantly increased renal secretory clearance of carnitine and increased renal clearance of the xenobiotic tetraethylammonium was observed, compared with wild-type mice. Moreover, distribution of carnitine to several organs, including heart, lung, liver, brain, gut, spleen and muscle, was significantly reduced in jvs mice. Therefore, OCTN2 plays a crucial role in maintaining appropriate systemic and tissue concentrations of carnitine, essentially by regulating its renal reabsorption and tissue distribution, and, to a lesser extent, by regulating its membrane transport during intestinal absorption. Considering that OCTN2 has been reported to transport also other substrates, including clinically used drugs like verapamil, quinidine and several β-lactam antibiotics, it has been hypothesized that OCTN2 may alter disposition, efficacy and toxicity of substrate drugs. After intravenous administration of cephaloridine, a cephalosporin antibiotic, in jvs mice the plasma concentration profile and kidney-to-plasma concentration ratio were higher compared with wild-type mice, whereas renal clearance in jvs mice was significantly

reduced and could be accounted for by glomerular filtration.[642] Expression and activity of OCTN2 has also been reported to determine tissue-dependent disposition of specific substrates, therefore allowing transporter-mediated drug delivery to specific tissues. For instance, the expression of OCTN2 protein in vascular endothelium in heart is likely to play a role in the pharmacological activity of mildronate, a carnitine precursor clinically used for treatment of angina and myocardial infarction due to its anti-ischemic properties, which has been reported to be a good substrate of OCTN2.[643,644] In addition, platinum accumulation within the dorsal root ganglion and its sensory neurons has been reported as the major determinant of the neurotoxicity of oxaliplatin. In *in vitro* studies oxaliplatin is efficiently transported by OCTN1 and OCTN2 and by OCT1-3. However, OCTN1 appears to be the major transporter mediating the neuronal accumulation and resulting toxicity of oxaliplatin in dorsal root ganglions, contributing to approximately 50% of the uptake of oxaliplatin, with a lesser contribution from OCTN2 and no significant uptake mediated by OCT1-3. If further confirmed in clinical studies, these findings of OCTN-mediated neuronal uptake of oxaliplatin responsible for neurotoxicity could be employed to design strategies for targeting OCTNs with pharmacological inhibitors in order to reduce dose-limiting oxaliplatin associated neurotoxicity. Theoretically this strategy is feasible, as 1) inhibition of deletion of Octn1 gene in mice appears to be tolerable (Ocnt1 knockout mice do not present any obvious deficiencies), 2) only a transient inhibition of OCTN1 would be required to protect against oxaliplatin induced neuropathy due to the very short half-life of oxaliplatin, and 3) tissue selective expression of oxaliplatin transporters suggest that other transporters (in particular OCTs, MATEs) rather than OCTN1 contribute to oxaliplatin disposition and antitumor activity at least in colorectal carcinoma, the major current clinical indication for oxaliplatin. In effect, in preclinical studies co-incubation of oxaliplatin with ergothioneine, an OCTN1 competitive substrate, resulted in reduced oxaliplatin uptake and accumulation in rat dorsal ganglion neurons and neurons viability appeared more preserved. Moreover in some clinical studies administration of OCTN substrates (e.g., acetyl-L-carnitine, ergothioneine) has been associated with a reduction in platinum-induced neurotoxicity, although a subsequent study was not able to confirm such finding.[617,645-647] However, whether oxaliplatin-induced neurotoxicity could be prevented in humans using OCNT1 inhibitors without reducing antitumor activity and/or causing additional side effects should be further investigated, as OCTN1 could be involved in the excretion of oxaliplatin and therefore its inhibition might lead to a pharmacokinetic drug-drug interaction resulting in increased toxicity.[617]

The expression of OCTN2, in human bronchial epithelial cells has been postulated to be responsible for the pharmacological activity of ipratropium bromide, an anticholinergic drug used in the treatment of asthma and chronic obstructive pulmonary disease, when administered via inhalation. Indeed, distribution parameters after ipratropium inhalation indicate that active transport is involved and preclinical experiments confirm transport of ipratropium and tiotropium by OCTN2 and, to a lesser extent by OCTN1, suggesting a potential role of OCNTs in the airway absorption of such drugs. The strategy to use OCTNs for selective delivery of drugs to specific pharmacological targets has been the basis for the recent synthesis of a carnitine ester pro-drug of prednisolone aimed to selectively target bronchial epithelial cells.[648]

Genetic variation in OCTN1 and OCTN2 has been described and several single nucleotide polymorphisms have been associated with increased incidence of Crohn's disease,

rheumatoid arthritis, diabetes and asthma, but the mechanisms involved remain unknown.[648] As reported above, specific non-functional mutations of OCTN2 gene have been associated with development of primary carnitine deficiency.

Drug-drug interactions

Secondary systemic carnitine deficiency in humans has been associated with long-term treatment with the antiepileptic valproic acid, the β-lactam antibiotic pivampicillin, the emetic and antibiotic emetine, and the nucleoside analog zidovudine. The competitive inhibition of OCTN2-mediated carnitine transport by at least some of these compounds (e.g., valproic acid, emetine) is considered the major mechanism responsible for this interaction. Similarly, the inhibitory effect of valproic acid and other antiepileptic drugs on the transport of carnitine mediated by OCTN2 may cause the fetal anticonvulsant syndrome, of which some symptoms are similar to the primary fetal carnitine deficiency phenotype.[650,651] In effect, carnitine supplementation has been described to reduce the adverse reactions associated with long-term valproic acid therapy.[652]

Other clinically relevant drug-drug interactions mediated by OCTNs can be postulated as a number of clinically used drugs have been reported as OCTNs substrates and/or inhibitors. For instance, OCTN1 has been reported to transport verapamil, quinidine, pyrilamine, oxaliplatin, mitoxantrone, doxorubicin, gabapentin and ipratropium. Reported substrates of OCTN2 include mildronate, cephaloridine, oxaliplatin, imatinib, valproate, verapamil, quinidine, and spironolactone, whereas several anticancer drugs (i.e., etoposide, vincristine, vinorelbine), zidovudine, levofloxacin and grepafloxacin appear to inhibit OCTN2 activity. However, for several of these drugs contrasting results have been reported in the literature.[643,648,652] Therefore, further investigations are warranted in order to better establish the role of OCTNs in drug interactions.

In **table 3**, examples of clinical drug-drug interactions probably mediated by organic anion and cation transporters are reported.

Table 3. Examples of the possible involvement of organic anion and cation transporters in clinical drug-drug interactions

Drug	*Inhibitor/inducer*	*Measured effect/ toxicity*	*Putative mechanism*	*References*
Penicillin	Probenecid	Decreased renal clearance, prolonged half-life	Inhibition of OATs	112, 504-506
ACE inhibitors	Probenecid	Decreased renal clearance, prolonged half-life	Inhibition of OATs	973-978
Methotrexate	Penicillin	Decreased renal clearance, increased toxicity	Inhibition of OATs	973, 979-982
Methotrexate	NSAIDs	Decreased renal clearance	Inhibition of OATs	467, 495-497 503, 983
Methotrexate	Probenecid	Decreased renal clearance	Inhibition of OATs	467, 492
Adefovir/ Cidofovir	Probenecid	Reduced nephrotoxicity	Inhibition of OATs	984-987
Cephalosporins	Probenecid	Increased peak plasma concentration and terminal half-life	Inhibition of OATs	504, 988-990
Furosemide	Probenecid	Decreased renal clearance	Inhibition of OATs	513
Zidovudine	Cimetidine	Decreased renal clearance	Inhibition of OCTs, OATs	991,992
Zidovudine	Probenecid	Decreased renal clearance	Inhibition of OATs	992-994
Famotidine	Probenecid	Increased plasma concentration, decreased renal clearance	Inhibition of OATs	995
Oxypurinol	Benzbromarone	Decreased plasma level, increased renal clearance	Inhibition of URAT1	511
Digoxin	Amiodarone	Increased plasma levels and toxicity	Inhibition of OATPs, MDR1	548, 996-999
Fexofenadine	Fruit juices (grapefruit, orange, apple)	Decreased plasma AUC and maximum plasma concentration	Inhibition of OATPs, MDR1	526, 1000
Fexofenadine	Erythromycin	Increased plasma levels	Inhibition of OATPs, MDR1	1001
Fexofenadine	Cimetidine	Decreased renal clearance	OATPs, MATEs	762
Repaglinide	Cyclosporin A	Increased plasma AUC	Inhibition of OATPs, MDR1, CYP	1002-1004
Bosentan	Cyclosporin A	Increased plasma levels	Inhibition of OATPs	1005

Table 3. ***(continued)***

Drug	*Inhibitor/inducer*	*Measured effect/ toxicity*	*Putative mechanism*	*References*
Statins	Cyclosporin A	Increased plasma AUC and maximum plasma concentration	Inhibition of OATPs, CYP3A4, CYP2C8, CYP2C9	541
Pravastatin	Cyclosporin A	Increased AUC and plasma levels	Inhibition of OATPs	1006
Cerivastatin	Cyclosporin A	Increased AUC and plasma levels	Inhibition of OATPs, CYP2C8/3A4	542, 1007
Atorvastatin	Cyclosporin A	Increased AUC and plasma levels	Inhibition of OATPs, CYP3A4	1008
Pitavastatin	Cyclosporin A	Increased AUC and plasma levels	Inhibition of OATPs	1009
Statins	Gemfibrozil	Increased myotoxicity	Inhibition of OATPs, MRP2, BCRP, CYP2C8, CYP2C9	1010-1011
Simvastatin	Gemfibrozil	Increased AUC and plasma levels	Inhibition of OATPs	1012
Cerivastatin	Gemfibrozil	Increased AUC and plasma levels	Inhibition of OATPs, CYP2C8/3A4	1010, 1013, 1014
Lovastatin	Gemfibrozil	Increased AUC and plasma levels	Inhibition of OATPs	1015
Rosuvastatin	Gemfibrozil	Increased AUC and plasma levels	Inhibition of OATPs, CYP2C9	1016
Metformin	Cimetidine	Decreased renal clearance, increased AUC	Inhibition of OCTs, OATs, OATPs, MATEs	575
Procainamide	Cimetidine	Decreased renal clearance, increased AUC	Inhibition of OCTs, OATs, OATPs,MATEs	1017
Dofetilide	Cimetidine	Decreased renal clearance, increased AUC	Inhibition of OCTs, OATs, OATPs	1018
Pilsicainide	Cimetidine	Decreased renal clearance, increased AUC and half life	Inhibition of OCTs, OATs, OATPs	1019
Pilsicainide	Cetirizime	Mutually reduction of renal clearance	Inhibition of OCTs, MDR1	608
Theophylline	Erythromycin	Increased plasma levels	Inhibition of OAT, CYPs	1020

4 - INTERACTIONS MEDIATED BY PEPTIDE TRANSPORTERS (PEPTS, SLC15)

The mammalian peptide transporter proteins belong to the Proton-coupled Oligopeptide Transporter (POT) superfamily, also known as Peptide Transporter family (PTR). Currently, the mammalian members of the POT family are divided into two subfamilies, represented

by the peptide transporters PEPT1 (SLC15A1) and PEPT2 (SLC15A2) and the subsequently discovered peptide/histidine transporters PTH1 (SLC15A4) and PTH2 (SLC15A3).[653]
Peptide transporters are predicted to contain 12 transmembrane domains (TMD) with both amino and carboxy termini localized intracellularly. In general they are proton/peptide transporters able to translocate di- and tripeptides and peptido-mimetic compounds against a substrate concentration gradient. In addition, PHT1 and PHT2 can also transport the amino acid histidine. PEPT1 transports neutral and cationic oligopeptides with a 1:1 proton to substrate stoichiometry ratio and charged anionic substrates with a 2:1 ratio.[654-656] Neutral substrates are translocated by PEPT2 with a 2:1 stoichiometry ratio and charged compounds with variable coupling ratios.[657] Furthermore, the extracellular pH has been reported to affect the affinity of peptide transporters for charged substrates.
PEPT1 is abundantly expressed at the apical membrane of enterocytes in the small intestine, in the renal proximal tubule (pars convolute, segment S1) and in epithelial cells of the bile duct.[658-661] PEPT1 is also identified in the pancreas and there is also some evidence for the presence of peptide transporters in the placenta.[662,663] PEPT2 is more widely expressed and is found in the segment S2 and S3 (pars recta) of the renal proximal tubule, in bronchial epithelial cells, type II pneumocytes, mammary gland, Muller cells in the retina, and in cells in dorsal root ganglia.[664-668] In the central nervous system of animal models PEPT2 is observed in astrocytes and in cells contributing to the blood-brain and blood cerebrospinal barriers (such as choroid plexus, ependyma and subependyma).[669-671]
Less is known about the tissue distribution of the putative peptide/histidine transporters (PHT1 and PHT2). Both PHT1 and PHT2 are found in human placenta and in gastrointestinal mucosa [Herrera-Ruiz D 2001]. The tissue distribution of peptide transporters suggests that these transporters play an essential role in the maintenance of body homeostasis. The main physiological role of PEPT1 has been reported to be the absorption of di- and tripeptides from the intestinal lumen. PEPT2, together with PEPT1, has been suggested to contribute to the renal tubular reabsorption of di- and tripeptides, avoiding their loss into urine. In brain tissue, there is some evidence that peptide transporters contribute to antioxidant mechanisms (e.g. mediated by glutathione) and in the synaptic removal of certain neurotransmitters, such as N-acetyl-L-aspartyl-L-glutamate.[672-674] PEPT2 in the choroid plexus epithelium was also found to contribute to the removal of neuropeptides, peptide fragments and peptidomimetics from the brain extracellular space into the cerebrospinal fluid. PEPT2 activity may also have a nutritional function by supplying small peptides from the blood circulation to tissues of the choroid plexus and of the cerebrospinal fluid.[675,676]

Main substrate classes, Inhibitors and Inducers

- *Main substrate classes.* There are some differences in substrate specificity and affinity as well as in transport capacity, between PEPT1 and PEPT2. PEPT1 is considered a low affinity, high capacity transporter whereas PEPT2 is a high affinity, low capacity system. The physiological substrates of PEPT1 and PEPT2 include all 400 different dipeptides and around 8000 possible tripeptides derived from the proteogenic L-α-amino acids. These transporters are stereoselective as they display higher affinity for oligopeptides containing L-enantiomers of amino acids residues than peptides with D-enantiomers. Moreover, as recently PEPT1 has been reported to transport simple ω-amino fatty acids that do not contain a peptide bond, several studies were performed to identify the

essential features of PEPT substrates. The minimal structure of PEPT substrates consists of two charged moieties at opposite ends (carboxyl and amino groups) separated by at least four methylene groups.[678] Furthermore, PEPT1 and PEPT2 effectively transport a significant number of peptidomimetic drugs, including penicillin β-lactam antibiotics (e.g. ampicillin, cyclacillin, amoxycillin) and cephalosporins (e.g. cefadroxil, cefixime, ceftibuten, cephalexin, cepharadine), anticancer drugs (e.g., didanosine, decitabine), angiotensin-converting enzyme (ACE) inhibitors (e.g. captopril, fosinopril and enalapril), antiviral nucleosides (e.g., zidovudine, gancyclovir, valacyclovir, oseltamivir), and the aminopeptidase inhibitor bestatin (also named ubenimex). PEPT substrates include also some pro-drugs, such as 3,4-dihydroxy-L-phenylalanine (L-DOPA, used in the treatment of Parkinson disease), and the antiviral L-valyl ester of acyclovir (called valaciclovir). Several non peptidic compounds are also transported by PEPTs, such as the photosensitizing agent 5-aminolevulinic acid (δ ALA), widely used in photodynamic therapy, and sulpiride, a selective dopamine D2 receptor antagonist.[677-687]

Less is known about PHT1 and PHT2 substrates. Both are reported to mediate the proton-dependent uptake of histidine and several di- and tripeptides.[688,689]

- *Inhibitors*. Several inhibitors of peptide transporters were identified during screening for substrates of these transporters, whereas others were synthesized more recently by a rational approach. 4-Aminomethylbenzoic acid (4-AMBA) is the first competitive not-transported PEPT1 inhibitor described.[689] Other clinically relevant non-competitive PEPT1 inhibitors are the orally active sulfonylurea-antidiabetic drugs nateglinide and glibenclamide as well as tolbutamide and chlorpropamide.[691,692] Recently, several high affinity competitive inhibitors of PEPT1 and PEPT2 have been developed: they are lysine-containing dipeptide derivatives starting from lysyl-4-nitro-benzyloxycarbonyl-proline (Lys[Z(NO2)]-Pro).[693,694] Moreover, certain compounds with a structure related to cephalosporins and ACE inhibitor-ester pro-drugs, such as quinalapril and quinalaprilat, fosinoprilat and enalaprilat, have been reported to block peptide transporter activity with low affinity.[695-697] Anti-hypertensive sartans like losartan, valsartan, irbesartan and eprosartan, are also reported to inhibit PEPTs.

-*Inducers.* The activity of peptide transporters can be modulated by several substances and in pathophysiological conditions. In particular, PEPT1 may be modulated at the transcriptional level or at the level of translocation of transporter proteins to the plasma membrane. Treatment with insulin, leptin, human growth hormone, pentazocine, clonidine or Ca^{2+} channel blockers as well as addition of high amounts of dipeptides in medium of cultured (Caco 2) cells expressing PEPT1 were reported to induce PEPT1 expression.[698-702] Dietary conditions and several pathophysiological states can modulate PEPT expression: in rats, a brief period of fasting or starvation or administration of a diet enriched in certain free amino acids and peptides was associated with up-regulation of PEPT1 expression.[704-707] A circadian regulation of intestinal PEPT1 expression has also been described in rats.[708,709] Diabetes, induced by treatment of rats with streptozotocin, resulted in an increased expression of PEPT1 in the small intestine.[710] Unusually high intestinal colonic levels of PEPT1 have been identified in patients affected by short-bowel syndrome, chronic ulcerative colitis, Crohn's disease or acute cryptosporidiosis, whereas PEPT1 is virtually absent in the colon of healthy subjects.[711-714] In contrast, endotoxin and lipopolysaccharide are associated with down-regulation of PEPT1 in animal models. Less is known regarding modulation of PEPT2 expression: up-regulation of PEPT2 has been described in rat remnant kidney after unilateral nephrectomy.[715-717]

Pharmacological and Toxicological Function

The physiological activity and the pharmacological and pathophysiological role of peptide transporters PEPT1 and PEPT2 have been studied by generation of Pept1 and Pept2 knockout mice. Pept1, as well as Pept2 knockout mice are healthy and viable and do not display any obvious phenotype. However, Pept1 deletion dramatically reduced the intestinal uptake and effective permeability of the model dipeptide PEPT substrate glycylsarcosine (i.e., by at least 80%), and its oral absorption following gastric gavage (i.e., by about 50%). In contrast, no significant difference in glycylsarcosine plasma concentrations was observed between the two mice strains after intravenous dosing. These findings indicate that PEPT1 plays a major role in the oral absorption of dipeptides and therefore may potentially affect the oral bioavailability of substrate drugs.[716] This was also anticipated by the already known function and tissue distribution of PEPT1 in humans and rodents, as PEPT1 is located on the brush border membrane of the small intestine, where it works as a high-capacity and low affinity transporter responsible for the absorption of small peptide fragments from the digestion of dietary proteins. In humans compounds that are structurally similar to oligopeptides and are PEPT1 substrates have shown good oral bioavailability, whereas class analogs not transported by PEPT1 display worse absorption after oral administration. In a study comparing several penicillins and cephalosporins, a significant correlation was found between the *in vitro* affinity for PEPT1 and their reported oral availability. In particular, aminopenicillins and aminocephalosporins that displayed higher affinity and higher *in vitro* transport rates than other (β-lactam antibiotics showed higher absorption rates in vivo.[717-719] Based on these findings, a strategy has been developed to improve the oral bioavailability of drugs by coupling an amino acid residue to a drug to obtain a peptide or a peptidomimetic compound transported by peptide transporters. For example, L-dopa-L-Phe, a peptide derivative developed as a pro-drug of L-dopa, displayed an around 40-fold higher intestinal uptake than that of free L-dopa, a drug widely used in the treatment of Parkinson's disease.[720] Analogously, valacyclovir, the L-valyl ester of acyclovir, a well-known antiviral drug, showed 3- to 5-fold higher oral bioavailability than that of free acyclovir (not transported by PEPT1 in *in vitro* models).[721-724] Similar results have been obtained with production of the L-valine ester of ganciclovir and zidovudine (used in the treatment of HIV infection). This transporter (PEPT1) mediated pro-drug approach is being increasingly used to improve the pharmacological profile of different kind of compounds ranging from antiviral nucleosides (oseltamivir), anticancer drugs (didanosine, decitabine), antibiotics (carbapenems), etc.[680-682,724-729] This novel "targeted-prodrug" strategy employing carrier-mediated transport could offer promising opportunities for precise and efficient drug delivery and for improving therapeutic efficacy and safety.

Recently, a pathophysiological role of PEPT1 in inflammatory bowel disease (e.g., chronic ulcerative colitis, Crohn's disease) has been hypothesized. Up-regulation of PEPT1 has been reported in the colon of patients with chronic bowel inflammation and *in vivo* and *in vitro* findings suggest that PEPT1 can transport various bacterial di/tripeptides into colon cells leading to activation of pro-inflammatory responses mediated by interactions with immune receptors. These findings could be of importance for the development of treatment strategies targeting PEPT1 in order to alleviate inflammation in patients with chronic inflammatory bowel disease. In effect, in mouse models with induced colitis, treatment with the anti-inflammatory PEPT1 tripeptide ligand Lys-Pro-Val, also in the

form of nanoparticles, resulted in clinical and pathological attenuation of intestinal inflammation.[729]

The physiological and pharmacological function of PEPT2 was anticipated by its tissue distribution and substrate recognition, as in humans and rodents it functions as a high-affinity and low capacity transporter, essentially located in the proximal tubule of the nephron and on the apical membrane of choroid plexus epithelial cells at the blood cerebrospinal fluid barrier. However, studies performed with Pept2 knockout mice have demonstrated that PEPT2 plays a major role in renal handling and reabsorption of peptide substrate and peptide-mimetic drugs, as well as it is involved in neuropeptide homeostasis and removal of neurotoxins from the brain. Indeed, in Pept2 knockout mice the clearance of the model substrate glycylsarcosine was significantly (2-fold) increased, resulting in a lower systemic exposure to the compound. Moreover, renal reabsorption was almost abolished and glycylsarcosine was eliminated almost exclusively by glomerular filtration. Of the 46% of glycylsarcosine reabsorbed in wild-type mice, PEPT2 accounted for 86% and PEPT1 for 14% of reabsorbed substrate. Pept2 (-/-) mice displayed lower choroid plexus concentrations of glycylsarcosine and a 5-fold lower choroid plexus-to-cerebrospinal fluid ratio compared with wild-type mice at 60 min.[730] Similarly, intravenous administration of cefadroxil, an aminocephalosporin antibiotic, at different doses, resulted in nonlinear disposition over the dose range studied, due to both saturable renal tubular secretion and reabsorption. At a clinically relevant dose in Pept2(-/-) mice cefadroxil clearance was 3-fold higher and the plasma concentrations were 3-fold lower compared with wild-type mice. Renal reabsorption of the drug was also almost completely abolished in knockout compared with control mice (i.e., 3% versus 70%, respectively). Of the 70% of cefadroxil reabsorbed in wild-type mice, 95% was mediated by PEPT2 and only 5% by PEPT1. Importantly, despite the lower systemic exposure, the cerebrospinal fluid-to-blood concentration ratio of cefadroxil was 6-fold higher in Pept2 knockout compared with control animals. As PEPT2 appears to significantly limit the exposure of cefadroxil in the cerebrospinal fluid, it can be hypothesized that the drug (and possibly other aminocephalosporins substrate for PEPT2) may be ineffective for the treatment of meningitis, due to sub-therapeutic concentrations of the drug at the active site of action. In theory, the development of drugs with limited PEPT2 affinity or the design of selective PEPT2 inhibitors could be a useful strategy to improve brain delivery of drugs aimed to treat brain disorders. However, subsequent experiments performed with Pept2 knockout mice suggest that modulation of PEPT2-mediated effect on drug disposition in the brain could translate into significant changes in drug-related toxicity. Intravenous administration of the PEPT1 and PEPT2 substrate 5-aminolevulinic acid (5-ALA, a precursor of cellular porphyrin synthesis and widely used as a photosensitizer in the photodynamic therapy of several tumors) to Pept2 (-/-) mice resulted in a 2-fold higher clearance, a 2-fold lower systemic exposure, a 5-fold greater concentration in the cerebrospinal fluid, and a 8-fold higher cerebrospinal fluid-to-blood concentration ratio compared with wild-type mice. This was associated with a significant increase in neurotoxicity. Indeed, after chronic administration of 5-ALA knockout mice presented significantly higher neuromuscular dysfunction (i.e., reduced ability to maintain balance on a rotating rod) and shorter survival (at higher dose administered) compared with wild-type mice. These results indicate a clinically relevant neuroprotective role of PEPT2. Moreover, the observed ability of PEPT2 to limit 5-ALA exposure in cerebrospinal fluid suggests that it has a role as

secondary genetic modifier in the brain sensitivity of diseases (e.g., hepatic porphyria and lead poisoning) where metabolism of 5-ALA is impaired.[730] These data clearly show a role of PEPT1 and PEPT2 beyond drug disposition, where, in particular PEPT2, modulates also the pharmacodynamics and toxicodynamic effects of drug substrates.

Although high sequence homology and similar tissue distribution and substrate specificity has been observed between mouse and human PEPT1 and PEPT2 proteins, additional studies are warranted in order to evaluate the translation of the results observed in mice to the human situation. In addition, although several genetic variants of PEPT1 and PEPT2 have been described (some with impaired or abolished function), it is unclear at this time whether and to what extent genetic polymorphism in such transporters affects drug disposition, dynamics and toxicity and could explain inter-individual variability in drug therapy. Few studies have reported associations of specific PEPT1 polymorphisms with development of inflammatory bowel disease, but results are contrasting and need further validation.[729] To date no clinical consequences for any PEPT1 and PEPT2 variants have been found. This could also be related to compensatory increased expression of other transporters/mechanisms with overlapping substrate specificity and function.[681,729]

Finally, PEPTs have been found on the cell membrane of a range of cancer tissues (e.g., pancreatic, gastric, colon and prostate cancer). A PET tracer targeted to PEPTs (11C-glycylsarcosine) has been tested in mice with xenograft tumors with promising results and potential advantages compared with the standard 18F-FDG due to the lack of affinity for inflammatory tissues.[731] However, their role is poorly understood and it is currently unknown whether targeting this expression would affect the outcome of anticancer drug therapy or whether these transporters would represent a useful target for tumor-specific drug delivery.

Drug-Drug Interactions

Clinically relevant drug-drug interactions mediated by peptide transporters have been reported between different β-lactam antibiotics and/or inhibitors of these transporters. Oral co-administration of cefadroxil and cephalexin has been shown to delay and decrease the time to maximal plasma concentration and decrease the AUC, respectively, of cefadroxil, presumably due to competitive inhibition by cephalexin of the intestinal PEPT1.[733] Similarly, altered pharmacokinetics of the β-lactam ampicillin and amoxycillin were described after oral administration, together with cyclacillin, to healthy volunteers. Competitive inhibition at the intestinal PEPT1 site is the hypothesized mechanism for this interaction.[734,735] Nifedipine, a widely used Ca^{2+} antagonist, has been reported to increase the bioavailability of orally administered amoxycillin and cefixime in humans and cephalexin in rats. One of the mechanisms proposed to explain these drug-drug interactions involves a change in the intestinal surface pH mediated by nifedipine (due to a decreased concentration of intracellular Ca^{2+}), thereby increasing the driving force for β-lactam drug transport mediated by PEPT1.[702,736-739]

In view of the wide range of clinically used drugs and pro-drugs which are transported by PEPTs (including but not limited to penicillin β-lactam antibiotics (e.g., ampicillin, cyclacillin, amoxycillin), cephalosporins (e.g., cefadroxil, cefixime, ceftibuten, cephalexin, cepharadine), antiviral nucleosides (e.g., zidovudine, gancyclovir, valacyclovir, oseltamivir)) and/or are reported to inhibit PEPTs in a competitive or not-competitive fashion (e.g., ACE inhibitors like zofenopril and fosinopril; sartans like losartan, valsartan, irbesartan

and eprosartan; sulfonylureas like nateglinide, glibenclamide and tolbutamide) several clinically relevant drug-drug interactions may be postulated.
On the other hand, taken in consideration the postulated involvement of PEPT1 in intestinal inflammation and oral drug absorption, and the activity of PEPT2 at the renal and at the blood cerebrospinal barrier, selective targeting of PEPTs could be a beneficial strategy improving drug delivery and therapeutic efficacy, but avoiding toxicity.

5 - INTERACTIONS MEDIATED BY MULTIDRUG AND TOXIN EXTRUSION TRANSPORTERS (MATES, SLC47)

Multidrug And Toxin Extrusion (MATE; SLC47A) proteins are efflux transporters acting as proton/cation antiporters at the brush-border membrane of proximal tubule cells in the kidney and in canalicular membranes of hepatocytes, thereby contributing to excretion of cationic endogenous substances and xenobiotics. Mammalian orthologs have been identified in humans, mice, rats and rabbits.[740-742] Mammalian MATE transporters can be classified in three phylogenetic subgroups: class I, which includes human (h), mouse (m), rat (r) and rabbit (rb) MATE1; class II, which comprises hMATE2, hMATE2-K, hMATE2-B, and rbMATE2-K; and class III, which includes rodent MATE2.[743,744] Transcripts of hMATE1 appear to be ubiquitously expressed in the body, with highest expression in kidney, liver, adrenal gland, and skeletal muscle.[740] MATE2, MATE2-K and MATE2-B transcripts are mainly detected in the kidney, with lower levels in other various human tissues.[745,746]. At the protein level, human and mouse MATE1 and hMATE2 are localized at the luminal (apical) membrane of proximal tubule cells in the kidney and MATE1 also in the canalicular (apical) membrane of hepatocytes.[740] The observed localization of MATE transporters in kidney and liver suggests a role in excretion of endogenous compounds and xenobiotics, including drugs, into urine and bile. Moreover, their expression in testis, adrenal and thyroid gland support the hypothesis of an involvement in secretion of hormones.[743,747,748]

Main substrate classes and Inhibitors

In in vitro studies MATE1 and MATE2-K present similar inhibitor and substrate specificities partially overlapping with those of OCTs. Endogenous substrates include the organic cations guanidine, thiamine, creatinine, estrone-3 sulfate and methylnicotinamide.[112] Several drugs have been reported to be transported by MATEs at clinically relevant concentrations. They include, but are not limited to, acyclovir, ganciclovir, metformin, levofloxacin, cephalexin, oxaliplatin, topotecan, fexofenadine, and procainamide. A number of drugs with inhibitory activity have been also observed (e.g., cimetidine, cetirizine, ciprofloxacin, levofloxacin, clonidine, clotrimazole, ketoconazole, erlotinib, gefitinib, dasatinib, nilotinib, sunitinib, imatinib, diltiazem, methotrexate, famotidine, omeprazole, ondansetron, ranitidine, procainamide, ritonavir, thrimethoprim, tolbutamide).[743,748,749]

Pharmacological and toxicological functions

The pharmacological and physiological functions of MATEs were partly elucidated by generation of Mate1 knockout mice.[750,751] Mate1$^{(-/-)}$ mice are viable, fertile and do not display any obvious physiological abnormalities. Therefore, compensation of Mate1 deficiency by other transporters can be assumed. In a model of chronic renal failure

(induced by nephrectomy) the expression of rMATE1 protein was reduced in the proximal tubules of rat kidney, whereas rOCT2 expression was unchanged. This resulted in a markedly decreased tubular secretion of cimetidine (proportional to the level of MATE1) suggesting that expression of MATE1 can be influenced by pathological conditions and that represents a crucial factor in tubular secretion of cimetidine.[752] In an acute kidney injury model (induced by ischemia/reperfusion), the protein expressions of basolateral rOCT2 and luminal rMATE1 were down-regulated in rat kidneys. This resulted in a 6-fold increase in the area under the plasma concentration curve for tetraethylammonium, a substrate of rMATE1, rOCT1 and rOCT2.[753] Therefore, it can be hypothesized that renal excretion in the urine of cationic drugs of endogenous compounds, like cimetidine or tetraethylammonium, may be influenced by an altered MATE function, and that several pathophysiological conditions (like acute or chronic renal failure) are able to influence the expression of MATE and other transporters with potentially serious consequences for the pharmacokinetics of substrate drugs. On the other hand, the expression of rMATE1 in the kidney, contrary to rOCT2, did not appear to be influenced by acute cholestasis, as induced by bile duct ligation in rats.[743,754]

The potential pharmacological role of MATE1 in drug disposition and elimination has been illustrated by the altered pharmacokinetics of several substrate drugs observed in Mate1 knockout mice. After a single intravenous administration of the antimicrobial drug cephalexin (5 mg/kg) a 1.5 fold increase of plasma drug concentration in MATE1(-/-) compared with wild type mice was observed. The renal clearance of cephalexin was also reduced to approximately 60%, whereas non-renal clearance remained unchanged. In contrast, there were no significant differences between both mice strains in the pharmacokinetics of anionic cefazolin, which is not a substrate for MATE1.[755] Similarly, after a single intravenous administration of the antidiabetic drug metformin (5 mg/kg), a 4.2-fold increase in plasma concentrations and 2-fold increase in the area under the blood concentration-time curve of metformin in Mate1(-/-) mice compared with wild type mice was observed, associated with a reduction in renal clearance of the drug to 18% and with a significantly reduction of urinary excretion of metformin. As a consequence of impaired excretion, renal tissue concentration of metformin was increased in knockout mice. Of note, the mRNA levels of other organic cation transporters such as Octs did not differ significantly between wild-type and Mate1 knockout mice, further supporting the role of MATE1 in distribution and systemic clearance of metformin.[750] another study, hepatic tissue concentrations of metformin were significantly increased in Mate1 knockout mice and led to lactic acidosis, suggesting a role of MATE1 in biliary excretion of metformin. However, metformin pharmacokinetics was not altered in heterozygous Mate1(+/-) mice.[756,757] These results suggest that homozygous dysfunctional MATE1 variants could be one of the risk factors for metformin-induced lactic acidosis. However, extrapolation to the human situation should be made with caution, due to differences in species specificity of MATE transporters, in particular regarding their tissue distribution. Indeed, MATE1 represents the only MATE transporters expressed in the mouse/rat kidney, whereas in human kidney expression of MATE2 is also present.[743]

MATE transporters have recently also been associated with cisplatin-induced nephrotoxicity.[615,758] Significantly increased plasma and renal tissue concentrations were observed in Mate1(-/-) compared with wild type mice after intravenous administration of cisplatin. Moreover, a significant rise in creatinine and blood urea nitrogen (BUN)

levels was observed in cisplatin-treated Mate1(-/-) mice in comparison to Mate1(+/+) after intraperitoneal administration of the drug. Similarly, in wild type mice co-administration of cisplatin with pyrimethamine, a selective MATE inhibitor, resulted in an increase in creatinine and BUN levels compared to cisplatin alone suggesting that not only a congenital deficiency but also a temporary functional deficiency of MATE1 could potentiate cisplatin nephrotoxicity.[615,743] Active tubular excretion of cisplatin appears to be supported by several pre-clinical and clinical reports where the renal clearance of cisplatin exceeded the glomerular filtration rate in humans and rats, suggesting cisplatin excretion across renal tubular cells.[615] In several articles reported in the literature, it is hypothesized that the nephrotoxicity of platinum agents, associated with the amount of platinum accumulated in the kidneys, is correlated to the affinity of the different platinum compounds for organic cation (OCT1-3) and MATE transporters. The renal toxicity of cisplatin would be the result of extensive accumulation by hOCT2 from the circulation and weak tubular excretion in the urine by hMATE1. In contrast, the lower renal accumulation of oxaliplatin and the reduced risk of nephrotoxicity could be related to more extensive transport of the drug by hOCT2, hOCt3, hMATE1, and hMATE2-K. The lack of affinity of carboplatin and nedaplatin for organic cation and MATE transporters could explain the absence of a nephrotoxic effect associated with administration of these drugs.[747]

Drug-Drug interactions

A number of drug-drug interactions probably mediated by MATEs have been described.[743,747-749] The importance of interactions mediated by MATE transporters is underlined by the fact that, despite the recent discovery and characterization, the evaluation of an interaction of new compounds mediated by MATE1 and MATE2 transporters is recommended by European and American regulatory authorities in guideline documents.[68,69]

Cimetidine, a H2-receptor blocker with MATEs and OCT2 inhibition activity, has been reported to reduce renal elimination of procainamide[759], cephalexin[760], metformin[761], and fexofenadine[762], all well-known MATE substrate compounds, in humans.[743] Moreover, pyrimethamine, a specific MATE-inhibitor, increasingly employed in clinical practice for the treatment of malaria due to chloroquine resistance in African countries, significantly increased the renal and hepatic concentration of metformin at clinically relevant doses in mice.[763] In healthy volunteers pyrimethamine significantly reduced the renal clearance of metformin by 35% and increased the maxima plasma concentration and AUC of metformin (142 and 139% of control values, respectively) at therapeutic doses.[764] Similarly, in view of the above mentioned pre-clinical studies, co-administration of pyrimethamine and the anticancer drug cisplatin is expected to potentiate cisplatin-induced nephrotoxicity. Of note, several MATE substrates and inhibitors also interact with organic cation transporters (OCTs), which mediate the uptake of organic cations from blood into hepatocytes and proximal tubular epithelial cells. This partial overlap in substrate specificity between MATE and organic cation transporters (OCTs) is functionally complementary, as for instance it allows the secretion of organic cations across the renal epithelial cells via OCT2 (expressed at the basolateral side) and MATEs (located at the apical cell side). However, the affinity of substrates and/or inhibitors of such transporters may vary substantially, with potentially clinically relevant consequences. For instance, pyrimethamine is a high affinity MATE1 and MATE2 inhibitor with Ki values in the nanomolar range, whereas the Ki for OCT2 is

in the micromolar range (10 μM). This may lead to an impairment of renal elimination of substrate compounds even at low inhibitory drug concentrations when luminal MATE –mediated efflux is already blocked but the OCT2-mediated basolateral uptake is still unaffected. Similarly, the inhibitory potency of cimetidine for MATE transporters appears to be stronger (and in the range of clinically reached plasma concentrations) than for OCT2. Renal excretion of cisplatin has been reported to be mediated by OCT2 and MATE transporters, which are considered responsible for the determination of cisplatin-induced nephrotoxicity. It was therefore hypothesized that administration of an OCT2 inhibitor (cimetidine or imatinib) would have been able to reduce the risk of nephrotoxicity. Actually an excess of cimetidine, as achieved in preclinical models, was able to inhibit cisplatin nephrotoxicity.[612] However, in view of the stronger inhibitory effect of cimetidine of MATE than of OCTs, a clinical dose of cimetidine could actually potentiate cisplatin-induce nephrotoxicity. In contrast, the inhibitory potency of imatinib appears to be similar for OCT2 and MATEs, and therefore a clinically administered dose of imatinib could to decrease renal accumulation and toxicity of cisplatin.[614,747,748] When trying to implement a drug as renoprotective agent for cisplatin-induced nephrotoxicity, inhibitory potency for different transporters, blood and renal concentrations of the drug should be considered.

Another clinically relevant drug-drug interaction potentially mediated, at least in part, by MATE transporters, has been reported between the antimalarial drug quinine and the antiviral agent ritonavir. Concurrent administration of ritonavir in healthy volunteers led to about 4-fold increase in both maximal plasma concentration and AUC of quinine. Clearance of quinine was also about 4.5-fold reduced. Although the interaction has been considered mediated essentially by CYP3A4 metabolizing enzymes, involvement of MATE transporters cannot be excluded, as at the dose administered quinine reached plasma concentrations able to inhibit MATEs and ritonavir is a well-known substrate of MATEs.[749,765]

Recently, a clinical case report concerning a fatal lactic acidosis induced by a drug-drug interaction between erlotinib (EGFR tyrosine kinase inhibitor) and metformin in a patient with lung cancer has been described.[766,767] Of note, metformin is a substrate of MATEs and several tyrosine kinase inhibitors (i.e., erlotinib, gefitinib, imatinib, sunitinib, dasatinib and nilotinib) and their major metabolites have been reported to inhibit MATE and OCT transporters *in vitro* at clinically relevant concentrations. A drug interaction resulting in increased levels of metformin with increased patient susceptibility to lactic acidosis cannot be ruled out. Clinical interactions studies are warranted in order to better explore such potentially life-threatening interactions, as several studies combining tyrosine kinase inhibitors with metformin and with anticancer drugs like oxaliplatin, which is a good substrate for MATEs, are being performed in patients.[767]

In addition, several clinically used drugs (e.g., acyclovir, ganciclovir, levofloxacin, cephalexin, oxaliplatin, topotecan, fexofenadine, procainamide, etc.) have been reported to interact with hMATE transporters, frequently at clinically relevant concentrations. Other compounds with inhibitory activity have been also observed (e.g., cimetidine, cetirizine, ciprofloxacin, levofloxacin, clonidine, clotrimazole, ketoconazole, erlotinib, gefitinib, dasatinib, nilotinib, sunitinib, imatinib, diltiazem, methotrexate, famotidine, omeprazole, ondansetron, ranitidine, procainamide, ritonavir, thrimethoprim, tolbutamide).[743,748,749] However, *in vivo* clinical interaction studies are considered necessary to explore the clinical relevance of potential drug-drug interactions mediated by MATEs, and their interplay with other transporters and metabolizing enzymes, resulting in altered pharmacodynamic

processes. Moreover, although several human genetic variants of the SLC47A1 and SLC47A2 gene have been identified, only limited data are available exploring the effect of single nucleotide polymorphisms in SLC47A genes on the interindividual variability of drug disposition, response and toxicity. For instance, conflicting results have been published regarding the association of a genetic variant (c.922-158G>A) with an increased glucose-lowering effect of metformin, possibly due to decreased efflux and increased liver intracellular concentrations of metformin.[743,768-770]

Affinity of specific substrate drugs for MATE transporters could result in an unbeneficial pharmacokinetic profile, seriously limiting the clinical development of the drug. For instance, the clinical development of a novel oxazolidinone antibiotic (PFU-288034) was early terminated due to insufficient exposure in a phase I study. This was attributed to extensive renal secretion essentially coordinated by OAT3 in the basolateral membrane and MATE1 in the luminal membrane of renal proximal tubule cells, as supported by compelling *in vitro* studies.[749,771]

6 - INTERACTIONS MEDIATED BY MONOCARBOXYLATE TRANSPORTERS (MCTS, SLC16)

The monocarboxylate co-transporter family (MCT, SLC16 gene family) is composed of 14 membrane transporters identified as MCT1-9, MCT11-14 and T-Type amino-acid transporter-1 (TAT1).[772] Of this family, only the first four (MCT1-MCT4) have been characterized in detail, therefore only these will be reviewed in this chapter. In 1994 the first monocarboxylate transporter (named MCT1) was cloned from a mutated Chinese hamster ovary cell line.[773-776] Subsequently, all the other members of the family have been identified.[777]

Topology studies and sequence analysis revealed that MCTs consist of 12 putative transmembrane spanning domains with a large intracellular loop between the transmembrane segments 6 and 7 and intracellular N- and C-termini.[778] MCT1 and MCT4 need an ancillary protein (e.g., GP70 in humans), for their trafficking, localization and functional expression.[779-782]

MCT1-4 mediate membrane transport of monocarboxylates (such as lactate, pyruvate, and ketone bodies like hydroxybutyrate, acetoacetate, etc.) with a 1:1 coupling between substrate molecule ad proton (H^+) fluxes.[783-786]

MCTs have different tissue distribution: MCT1 was found in almost all tissues, including skeletal muscle, heart, brain (in particular in astroglial cells and capillary endothelium of cerebral microvasculature), kidney, colon, retina, the head region of spermatozoa, stomach, liver and placenta. In skeletal muscles MCT1 expression seems to correlate with the mitochondrial content and the presence of type I fibers.[787-791] MCT2 is expressed less widely and shows substantial species specificity in its tissue distribution: in mouse, rat and hamster MCT2 was found in the kidney, liver, brain and sperm tails whereas in humans it was barely or not expressed in a wide range of tissues.[777,792,793] MCT3 was found only on the basolateral membrane of the retinal pigment epithelium.[794-799] MCT4 is particularly abundant in tissues with high glycolytic metabolism, such as white skeletal muscle fibers (in particular in type II muscle fibers), white blood cells, tumor cells, and in placenta.[777,788,800,801]

In skeletal muscle it has been hypothesized that MCT1 is responsible for influx of lactic acid, whereas MCT4 has been reported to catalyze the efflux of lactic acid.[802] MCTs are also responsible for transport of lactic acid into tissues, such as the liver and kidney, that extract it for gluconeogenesis and lipogenesis, and into the heart, brain, spermatozoa and red muscles, that oxidize lactic acid and ketone bodies for respiratory fuel.[772] In the gastrointestinal tract, expression of MCT1 appears to contribute to the uptake of short-chain fatty acids (e.g. butyrate and acetate).[803]

Main substrate classes, Inhibitors and Inducers

- *Main substrate classes*. MCT1 substrates consist of a broad range of short-chain monocarboxylates, especially the ones substituted in the C-2 and C-3 position, such as lactate, pyruvate, acetoacetate, β-hydroxybutyrate, some branched-chain keto-acids (e.g. α-ketoisocaproate) and also acetate, proprionate and butyrate.[772,804] MCT1, as well as other MCTs, is also able to transport some exogenous or pharmacologically active compounds, usually consisting of monovalent weak organic acids with the carboxyl bound to a lateral small hydrophobic or hydrophilic group. Putative clinically relevant MCT1 substrates include salicylic acid, benzoic acid, nicotinic acid, foscarnet and R- and S-mandelic acid.[805-807] MCTs in general, and MCT1 in particular, have been implicated in the transport of some β-lactam antibiotics (e.g. phenethicillin, propicillin, carindacillin).[808,809] and HMG-CoA reductase inhibitors (e.g. simvastatin, lovastatin, pravastatin, atorvastatin) at the blood brain barrier.[810-812] As MCT1, MCT2 has been found to transport a wide range of monocarboxylates with substantially higher affinity than MCT1, in particular for pyruvate, whereas MCT4 displayed lower affinity for most MCT1 substrates.[802,813-815]

-*Inhibitors (competitive, non-competitive)*. Thus far, several MCT1 inhibitors have been described, but none of them is specific. MCT1 inhibitors can be divided in several groups: the first is composed of substituted bulky or aromatic monocarboxylates (including 2-oxo-4-methylpentanoate, phenyl-pyruvate) and cyanocinnamate derivatives (e.g. α-cyano-4-hydrocinnamate (CHC)) that act as competitive inhibitors. The second group consists of amphiphilic compounds with different structures that inhibit also the anion exchanger AE1 and other membrane transporters. This group includes bioflavonoids, such as phloretin and quercetin, and anion transport inhibitors such as niflumic acid and 5-nitro-2-(3-phenyl-propylamino)-benzoate (NPPB). 4,4′-dibenzamidostilbene-2,2′-disulphonate (DBDS) appears to block irreversibly MCT1 activity on prolonged exposure.[816] Other irreversible inhibitors are a broad range of amino reagents (e.g. phenyl-glyoxal, pyridoxal phosphate) and the organomercurial thiol reagent p-chloromercuribenzene sulphonate (pCMBS).[772,802,804] MCT2 is more sensitive to inhibition by a range of MCT blockers including CHC, DBDS and DIDS, but is insensitive to pCMBS. MCT3 is reported to be insensitive to CHC, pCMBS and phloretin, MCT4 exhibits less affinity for most inhibitors than MCT1.[802,812-815,818]

-*Inducers.* Several studies suggested that in human muscles the lactate/H^+ transport capacity and MCT expression could be enhanced by training.[819-822] Conversely, lactate transport capacity is reduced and MCTs are down-regulated after denervation of muscle or spinal injury.[822-824] Expression of MCT1 and MCT4 appears to be reduced in patients type 2 diabetes compared with healthy subjects.[825] In adult rats a ketogenic diet as well as ischemic conditions were reported to induce brain MCT1 expression.[826,827] In retinal explants, MCT1 was induced by hypoxia and vascular endothelial growth factor.[806,828],

whereas in cultured macrophages lipopolysaccharides and tumor necrosis factor-α determine increased expression of MCT1.[829]

Pharmacological and Toxicological Function

The majority of studies performed to date have been focused on the understanding of the physiological role of monocarboxylate transporters (MTCs). MCTs play a crucial role in homeostasis of lactate, pyruvate and of other monocarboxylic acids, which are involved in vital cellular functions. Mutations of MCTs have been reported to cause lactate transport deficiency with important pathophysiological effects. Some decades ago an otherwise healthy patient who developed severe chest pain and elevated serum creatine kinase levels after heavy exercise has been described. He was reported to have a defective red cell lactate efflux and a delayed reduction in muscle lactate after exercise.[830] In a subsequent study, genetic analysis of this patient and other subjects with reported defective lactate transport led to the identification of missense mutations of the MCT1 gene with altered protein function. Recently, mutations of the MCT8 gene (a MCT that has been shown to participate in thyroid hormone transport and metabolism) have been identified, and several of them have been associated with the Allan-Herndon-Dudley syndrome (AHDS), a X-linked condition characterized by severe mental retardation, neurological dysfunction, and elevated serum triiodothyronine (T3) levels.[831-833]

The cellular location and tissue distribution of MCTs in liver, kidney, intestine and brain, suggest a possible role for MCTs on drug pharmacokinetics. As MCT1 is expressed at the apical membrane of intestinal cells, its activity has been implicated in the intestinal absorption of some β-lactam antibiotics (e.g., cefdinir, phenethicillin, propicillin, carindacillin), reported to be good MCT substrates *in vitro*.[806,807,834] A possible contribution of MCTs to the intestinal absorption of atorvastatin, a HMG-CoA reductase inhibitor, has been speculated but other mechanisms and/or transporters may be involved as well.[811] This concept has been employed in the development of XP13512, a gabapentin pro-drug, which was designed to be a substrate of MCT1, specifically to overcome the poor intestinal absorption of gabapentin. In preclinical *in vivo* studies oral absorption and bioavailability of XP13512 were significantly higher when compared to gabapentin.[834]

MCT1 expression at the blood-brain barrier may play a crucial role in the efflux of certain drugs from the brain: the low distribution of probenecid and 6-mercaptopurine in the brain has been proposed to depend, at least in part, on their MCT-mediated efflux.[835,836] The tissue-specific expression of certain MCTs may also explain the development of certain side effects observed with specific MCT substrate drugs. For instance, some of the CNS side effects (predominantly sleeplessness) associated with administration of the lipophilic HMG-CoA reductase inhibitors lovastatin and simvastatin, which present a carboxylic acid moiety, have been correlated with MCT mediated transport of the drugs across the blood-brain barrier.[809,837]

Drug-drug interactions

To date, no clinically relevant drug-drug interactions mediated by MCTs have been described. However, because MCTs are involved in important pathophysiological conditions (hypoglycemia, diabetes, starvation, cerebral and cardiac ischemia), modulation of MCTs expression and activity may be a useful strategy to protect some tissues from ischemic or metabolic damage. In pre-clinical studies transfection of MCT2 in

cultured neurons has been reported to confer neuroprotection against acute neurological insults.[838] Administration of MCT inhibitors has been recently proposed as a possible clinical treatment strategy for γ-hydroxybutyrate overdose. γ-Hydroxybutyrate is a short-chain fatty acid formed from γ-aminobutyric acid (GABA). It is used in the treatment of narcolepsy, alcohol withdrawal or by drug abusers. Inhibition of renal reabsorption by blocking MCT activity is expected to result in increased renal clearance and decreased drug exposure. In addition, inhibition of MCT-mediated intestinal absorption may substantially reduce drug bioavailability. In animal studies, administration of MCT inhibitors (e.g., L-lactate, the flavonoid luteolin) increased total and renal γ-hydroxybutyrate clearance and improve sedation, respiratory depression, and mortality. Similarly, in a pilot clinical study, MCT inhibition with L-lactate administered with osmotic diuresis increased the renal clearance of γ-hydroxybutyrate in humans.[834,837,839-841]
Further studies need to be undertaken to further unravel the role of MCTs in clinically relevant drug-drug interactions.

7 - INTERACTIONS MEDIATED BY NUCLEOSIDE (CONCENTRATIVE & EQUILIBRATIVE) TRANSPORTERS (CNTs/ENTs, SLC28/29)

During the last two decades, two major classes of nucleoside transport systems have been identified in mammalian tissues and cell lines, the equilibrative (ENT, SLC29) and the concentrative (CNT, SLC28) nucleoside transporters.
ENTs mediate facilitated diffusion of nucleosides and nucleobases across the plasma membrane bidirectionally, depending on the substrate concentration gradient. Before the identification of their genes, ENTs were classified, based on their sensitivity to inhibition by nitrobenzylthioinosine (NBTI), as es (equilibrative sensitive) and ei (equilibrative insensitive).[842] To date, four equilibrative nucleoside transporters (ENT1, ENT2 ENT3, ENT4) have been cloned and characterized.[843-848] Topological studies and sequence analysis indicate that ENTs consist of 11 putative transmembrane spanning domains with two large loops (one cytoplasmatic and one extracellular and glycosylated, respectively), an intracellular N-terminus and an extracellular C-terminal tail.[844,849-851]
CNTs are influx active transporters: CNTs couple the inward flux (transport) of nucleosides and nucleoside analogs to the inwardly directed Na+ gradient (created by the Na-K+ ATPase). Five Na-dependent nucleoside transport subtypes were identified, but currently only three genes encoding nucleoside transporter (CNT1, CNT2, CNT3) belonging to the concentrative nucleoside transporter family have been cloned.[852-855] CNTs are predicted to consist of 13 transmembrane spanning domains with a cytoplasmatic N-terminal region and an extracellular glycosylated C-terminal part.[852,854,856-858] The Na+/nucleoside coupling ratio is reported to be 1:1 for CNT1 and CNT2, and 2:1 for CNT3.[855,859]
In general, ENTs are widely distributed in mammalian organs although with different abundance in different tissues (e.g. ENT2 is reported to be particularly abundant in human skeletal muscle)[860] and when expressed in polarized epithelial cells, ENT1 and ENT2 are predominantly detected in the basolateral membrane.[861]
CNT1 is localized primarily at the apical membrane of specialized epithelia, such as the intestine, kidney and liver[853,862,863], whereas CNT2 and CNT3 are more broadly distributed: CNT2 has also been detected in the spleen, heart, placenta, pancreas, brain and skeletal

muscle[864], and CNT3 is found in the pancreas, trachea, bone marrow, mammary gland, duodenum, prostate and lung too.[855]
The co-expression in some epithelia (e.g. intestine and kidney) of ENTs and CNTs at different cellular sides (basolateral and apical, respectively), together with the results of vectorial studies performed in cells co-expressing ENT1 and CNT1, support the hypothesis that ENTs and CNTs may cooperate to mediate the transepithelial nucleoside transport.[860,865] For instance, in the kidney ENTs and CNTs are supposed to work in concert to mediate the active reabsorption of nucleosides and nucleoside analogs from the tubular filtrate, and ENTs may be involved also in the renal excretion of substrates.[861,866] Nucleoside transporters play crucial roles in nucleoside salvage pathways maintaining the nucleoside homeostasis at both tissue and cellular level. Due to their specific tissue distribution and their ability to transport adenosine, ENTs may influence the concentration of adenosine intracellularly or at specific receptors, thus affecting critical physiological processes such as cardiac function, renal vasoconstriction, coronary blood flow, inflammation, platelet aggregation, lipolysis and nociceptive neurotransmission.[860]

Main substrate classes, Inhibitors and Inducers

-*Main substrate classes*. In general most nucleosides, nucleobases and their analogs used in anticancer and antiviral therapy are substrates of the nucleoside transport systems. There are many similarities between the spectrum of compounds transported by ENTs and CNTs, although there is no complete overlap.
ENT1 displays broad substrate specificity for purine and pyrimidine nucleosides, with higher affinity for adenosine and lower affinity for cytidine, but it is reported not to transport nucleobases.[844,867-871] Anticancer and antiviral drugs transported by ENT1 include gemcitabine, cytarabine, fludarabine, cladribine and ribavirin, whereas the nucleoside analogs 2′,3′-dideoxycytidine (zalcitabine) and 2′,3′-dideoxyinosine (didanosine) are only weak substrates.[844,867-869,871-875]
ENT2 transports a broad range of substrates, including purine and pyrimidine nucleosides, nucleobases (hypoxantine, adenine, guanine, uracil, thymine and cytosine) and possibly cyclic nucleotides too.[845,851,867-869,876,877] In contrast with ENT1, ENT2 can transport zidovudine (3′-azido-3′-deoxythymidine) and displays much greater affinity for zalcitabine and didanosine.[868-869,874-875] The anticancer drugs gemcitabine and fludarabine are substrates of ENT2 too.[877-880] Characterization of the substrate specificity of ENT3 and ENT4 is currently in progress. ENT3 appears to be able to transport several nucleosides (with the exception of hypoxantine) and antiviral nucleoside analogs such as zidovudine, zalcitabine and didanosine. Adenosine is reported to be a weak substrate for ENT4.[860,880]
CNT1 selectively transports pyrimidine nucleosides (cytidine, thymidine, uridine) and adenosine.[853,881,882] CNT1 substrates include also the antiviral nucleoside analogs zidovudine, lamivudine, zalcitabine, the anticancer drugs gemcitabine (2′,2′-difluorodeoxycytidine), cytarabine ((1-β-D-arabinofuranosyl)cytosine) and 5′-deoxy-5-fluorouridine (5′-DFUR, which is the active metabolite of capecitabine).[852,874,881-883] In contrast, CNT2 selectively transports purine nucleosides (adenosine, guanosine, inosine), uridine and formycin B.[852,881,885-888] CNT2 substrates include also didanosine (2′,3′-dideoxyinosine) and ribavirin.[852,853,886-889] CNT2 does not appear to transport other antiviral drugs such as zidovudine, zalcitabine nor the anticancer nucleoside analogs currently used in anticancer chemotherapy.[875,887,889]

CNT3 is widely selective for nucleoside substrates, accepting both purine and pyrimidine nucleosides. CNT3 transports also several nucleoside analogs, such as fludarabine, cladribine, zebularine, 5-fluorouridine, gemcitabine, zidovudine, zalcitabine, didanosine, the latter although weakly.[855,889-891]

-*Inhibitors (competitive, non-competitive)*. Inhibition activity of nitrobenzylthioinosine (NBTI) was historically used to classify ENTs.[843,891,892] Coronary vasodilators such as dipyridamole, dilazep, lidoflazine and the lidoflazine-related compounds, mioflazine, soluflazine, R75231 and draflazine are reported to be potent ENT1 competitive inhibitors, whereas ENT2 is much less susceptible to inhibition by these compounds.[860,868,880,893,894] A large number of protein kinase inhibitors, including some inhibitors of serine/threonine or tyrosine kinases, such as imatinib mesylate, protein kinase C inhibitors (e.g. staurosporine, arcyriarubin A), rapamycin, kinase inhibitors against p38 MAPK, EGFR kinase and others have been reported to inhibit ENT1 and/or ENT2 in a human erythroleukemia cell line.[895-897] The antidiabetic troglitazone, a thiazolidinedione, was able to competitively inhibit ENT1 whereas it did not have any effect on ENT2 activity.[898] *In vitro*, ethanol is suggested to selectively inhibit ENT1 after acute administration and to down regulate ENT1 after chronic exposure.[899,900] Lately, a range of 6-benzylthioinosine analogs, currently studied as potential agents against Toxoplasma gondii, have been described as inhibitors of ENT1.[901] Potential clinical consequences of these recent observations warrant further investigations.

-*Inducers*. Expression of nucleoside transporters appears to be regulated by several cellular and systemic events, as expression of nucleoside transporter is cell cycle-dependent and varies with cell differentiation and with the cellular deoxynucleotide levels.[900-903] In several preclinical studies, various pathophysiological conditions (e.g., starvation, nucleotide supplemented diet, hepatectomy, dexamethasone, concentration of glucose, glucagon, and insulin, thyroid hormone T3) were able to affect expression of nucleoside transporters.[863,904-916]

Pharmacological and Toxicological Function

The solute carrier (SLC)28 and SLC29 families of human equilibrative (ENT 1-4) and concentrative (CNT1-3) nucleoside transporters are responsible for cellular transport of nucleosides, endogenous compounds which play a key role in several physiological cellular processes, including proliferation, metabolism and signaling. Moreover, by regulating the concentrations of adenosine available to cell surface receptors, they modulate several physiological processes ranging from neurotransmission to cardiovascular activity. The cellular location of ENTs and CNTs in most normal and several cancer tissues suggests that they may influence the intracellular bioavailability and the therapeutic and toxicological profiles of nucleoside analog substrate drugs. Several nucleoside analogs are employed in the treatment of cancer (e.g., gemcitabine, fludarabine, clofarabine, cladribine, 5-fluorouridine, 2-deoxy-5-fluorouridine, decitabine and azacytidine) and viral diseases (e.g. zalcitabine, zidovudine, didanosine).[681] Variation in nucleoside transporter expression in target cells may contribute to variation in drug efficacy and drug resistance. Decreased nucleoside transporter expression and/or activity in tumor tissues and in virally infected cells that are targets for nucleoside antiviral drugs has been correlated with reduced drug-uptake and the development of drug resistance. Expression levels of hENT1 in tumor tissues of patients with pancreatic cancer has been reported to predict response

to gemcitabine and correlated with survival.[681] Cytotoxicity of cytarabine, fludarabine, and other nucleoside analogs has been correlated to expression and functional activity of nucleoside transporters in several in vitro models.[876-878,884,915-920]
Moreover, the location of nucleoside transporters in the intestine, kidney, and liver suggests that these transporters may affect the pharmacokinetics of endogenous compounds and of nucleoside analog substrate drugs. The study of the pathophysiological role of nucleoside transporters and their role in drug disposition is hampered by the lack of good pre-clinical models. To date only Ent1 knockout mice have been generated.[895,897] The absence of animal models knocked out for several nucleoside transporter genes, together with the overlap in substrate specificity between the various nucleoside transporters and between other membrane transporters able to translocate nucleoside analogs, makes it difficult to evaluate the contribution of individual nucleoside transporters to the in vivo disposition of nucleoside analogs. However, the large distribution volume of ribavirin has been ascribed to the broad tissue distribution of ENT1, whereas absorption after oral administration of ribavirin seems to correlate to expression of CNT2, together with ENT1, in the gastrointestinal tract.[871,921] Compared with wild type mice, in Ent1(-/-) mice the plasma exposure to ribavirin was decreased about 3-4 fold after oral administration, whereas the ribavirin erythrocyte exposure was decreased by approximately 3-fold in Ent1 (-/-) mice after intravenous administration. Moreover, saturation of ribavirin intestinal absorption was reported in mice at high administered doses, similarly to observations described in humans. All these experiments suggest that Ent1 and possibly also Cnt2/Cnt3 (expressed at the basolateral and apical side of enterocytes, respectively) play a critical role in ribavirin absorption.[922] The higher accumulation of ribavirin and metabolites in erythrocytes of Ent1 knockout mice compared with wild type ones, suggest a contribution of Ent1 in toxicity of ribavirin *in vivo*.[923] Nucleoside transporters widely expressed in the kidney appears to influence the renal disposition of natural nucleosides and analogs.[861,862]
Genetic variability in nucleoside transporters leading to reduced function or nonfunctional transporter proteins has been described in humans, but no association with pathophysiological conditions has been reported. However, it has been hypothesized that genetic variability in nucleoside transporters could be responsible for the observed large inter-patient variability in systemic and intracellular levels of nucleoside analogs and in activity of anticancer and antiviral nucleoside analogs. In general, ENTs appear to have less genetic and functional diversity than CNTs, suggesting that they are more critical for viable human life. CNT1 and CTN2 display more genetic diversity than CTN3, possibly due to overlapping tissue distribution and substrate specificity between CTN1 and CTN2. Two non-functional CTN1 variants and one variant (CNT1-Vall8911e) with reduced affinity for the anticancer nucleoside analog gemcitabine have been described.[924] A non-synonymous CNT2 variant (CNT2-F355S) with altered specificity for inosine and uridine was identified in the African-American population.[925] Genetic variants of hENT1 and hENT2 have been reported too, but due to the similar functional activity compared with the reference ENT1 and the low frequency reported, respectively, the potential clinical consequences of such genetic polymorphism are uncertain at this time.

Drug-drug interactions
Recently a drug-drug interaction mediated by nucleoside transporters has been documented in healthy volunteers between ribavirin, a guanosine analog substrate of

hENT1 and used for treatment of hepatitis C and dipyridamole, an ENT1 inhibitor clinically employed in several cardiovascular disorders.[926] Oral co-administration of the two drugs resulted in a significant ($p<0.05$) 23% and 17% reduction in the AUC of ribavirin in plasma and erythrocytes, respectively, which was proportional to the mRNA expression of ENT1 in peripheral blood mononuclear cells. The results suggest reduced gastrointestinal and erythrocyte absorption of ribavirin due to ENT1 inhibition mediated by dipyridamole.

Although no other clinically relevant drug-drug interactions mediated by nucleoside transporters have been described to date, the knowledge about nucleoside transporters and their inhibition is being employed to develop various clinical therapeutic strategies for several pathological conditions.[842,843,927-935]

The modulation of extracellular concentrations of adenosine in several tissues through administration of ENT inhibitors could potentiate adenosine-mediated protective cellular processes. The beneficial effect of several compounds, like dipyridamole, dilazep and draflazine, in cardiovascular diseases could be explained, at least partly, by their ability to inhibit ENTs. In preclinical models the infusion of the ENT inhibitor R-75231 before coronary artery occlusion has been reported to increase myocardial adenosine levels, to reduce infarction size, and to enhance post-ischemic recovery in swine.[927] Similarly, the therapeutic benefit of dipyridamole during percutaneous transluminal coronary angioplasty in humans could be related by its ability to block the nucleoside/adenosine uptake into endothelial cells, thus prolonging and enhancing the cardioprotective effects of adenosine.[928,929]

Administration of nucleoside transporter inhibitors has also been reported as potentially beneficial in the treatment of ischemic neuronal damage. In rats, pre-ischemic administration of the phosphorylated pro-drug form of nitrobenzylthioinosine, an ENT1 blocker, increased brain adenosine levels and reduced ischemic neuronal death. Similarly, the xanthine derivative propentofylline has been studied as a neuroprotective drug for brain ischemic injury, due to its ability to block ENT1, ENT2, and CNT1, that would result in increased extracellular concentrations of endogenous adenosine via inhibition of its cellular uptake.[930-934]

Inhibition of nucleoside transporter activity may also be a potentially useful strategy in the treatment of chronic pain. Adenosine Al receptor activation in the spinal cord has antinociceptive effects,[937] and in mice, administration of nucleoside transporter inhibitors have been described to enhance the opioid-mediated antinociception. Moreover, ENT1 was found at significant levels in the dorsal horn of the rat spinal cord, and administration of the ENT1 inhibitor nitrobenzylthioinosine resulted in increased extracellular adenosine levels that modulated glutamate release at the presynaptic Al receptors, leading to suppression of nociceptive neurotransmission.[932-934]

Inhibition of nucleoside transporter activity via administration of selective inhibitors has also been suggested as potential clinical strategy to improve the activity of several nonnucleoside antimetabolite cytotoxic drugs by potentiating antifolate activity. In effect, in in vitro studies dipyridamole potentiated the cytotoxic effect of methotrexate, pemetrexed, lometrexol, and of certain thymidylate synthase inhibitors by preventing the nucleoside cellular salvage mediated by nucleoside transporters.[843,935-941] However, co-administration of dipyridamole and antimetabolite drugs failed to show improved efficacy in clinical trials, probably due to the presence of nucleobase transporters in cancer cells that are insensitive to nucleoside transport inhibitors and to insufficient

plasma concentrations of free dipyridamole in the plasma related to its avid binding to serum proteins.[891,943]

Moreover, as several protein kinases inhibitors, some of which (e.g. imatinib) are widely used in clinical practice as anticancer or immunosuppressive agents, have been demonstrated to block nucleoside transporter activity, clinically relevant unbeneficial drug-drug interactions between protein kinase inhibitors and nucleoside analogs can be speculated and should be explored to avoid potential antagonistic interactions. For instance, the combination of imatinib and hydroxyurea or methotrexate demonstrated antagonistic effects in several leukemia cell lines.[944] On the other hand, as nucleoside transporters (in particular ENTs) can mediate both cellular uptake and efflux of therapeutic nucleoside analogs, the chronomodulated co-administration of selective nucleoside transporter inhibitors with nucleoside analogs could improve the cytotoxicity of nucleoside analogs. In vitro, dipyridamole has been reported to increase intracellular ara-CTP levels by blocking cellular efflux of cytosine arabinoside in human leukemic myeloblasts.[942,945] Similarly, in cultured human leukemic lymphoblasts the cytotoxicity of cladribine (2-chlorodeoxyadenosine) has been shown to be improved by subsequent treatment with nitrobenzylthioinosine or dipyridamole.[946]
Finally, as it has been suggested that low expression of nucleoside transporters (in particular ENTs) in cancer cells or virus could be responsible for resistance to nucleoside analog drugs substrates of nucleoside transporters, knowledge about the structure and function of such transporters could help in developing nucleoside drugs that are not substrates of such transporters and are able to enter cells by other routes, thus circumventing resistance mediated by ENTs. This concept however needs thorough clinical validation especially as recently a study with the gemcitabine prodrug CO 1.01 versus gemcitabine in patients with hENT1-low advanced pancreatic cancer turned out to be negative.[947]

CONCLUSIONS

Over the past two decades transporters have been the subject of intensive research further elucidating their role in mediating drugs' pharmacokinetics, pharmacodynamics and toxicity. Membrane transporters are involved in processes, such as absorption, distribution and elimination of endogenous and exogenous compounds, in regulating cellular drug resistance by mediating decreased uptake or increased efflux in the target/therapeutic organ and in mediating clinically relevant drug-drug interactions. The increasing relevance of drug transporters in drug development and in clinical practice is has led to the foundation of the International Transporter Consortium (ITC), a group of academic, industrial and regulatory scientists focused on the role of transporters in drug disposition, particularly when related to drug development. Recommendations have been formulated by the ITC about transporters to be taken under consideration during drug development, for which also the present chapter aims to provide guidance. [948,949] Decision trees were drawn by the ITC to help in making choices when drug transporters should be studied, as well as which pre-clinical and clinical in vitro and/or in vivo models/studies/testing are considered most suitable to assess affinity of a new molecular entity for transporters and predict clinical consequences. Indications were also given on when

a potential drug-drug interaction mediated by transporters should be further explored in clinical studies. Recommendations for the best approach to study pharmacogenetics of transporters were also given. These recommendations have been reflected in guidance documents issued recently by regulatory authorities (i.e., EMA and FDA), with the intent to define and harmonize issues pertaining to the investigations of transporters and related drug interactions during drug development.[68,69,950] Several databases about drug transporters have been also developed (http://bts.ucsf.edu/fdatransportal; http://www.membranetransport.org;http://nutrigene.4t.com/translink.htm;http://pharmacogenetics.ucsf.edu; http://www.druginteractioninfo.org; http://lab.digibench.net/transporter; etc) in order to inform scientists, drug developers, and physicians about transporters that play a critical role in drug disposition, activity and toxicity and about clinically relevant drug-drug interactions mediated by transporters.[951]

In the recent years the understanding of the pathophysiological and pharmacological role of drug transporters has been significantly improved. New transporters (MATEs) which play a crucial role in disposition of affected drugs have been identified opening a new field of research.[740]

It is now much better understood that transporter polymorphisms are an important determinant in interindividual variation in drug response and toxicity.[952] Computational models predicting the functional effects of nonsynonymous SNPs, which use structure and include docking of substrates and inhibitors to binding sites in transporters, are expected to facilitate understanding of the effects of coding region variants on transporter function and predicting drug-genotype interactions.[952,953]

The synergistic interplay between different transporters and between transporters and drug metabolizing enzymes has been increasingly studied and is now recognized as possible etiology of drug-drug interactions.[23,948,954] An example is related to docetaxel, a well-known substrate of P-gp, MRP2 and CYP3A4. In pre-clinical in vivo studies, the systemic exposure to docetaxel in Mrp2-/- (knockout) mice was not significantly different compared with wild type mice, after either oral or intravenous administration. However, when compared to wild type mice, systemic docetaxel exposure after oral administration was 2.3-fold increased in Cyp3a/Mdr1a/b-/- mice, and 166-fold increased in Cyp3a/Mdr1a/b/Mrp2-/- mice, which was disproportionate comparing with the separate Cyp3a-/- (12-fold) and Mdr1a/b/Mrp2-/- (4-fold) mice. The oral bioavailability increased from 10% to 73% in wild type and Cyp3a/Mdr1a/b/Mrp2-/- mice, respectively, suggesting that, in the absence of CYP3A and Mdr1a/b activity, Mrp2 has a marked impact on docetaxel pharmacokinetics.[23,955]

Moreover, the impact of physiologic factors (age, gender, pregnancy) as well as pathologic conditions (chronic renal failure, Dubin-Johnson syndrome), on the expression and function of transporters is being increasingly studied.[46]

New in vitro and in vivo methodologies to study distribution and pharmacological properties of drug transporters as well as to support transport evaluation in drug discovery and development have been developed. Mice with genetic deletion of multiple transporter genes and/or metabolic enzymes, as well as transgenic mice expressing human transporters and/or drug metabolizing enzymes have been generated.[23] Suspended hepatocytes and sandwich-cultured hepatocytes are now routinely employed to evaluate hepatic uptake and/or biliary excretion of compounds. Translational modeling of transporter-mediated pharmacokinetics and drug interactions is being increasingly

applied. Along with the standard static models for transporter in vitro-in vivo extrapolation, a dynamic physiologically based pharmacokinetic modeling approach has been proposed and implemented.[949] Positron emission tomography (PET) and single-photon emission computed tomography (SPECT) using tracers represented by endogenous compounds, drug substrates, or inhibitors of transporters is expected to provide a useful tool for noninvasive, dynamic in vivo evaluation of tissue concentrations of specific transporters, or of drug substrates for specific transporters, in order to assess how efficiently drugs reach their target organs and accumulate not only in clearance organs, such as the liver and the kidney, but also in other organs and tumors. Penetration in sanctuary sites and tumors, as well as the expected effect of transporter inhibitors, can this way also be tested in vivo, in animals and humans.[36,149,150] These and possibly other new approaches and strategies in the upcoming years are expected to increase our understanding of the expression and function of drug transporters. This will lead to novel and probably better strategies for optimal dosage regimens and reduce the risk of adverse drug reactions as well as adverse drug-drug interactions. Inhibition of specific transporters able to mediate drug resistance at the cellular level could enable to improve activity of specific anticancer drugs. Moreover, modulation of transporter activity mediating drug disposition could be a useful strategy to improve a drugs' pharmacokinetics and efficacy. Development of new drugs that do not have affinity for drug transporters could lead to more active and safer drugs.

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4

Effect of the ATP-binding cassette drug transporters ABCB1, ABCG2, and ABCC2 on erlotinib hydrochloride (Tarceva®) disposition in *in vitro* and *in vivo* pharmacokinetic studies employing Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ (triple knockout) and wild-type mice

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Mol Cancer Ther 2008; 7(8): 2280-7.

ABSTRACT

We tested whether erlotinib hydrochloride (Tarceva®, OSI-774), an orally active epidermal growth factor receptor tyrosine kinase inhibitor, is a substrate for the ATP-binding cassette drug transporters P-glycoprotein (P-gp; MDR1, ABCB1), Breast Cancer Resistance Protein (BCRP, ABCG2) and Multidrug Resistance Protein 2 (MRP2, ABCC2) *in vitro* and whether P-gp and BCRP affect the oral pharmacokinetics of erlotinib hydrochloride *in vivo*.
In vitro cell survival, drug transport, accumulation and efflux of erlotinib were done using Madin-Darby canine kidney II [MDCKII; wild-type (WT), MDR1, Bcrp1, and MRP2) and LLCPK (WT and MDR1) cells and monolayers as well as the IGROV1 and the derived human BCRP over-expressing T8 cell lines. *In vivo*, the pharmacokinetics of erlotinib after p.o. and i.p. administration was studied in Bcrp1/Mdr1a/1b$^{-/-}$ (triple knockout) and WT mice.
In vitro, erlotinib was actively transported by P-gp and BCRP/Bcrp1. No active transport of erlotinib by MRP2 was observed. *In vivo,* systemic exposure (P=0.01) as well as bioavailability of erlotinib after oral administration (5 mg/kg) were statistically significantly increased in Bcrp1/Mdr1a/1b$^{-/-}$ knockout mice (60.4%) compared with WT mice (40.0%) (P=0.02).
Conclusions: erlotinib is transported efficiently by P-gp and BCRP/Bcrp1 *in vitro*. *In vivo*, absence of P-gp and Bcrp1 significantly affected the oral bioavailability of erlotinib. Possible clinical consequences for drug-drug and drug-herb interactions in patients in the gut between P-gp/BCRP-inhibiting substrates and oral erlotinib need to be addressed.

INTRODUCTION

The ATP-binding cassette drug efflux transporters P-glycoprotein (P-gp; MDR1, ABCB1), breast cancer resistance protein (BCRP; ABCG2), and multidrug resistance protein 2 (MRP2; ABCC2) are involved in multidrug resistance, as they actively extrude a wide variety of anticancer drugs from tumor cells (1–3). Besides various tumor tissues, these transporters are expressed in several normal tissues (such as the intestine, liver, blood-brain barrier, and placenta syncythiotrophoblast), where they exert a protective role. They limit the intestinal uptake and the brain and fetal penetration of xenobiotics, and due to their localization in liver and kidney, they may facilitate the elimination of toxic compounds. Similarly, they can affect the pharmacologic behavior (absorption, distribution, metabolism, excretion, and toxicity) of various (anticancer) drug substrates (4).
Erlotinib hydrochloride (Tarceva®, OSI-774, CP-358774) is a small-molecule, orally active, selective, and reversible epidermal growth factor receptor 1 tyrosine kinase inhibitor. Erlotinib, like its analogue gefitinib (Iressa, ZD1839), is a quinazoline derivative that competes with the binding of ATP to the intracellular tyrosine kinase domain of epidermal growth factor receptor, thereby inhibiting receptor autophosphorylation and blocking downstream signal transduction.
Several studies addressed the affinity of tyrosine kinase inhibitors, especially gefitinib, for the ABC drug transporters BCRP and P-gp (5). Gefitinib has been reported to inhibit BCRP and, to a lesser extent, P-gp function *in vitro* and *in vivo* (6, 7). Gefitinib was able to reverse resistance and enhance cytotoxicity of well-known BCRP/P-gp substrates, such as topotecan, mitoxantrone, irinotecan, and its active metabolite SN-38, in BCRP- or P-gp-overexpressing cells (8-12). Oral gefitinib has been reported to increase the oral

bioavailability of irinotecan and topotecan (6, 13) and to enhance the CNS penetration of topotecan in mice (14). Other studies support the hypothesis that gefitinib is a substrate for BCRP (7, 15, 16) and a functional variant of ABCG2 (BCRP) has recently been associated with greater gefitinib accumulation in humans, thus supporting the hypothesis that BCRP expression and activity may affect the pharmacokinetics of gefitinib (16).
In contrast, studies evaluating the affinity of erlotinib for ATP-binding cassette drug efflux transporters are limited. A recent study indicates that erlotinib reverses ABCB1- and ABCG2-mediated multidrug resistance in cancer cells through direct inhibition of the drug efflux function of MDR1 and BCRP (16). The authors speculate also on a possible transport of erlotinib by MDR1 and BCRP, supported by the publication of Li et al., showing that erlotinib is a substrate of BCRP at relatively low concentrations and a BCRP inhibitor at high concentrations *in vitro* (17). However, the *in vivo* implications of these findings have not been explored yet. In addition, the affinity of erlotinib for MDR1 and MRP2 has not been reported.
Moreover, recently several drug-drug interactions involving erlotinib have been described and others are expected: treatment with erlotinib after administration of rifampicin resulted in 67% decrease in the area under the plasma concentration-time curve (AUC) for erlotinib in healthy volunteers (18). In another study, coadministration of erlotinib with ketoconazole significantly increased the systemic exposure to erlotinib (19–21). Considering that erlotinib is metabolized primarily by the CYP3A4 enzyme system (22) and that rifampicin and ketoconazole are well-known CYP3A4 inducer and inhibitor, respectively, these drug-drug interactions have been considered as primarily CYP3A4 mediated. However, given that rifampicin has been shown to induce also intestinal P-gp (23, 24) and that ketoconazole is a P-gp inhibitor (25, 26), although the effects of these two drugs on CYP3A expression/activity are expected to be greater and therefore more clinically relevant compared with the modulation of P-gp, a potential contribution of ATP-binding cassette drug efflux transporters to these interactions cannot completely be excluded.
Here, we investigated the affinity of erlotinib for P-gp, BCRP, and MRP2 using a panel of *in vitro* models, including the Madin-Darby canine kidney II epithelia cells (MDCKII) transfected with human MDR1 and MRP2 and mouse Bcrp1, the IGROV1 human ovarian cancer cell line and the T8 BCRP-expressing subline, and the porcine kidney epithelial cells (LLCPK) transfected with human MDR1. Moreover, we evaluated the effect of P-gp and BCRP on the p.o. and i.p. pharmacokinetics of erlotinib using Bcrp1/Mdr1a/1b$^{-/-}$ (triple-knockout) mice.

MATERIALS AND METHODS

In vitro Studies

Chemicals and Reagents

Chemicals and Reagents. Erlotinib hydrochloride and [^{14}C]erlotinib hydrochloride (129 µCi/mg) were kindly provided by Roche (Drs. J.W. Smit and Ch. Funk): in all *in vitro* and *in vivo* studies, the hydrochloride form of erlotinib was employed. For all *in vitro* studies, erlotinib hydrochloride was solved in DMSO (10 mg/mL). [3H]inulin (0.78 Ci/mmol) was purchased from Amersham Biosciences. Pantoprazole (Pantozol 40 mg, Altana Pharma) was obtained

from the pharmacy of The Netherlands Cancer Institute. Elacridar (GF120918) was a generous gift from GSK and zosuquidar trihydrochloride (LY335979) was kindly provided by Dr. P. Multani (Kanisa Pharmaceuticals). All other chemicals and reagents were from Sigma and of analytical grade or better.

Cell Lines

Polarized MDCKII cells, wild-type (WT) and stably expressing human MRP2 (ABCC2), human MDR1 (ABCB1), or mouse Bcrp1 (Abcg2), were kindly provided by Dr. A.H. Schinkel (The Netherlands Cancer Institute) and cultured as described previously (27–29).

The IGROV1 human ovarian adenocarcinoma and the IGROV1-derived resistant T8 cell lines were developed and cultured as described previously. T8 cells were exposed to 950 nmol/L topotecan weekly for 1 h, which keeps the resistance level in T8 constant for at least 25 weeks (30).

The polarized porcine kidney epithelial cell line (LLCPK) WT and MDR1, which were a generous gift from Dr. P. Borst (The Netherlands Cancer Institute), were employed as indicated previously (31).

All cell lines were grown at 37°C with 5% CO2 under humidifying conditions.

Clonogenic Survival Assay

Exponentially growing MDCKII cells were trypsinized and plated (~100 per 3 mL medium/well for the MDCKII-WT, MDCKII-Bcrp1, and MDCKII-MRP2 cells; 150 per 3 mL medium/well for the MDCKII-MDR1 cells) in six-well microplates (~4 cm diameter/well; Costar) and allowed to attach for 8 h at 37°C under 5% CO2. IGROV1 and T8 cells were plated (~200 per 4 mL medium/well) in 5-cm diameter dishes and incubated for 8 h for the cells to attach. After this attachment period, erlotinib hydrochloride was added at different concentrations. Cells were allowed to form colonies for 7 days (when MDCKII cells were used) or 14 days (when IGROV1 and T8 cells were used); subsequently, they were fixed and stained by 0.2% crystal violet/2.5 glutardialdehyde. The number of colonies containing at least 50 cells was visually counted under a light microscope. In each experiment, two replicates at each concentration of erlotinib were evaluated; at least three independent experiments with each cell line were done.

Transport across MDCKII and LLCPK Monolayers

Transport experiments were done in Costar Transwell plates with 3 μm pore membranes (Transwell 3414, Costar) as described previously (32). At least three independent experiments for each cell line and/or combination were done.

Accumulation and Efflux Experiments

Intracellular accumulation and efflux of erlotinib hydrochloride were measured in IGROV1 and T8 cells. Cells were plated at a density of $1x10^6$ in cell culturing plates (ø 4.8 cm, Costar) in 5 mL complete medium and allowed to grow to ~80% to 90% confluency.

In accumulation studies, plates were incubated for 30 min at 37°C with 5 mL complete medium containing 0, 5, 10, 15, 20, and 25 μmol/L erlotinib hydrochloride. After incubation, cells were washed twice with ice-cold PBS, scraped immediately, collected in Falcon tubes, and centrifuged (2 min, 1,300 rpm, 0°C). Subsequently, the cells were resuspended in 1 mL of 0.1% acetic acid to lyse the cells. Protein concentrations were determined using

the Bio-Rad assay based on the Bradford method (33). The concentration of erlotinib in the samples was determined by a validated high-performance liquid chromatographic analysis.
In efflux studies, IGROV1 and T8 cells were loaded with 15 µmol/L erlotinib hydrochloride for 30 min at 37°C. Subsequently, medium was removed and replaced by fresh medium. Immediately after the end of the incubation and at several following time points (1, 3, 6, 10, and 15 min), intracellular concentrations of erlotinib were measured with the same method employed in accumulation studies.
Accumulation and efflux of erlotinib were determined in two replicates for each erlotinib concentration in at least three independent experiments.

In vivo Studies

Animals

Animals used were female WT and Bcrp1/Mdr1a/1b$^{-/-}$ (Bcrp1/P-gp knockout) mice [obtained by cross-breading Bcrp1$^{-/-}$ (34) and Mdr1a/1b$^{-/-}$ (35) mice], all with >99% FVB genetic background and between age 10 and 14 weeks. They were housed and handled according to institutional guidelines complying with Dutch legislation. Animals were kept in a temperature-controlled environment with a 12-h light/12-h dark cycle and received a standard diet (AM-II; Hope Farms) and acidified water *ad libitum*. To exclude a possible interaction with food in all experiments before the treatment of erlotinib mice were fasted for 4 h while kept in cages that would prevent coprofagy. All experiments involving animals were approved by the local animal ethics committee of our institute. At least 8 mice for each group were treated.

Drug Preparation and Administration

In *in vivo* studies, erlotinib hydrochloride was dissolved in DMSO and further diluted in acidified water (ratio 1:3) to obtain a final concentration of 0.25 mg/mL.
For pharmacokinetic studies, Bcrp1/Mdr1a/1b$^{-/-}$ and WT mice were treated p.o. or i.p. with 5 mg/kg erlotinib. The i.p. administration was chosen assuming good drug absorption and complete bioavailability. Sampling was done from the tip of the lateral tail vein in three series. During the first series, whole blood samples were collected at 15 min and 0.5, 1.5, 5, and 10 h after injection. Based on the results of this initial group, the sampling times of the two subsequent series were adapted to 5 and 15 min and 0.5, 1.5, 4, and 8 h after injection. After collection, the blood samples were immediately centrifuged and plasma was stored at -20°C until high-performance liquid chromatographic analysis took place.

High-Performance Liquid Chromatographic Analysis

Erlotinib hydrochloride was determined by reverse-phase high-performance liquid chromatography with UV detection at 330 nm. Separation was achieved using a Symmetry C18 column (150 x 2.1 mm, i.d.) and a mobile phase composed of 28% (v/v) methanol, 25% (v/v) acetonitrile, and 47% (v/v) 50 mmol/L potassium phosphate buffer containing 0.2% triethylamine (pH adjusted to 6.5 with hydrochloric acid). Sample pretreatment involved mixing of 200 µL plasma (standard, quality control, or unknown) with 50 µL internal standard (50 µg/mL midazolam in water) and 1 mL tert-butyl methyl ether for 5 min. After centrifugation (5 min, 5000 x *g*), the aqueous layer was frozen on dry ice/ethanol and the organic top layer was decanted into a clean tube and dried by vacuum.

Following reconstitution in 100 µL acetonitrile/water (20:80, v/v) by sonication/mixing, 50 µL were subjected to high-performance liquid chromatography. The calibration curve ranged from 20 to 1000 ng/mL. Samples higher than 1000 ng/mL were diluted with blank human plasma to fit into the dynamic range of the calibration curve. The lower limit of quantitation of the assay was 20 ng/mL when using 200 µL sample.

Pharmacokinetic and Statistical Analysis

Pharmacokinetic variables after administration of erlotinib hydrochloride were calculated using WinNonlin Professional (version 5.0, Pharsight). The AUC was calculated by employing the linear trapezoidal rule up to the last sampling point for each animal separately with extrapolation to infinity ($AUC_{0\text{-}inf}$) using the concentration at the last measured time point divided by the elimination rate constant *k*, which was obtained by log-linear regression analysis of data points of the elimination phase, by using WinNonlin. Data were accepted only if the contribution of the extrapolated area to the AUC0-inf was not greater than 20% of the total AUC.

Statistical analysis was done using Student's *t* test (two-tailed, unpaired). The bioavailability was calculated as the ratio of the AUC after p.o. and i.p. administration (assuming complete bioavailability after i.p. administration). The bioavailability of WT and Bcrp1/Mdr1a/1b$^{-/-}$ groups was compared after log transformation of the AUC data. Differences between two sets of data were considered statistically significant at $P < 0.05$.

RESULTS

Cytotoxicity of Erlotinib

As expected due its non-cytotoxic mechanism of action, the cytotoxicity of erlotinib hydrochloride in the cell lines tested employing the colony forming assay was low and in the micromolar range (Table 1). Moreover, visual inspection revealed differences in the dimension of the colonies of the applied cell lines and over the applied range of concentrations of the drug. At the same erlotinib concentration, the colonies were bigger in size in the MDR1- and BCRP/Bcrp1-expressing cells compared with WT cells and, in the same cell line, colonies were smaller (but still composed of at least 50 cells) at higher erlotinib concentrations. These results were interpreted as a sign that MDR1 and BCRP affect intracellular accumulation of erlotinib resulting in different growth characteristics in the colony forming assay.

Moreover, a small but statistically significant difference in IC_{50} was found between MDCKII-WT and MDCKII-MDR1 cells (Resistance Index (RI): 1.63, $P<0.05$) and between WT and -Bcrp1 cell lines (RI: 1.31, $P<0.05$). A significant difference in IC_{50} was also seen in the same assay employing IGROV1 and T8 cells (RI: 2.10; $P<0.05$).

In contrast, no significant difference in IC_{50} of erlotinib was observed between MDCKII-WT and MDCKII–MRP2 cell lines ($P>0.1$) (Table 1).

These studies provided first hints that P-gp and BCRP mediate the transport of erlotinib and thereby affect cell survival. Based on these results, transwell experiments were performed at much lower and non-cytotoxic concentrations of erlotinib.

Table 1. Cytotoxicity of erlotinib in IGROV1, T8 and MDCKII cell lines

	MDCKII-WT	MDCKII-Bcrp1		MDCKII-MDR1		MDCKII-MRP2		IGROV1	T8	
	IC_{50} (μM)[a]	IC_{50} (μM)[a]	RI[b]	IC_{50} (μM)[a]	RI[b]	IC_{50} (μM)[a]	RI[b]	IC_{50} (μM)[a]	IC_{50} (μM)[a]	RI[b]
Erlotinib	13 ± 0.6	17 ± 2.5	1.31[c]	21.8 ± 0.9	1.63[c]	12 ± 0.2	0.90	0.87 ± 0.2	1.82 ± 0.4	2.10[c]

[a] Assessed by colony forming assay after 7 days (in MDCKII cells) or 14 days (in IGROV1 and T8 cells) of drug exposure. Mean ± SD of at least three experiments.
[b] RI, resistant index: ratio between the IC_{50} values of the resistant and parental cell lines.
[c] Significant difference compared with its parental cells (P<0.05).

Transport Experiments in MDCKII and LLCPK Monolayer Cells

Transport of Erlotinib by Bcrp1

Transport of erlotinib hydrochloride by Bcrp1 was studied using epithelial monolayers of MDCKII –Bcrp1 as well as WT cells as controls. Bcrp1 transported erlotinib (1.3 μM) efficiently as can be seen by the more than 16-fold increased transport after 4 hours from the basolateral to the apical side (BA) and decreased transport from the apical to the basolateral side (AB) in MDCKII-Bcrp1 (ratio BA/AB: 17.09) compared with the WT monolayer (ratio BA/AB: 1.03; Fig.1). Furthermore, we demonstrated that active transport of erlotinib was inhibited in MDCKII-Bcrp1 monolayer in the presence of the BCRP/P-gp inhibitors pantoprazole (500 μM) or elacridar (5 μM) (Fig. 1).

Transport of Erlotinib by P-gp (MDR1)

Active transport of erlotinib (1.3 μM) was found in MDCKII-MDR1 and LLCPK-MDR1 monolayer cells. Indeed, transport of erlotinib was > 3-fold increased in the MDCKII-MDR1 compared to the WT cell line (ratio BA/AB after 4 hours was 3.7 vs 1.2, respectively; data not shown). Similar results were obtained using LLCPK-MDR1 and WT monolayers (Fig. 2). Furthermore, co-incubation with the selective P-gp inhibitor zosuquidar (LY335979, 5 μM, Fig. 2) or with pantoprazole (1.25 mM, data not shown) resulted in complete inhibition of active transport of erlotinib by P-gp in MDCKII- and LLCPK- MDR1 cell monolayers.

Transport of Erlotinib by MRP2

In contrast, no transport of erlotinib was found in transwell experiments using MDCKII-MRP2 cell monolayers (data not shown).

Accumulation and Efflux of Erlotinib in IGROV1 and T8 Cells

To further investigate the role of BCRP in the transport of erlotinib, we performed accumulation and efflux experiments in IGROV1 and T8 cells. Accumulation of erlotinib was ~ 1.4 fold lower in T8 compared with IGROV1 cells (data not shown).
In efflux experiments, a significantly increased initial efflux rate of erlotinib was found in the T8 cells as compared with the IGROV1 cells: at 1 and 3 min after culturing in drug-free medium ~ 78% and ~ 91.5% of intracellular erlotinib, respectively, effluxed from the T8 cells *versus* ~ 60% and 78%, respectively, from the IGROV1 cells (Fig. 3).

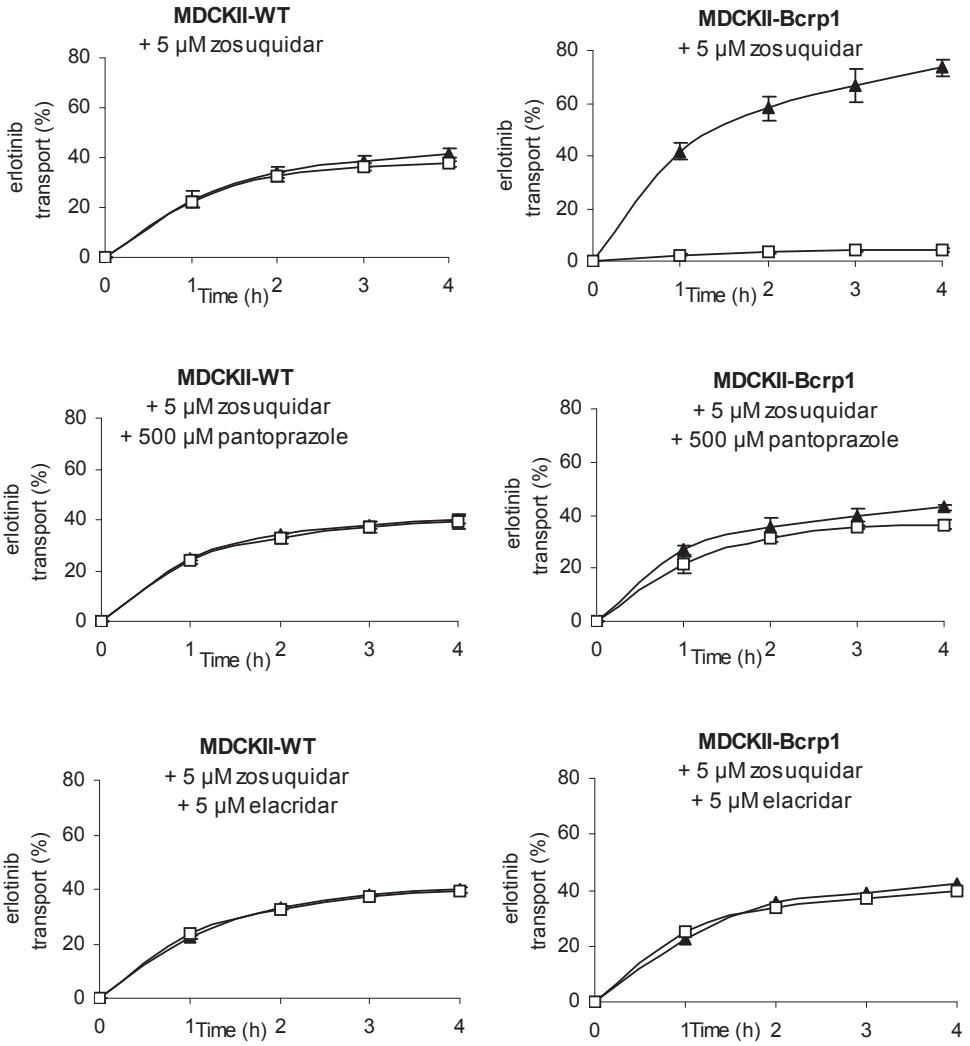

Fig. 1 Transport of [^{14}C]-erlotinib hydrochloride (1.3 µM) across MDCKII-WT and MDCKII-Bcrp1 cell monolayers in the absence or presence of the BCRP/P-gp inhibitors pantoprazole (500 µM) or elacridar (5 µM). Labeled drug was added to either apical or basolateral compartment and every hour up to 4 hours samples (200 µl) were obtained from both compartments. Appearance of radioactivity in the opposite compartment was measured and represented as the fraction (shown in percent) of total radioactivity added at the beginning of the experiment. Zosuquidar (LY335979, 5 µM) was added to inhibit endogenous P-gp levels. [^{3}H]inulin leakage (to check the integrity of the monolayer) was tolerated up to 2% of the total radioactivity over 4 hours. (▲)Translocation from basal to apical compartment; (□) translocation from apical to basolateral compartment. Mean ± SD of at least three experiments.

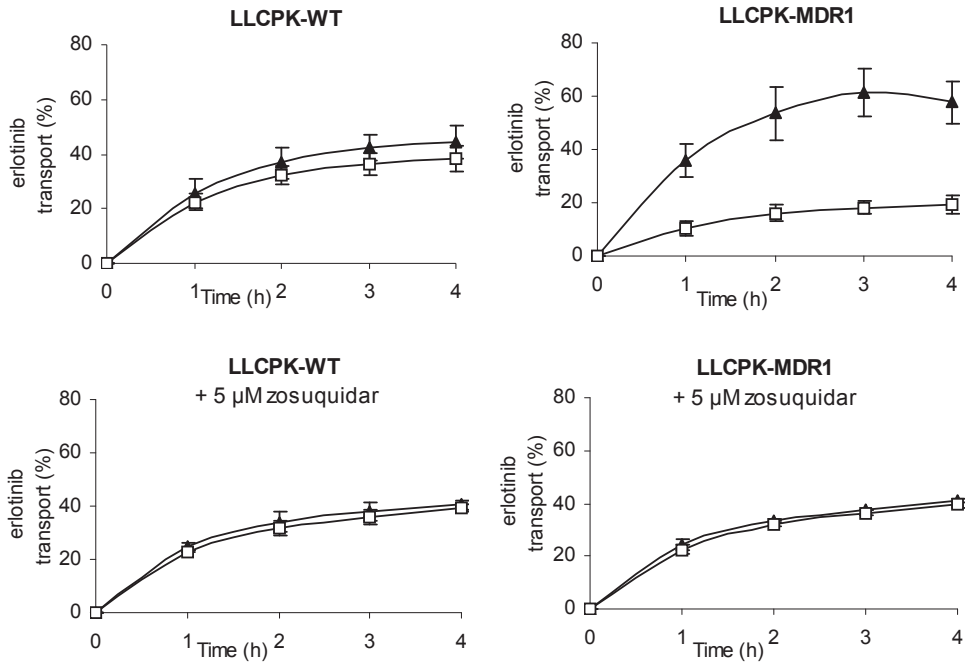

Fig. 2 Transepithelial transport of [^{14}C]-erlotinib hydrochloride (1.3 µM) across LLCPK-WT and LLCPK-MDR1 cell monolayers in the absence or presence of the selective P-gp inhibitor zosuquidar (LY335979, 5 µM). (▲)Translocation from basal to apical compartment; (□) translocation from apical to basolateral compartment. Mean ± SD of at least three experiments.

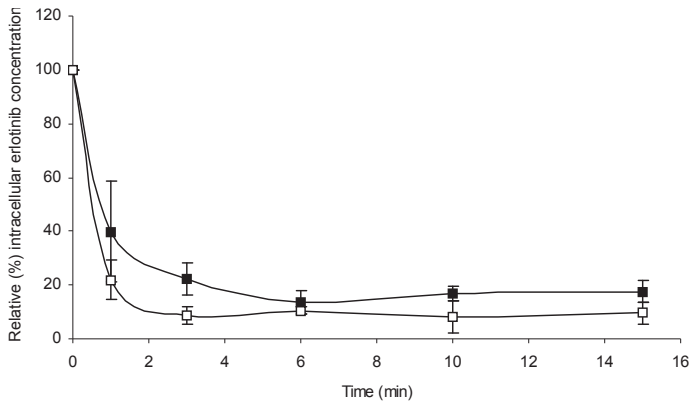

Fig. 3 Efflux of erlotinib hydrochloride from IGROV1 (■) and T8 (□) cells. Cells were loaded for 30 min at 37°C with 15 µM erlotinib hydrochloride. Subsequently, efflux of erlotinib from the cells was determined. Points, mean of three independent experiments; bars, SD.

In vivo Pharmacokinetics of Erlotinib in Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ and WT Mice

To explore whether the observed transport of erlotinib by P-gp and BCRP/Bcrp1 *in vitro* is also relevant *in vivo*, we administered erlotinib p.o. and i.p. to WT and Bcrp1/Mdr1a/1b$^{-/-}$mice.

At a dose of 5 mg/kg, there was a 1.49-fold statistically significant difference between the area under the curve in plasma ($AUC_{0\text{-}inf}$) after p.o. administration of erlotinib comparing Bcrp1/Mdr1a/1b$^{-/-}$ and WT mice (7419 ± 1720 versus 4957 ± 1735 ng*h/mL respectively, *P*=0.01, Table 2; Fig.4). No significant difference in the AUC after i.p. administration of erlotinib was found between Bcrp1/Mdr1a/1b$^{-/-}$ and WT mice (*P*>0.2; Fig.4). The observed difference in the AUC between p.o. and i.p. administration was significant, which can at least partly be explained by incomplete absorption of erlotinib from the gastrointestinal tract.

The total plasma clearance of erlotinib, assuming complete bioavailability after i.p. administration, was not significantly different between WT and Bcrp1/Mdr1a/1b$^{-/-}$ mice (11.0 ± 1.8 and 11.1 ± 2.9 mg/L*h, respectively, *P*>0.1).

The calculated apparent oral bioavailability was 60.4% (95% confidence interval: 48.6-75.0) and 40.0% (95% confidence interval: 27.8-57.7) for Bcrp1/Mdr1a/1b$^{-/-}$ and WT mice respectively, i.e., significantly increased in Bcrp1/Mdr1a/1b$^{-/-}$ mice (*P*=0.02, table 2).

Table 2. Pharmacokinetic variables of erlotinib after p.o. and i.p. administration in WT and Bcrp1/Mdr1a/1b$^{-/-}$ mice

	WT	**Bcrp1/Mdr1a/1b$^{-/-}$**	***P***
AUC p.o. (ng*h/mL)[a]	4957 ± 1735	7419 ± 1720	0.01
AUC i.p. (ng*h/mL)[b]	11873 ± 2779	12054 ± 1896	>0.2 (NS)
Oral Bioavailability (%)	40.0	60.4	0.02
(95% conf. interval)	(27.8 – 57.7)	(48.6 – 75.1)	

Mean ± SE. NS = not significant
[a] AUC up to infinity after oral administration of erlotinib hydrochloride.
[b] AUC up to infinity after i.p. administration of erlotinib hydrochloride.

DISCUSSION

Our *in vitro* data indicate that erlotinib hydrochloride is a substrate of P-gp and BCRP/Bcrp1, but not of MRP2. Also, data obtained *in vivo* support affinity for P-gp/Bcrp1, as the combined deletion of P-gp and Bcrp1 in the triple knockout (Bcrp1/Mdr1a/1b$^{-/-}$) model resulted in a significantly increased systemic exposure and bioavailability after oral administration of erlotinib.

The first indication of affinity of erlotinib for P-gp and BCRP was obtained in *in vitro* studies employing cells overexpressing BCRP and P-gp. A small but statistically significant difference in IC_{50} was found between BCRP/Bcrp1- or P-gp-over-expressing and WT cell lines, which is apparently in contrast with the high rates of transport of erlotinib observed in transwell experiments in Bcrp1- or P-gp-overexpressing cells. A first explanation for this discrepancy is that erlotinib is a growth factor inhibitor rather than a pure cytotoxic drug. This hypothesis is supported by the visual inspection of the plates obtained in the colony forming assays, revealing a significant difference in growth characteristics of the colonies between cell lines and at applied different concentrations of the drug. At the same erlotinib concentration, the colonies were bigger in size in the MDR1- and BCRP-expressing cells compared with WT cells, and in the same cell line, colonies were smaller (but still composed of at least 50 cells) at higher erlotinib concentrations. Therefore, the

difference in the IC_{50} value only may not be fully representative for the effect of MDR1 or BCRP overexpression on the cytotoxicity of erlotinib.
A second explanation may be that different concentrations of erlotinib were used in the different assays: in colony forming and in accumulation experiments the concentrations of erlotinb used were significantly higher compared with transwell experiments (between 25 and 5 μM versus 1.3 μM, respectively). It has recently been reported that gefitinib, an analog of erlotinib, is transported by BCRP at lower concentrations, whereas at higher concentrations it is an effective BCRP inhibitor (16, 17). Therefore, it may be speculated that erlotinib at the higher concentrations used inhibited BCRP and/or P-gp activity, thus reducing its own efflux from the cells. This mechanism could potentially reduce the difference in resistance between WT and BCRP/P-gp-overexpressing cells.
A third explanation could be that erlotinib, like other P-gp inhibitors, exerts a cytotoxic effect on P-gp-overexpressing cell lines at higher concentrations, interfering with the function of the plasma membrane.
Indeed, transwell experiments clearly showed a significant active transport of erlotinib by Bcrp1 and MDR1 in MDCKII and LLCPK cell monolayers. LLCPK cells were used because MDCKII cells show a low level of endogenous P-gp expression. Transport of erlotinib from the basolateral to the apical side in Bcrp1-expressing monolayers was ~ 17-fold increased compared with the translocation of the drug from the apical to the basolateral compartment. Transport of erlotinib in MDCKII and LLCPK cell monolayers expressing MDR1 was > 3-fold higher than in parental cells. Further evidence for the active transport of erlotinib by Bcrp1 and MDR1 was obtained in transwell experiments done by coincubation with elacridar or pantoprazole (BCRP and P-gp inhibitors) or zosuquidar (a selective P-gp blocker), respectively, which completely inhibited the transport of erlotinib. In contrast, no difference in cytotoxicity was observed between MDCKII-WT and MDCKII–MRP2 cells and no active transport of erlotinib was found in MDCKII-MRP2 monolayers. These results suggest that erlotinib is not a substrate for MRP2.
In order to quantitate the effect of the absence of P-gp and Bcrp1 on the *in vivo* pharmacokinetics of erlotinib hydrochloride, we investigated the pharmacokinetics after p.o. and i.p. administration of the drug in WT and in Bcrp1/Mdr1a/b$^{-/-}$ mice. Results obtained after oral administration revealed that there is a statistically significantly increased AUC of erlotinib in triple-knockout compared with WT-mice (P=0.01). In addition, the bioavailability of oral erlotinib was significantly increased in Bcrp1/Mdr1a/b$^{-/-}$ mice, considering also the small and non-significant difference in the systemic clearance of erlotinib found between Bcrp1/Mdr1a/b$^{-/-}$ and WT mice. Therefore, effective inhibition of P-gp/BCRP in patients may significantly increase the systemic exposure to erlotinib, assuming that these results obtained in mice are representative for the clinical situation. This needs to be confirmed. Indeed, the increase in erlotinib exposure observed in triple-knockout mice may overestimate what would be observed in humans with functionally or genetically related impaired expression and/or activity of P-gp and BCRP. Moreover, although an effective inhibition of these transporters has been obtained in the clinic (36, 37), a complete block as well as a genetic absence of both transporters is unlikely in humans.
On the other hand, besides P-gp/BCRP, other transporters (in particular uptake transporters in the gut) could be involved in erlotinib absorption, thus counteracting the activity of Bcrp1 and P-gp in the gut. This could explain the relatively high apparent

oral bioavailability of erlotinib found in WT mice that have proficient Bcrp1 and P-gp. Furthermore, it cannot be excluded that as a consequence of P-gp and Bcrp1 gene deletion other transporters and/or drug metabolizing enzymes involved in oral absorption of erlotinb are overexpressed in knockout mice, thus reducing the effect of Bcrp1/P-gp deletion on erlotinib pharmacokinetics. Indeed, considering that recently expression of CYP3A in the intestine has been shown to affect drug absorption (38) and that erlotinib has been reported to be susceptible to CYP3A-mediated metabolism (22, 39, 40), an altered expression/activity of CYP3A in our mice model might affect the magnitude of our findings. However, the bioavailability observed in WT-mice is of the same magnitude as observed in humans (41) and the experimental condition in this model may thus reflect the pharmacokinetic condition in patients. It has been reported previously that food can significantly increase the bioavailability of erlotinib. In humans, the bioavailability increased from ~ 60% to ~ 90% when erlotinib was taken with food (42). For this reason, in our experiments mice were fasted for four hours before the treatment with erlotinib.
The biotransformation of erlotinib is complex and extensive. Besides incomplete uptake of erlotinib from the gut also extensive biotransformation during first-pass could contribute to the observed incomplete bioavailability. Furthermore, it can as yet not be excluded that other drug transporters are involved in the oral pharmacokinetics of erlotinib. Finally, in extrapolating results from animal models to humans, it is important to take into account that there are species differences in type and expression of transporters.

CONCLUSION

The experiments indicate that *in vitro* erlotinib is a substrate for P-gp as well as for BCRP/bcrp1, whereas it does not seem to be transported by MRP2. In mice, a significantly increased systemic exposure and bioavailability after oral administration of erlotinib in Bcrp1/Mdr1a/1b$^{-/-}$ compared with WT mice was found. Complete inhibition of Bcrp1/Mdr1a/1b$^{-/-}$ resulted in an increase in the oral bioavailability of 66%.
In view of our results, potential implications of transporter pharmacogenetics on erlotinib pharmacokinetics variability and response (toxicity and efficacy) should be evaluated, because, as shown recently for another epidermal growth factor receptor tyrosine kinase inhibitor, gefitinib (17), functional variants of these ATP-binding cassette drug efflux transporters might be relevant to toxicity and antitumor activity of erlotinib. Moreover, possible clinical consequences of our results for drug-drug and herb-drug interactions at the intestinal level between oral erlotinib and P-gp/BCRP substrates and/or inhibitors warrant further investigation.

Acknowledgments:
We thank Drs J.W. Smit and Ch. Funkfor support, Dr H. van Tinteren (statistician at The Netherlands Cancer Institute) for statistical analysis, and D. Pluim (Department of Experimental Therapy, The Netherlands Cancer Institute) for technical assistance.

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5

In vitro transport of gimatecan (7-t-butoxyiminomethylcamptothecin) by breast cancer resistance protein, P-glycoprotein, and multidrug resistance protein 2

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Mol Cancer Ther 2007; 6(12): 3307-13.

ABSTRACT

Lipophilic campthothecin derivatives are considered to have negligible affinity for Breast Cancer Resistance Protein (BCRP; ABCG2). Gimatecan, a new orally available 7-t-butoxyiminomethyl-substituted lipophilic camptothecin derivative, has been previously reported to be not a substrate for BCRP. Using a panel of *in vitro* models, we tested whether gimatecan is a substrate for BCRP as well as for P-glycoprotein (P-gp, MDR1) or Multidrug Resistance Protein 2 (MRP2; ABCC2), ATP-binding cassette drug efflux transporters involved in anticancer drug resistance, and able to affect the pharmacokinetics of substrate drugs.
Methods: cell survival, drug transport, accumulation and efflux were studied in IGROV1 and (human BCRP overexpressing) T8 cells, Madin Darby canine kidney II (MDCKII-WT, MDCKII-Bcrp1, MDCKII-MDR1, and MDCKII-MRP2) and LLCPK (LLCPK-WT and LLCPK-MDR1) cells. Competition with methotrexate uptake was studied in Sf9-BCRP membrane vesicles.
Results: *in vitro*, expression of BCRP resulted in 8- to 10-fold resistance to gimatecan. In transwell experiments gimatecan was transported by Bcrp1 and transport was inhibited by the BCRP/P-gp inhibitors elacridar and pantoprazole. Efflux of gimatecan from MDCKII-Bcrp1 cells was faster than in WT cells. In Sf9-BCRP membrane vesicles, gimatecan significantly inhibited BCRP-mediated transport of Methotrexate. In contrast, gimatecan was not transported by MDR1 or MRP2.
Conclusions: Gimatecan is transported by BCRP/Bcrp1 *in vitro,* although to a lesser extent than the campthothecin analogue topotecan. Implications of BCRP expression in the gut for the oral development of gimatecan and the interaction between gimatecan and other BCRP substrate drugs and/or inhibitors warrant further clinical investigation.

INTRODUCTION

The ATP-binding cassette (ABC) drug efflux transporters, breast cancer resistance protein (BCRP; ABCG2) (1), P-glycoprotein (P-gp, MDR1, ABCB1), and the Multidrug Resistance Proteins (MRP) 1 to 5 (ABCC1 - ABCC5) are involved in resistance against anticancer drugs (2,3). Besides expression in various tumor tissues, these drug transporters are expressed in several normal tissues where they exert partly overlapping physiological functions. P-gp and BCRP are highly expressed at the luminal side of the intestinal epithelial cells, in the bile canalicular membrane, the syncythiotrophoblast, and at the vascular endothelial side of the blood-brain barrier (4,5). These drug transporters mediate the active (i.e., ATP-dependent) efflux of a wide range of chemical compounds with different physico-chemical characteristics. In the intestine, blood-brain barrier, and placenta, these transporters have a protective role as they limit uptake from the intestinal lumen into the body, the penetration of compounds into the central nervous system, or the exposure of the fetus by limiting penetration through the placenta (4).
The camptothecin-derived topoisomerase I inhibitors are substrates for BCRP and P-gp (6). The affinity of the camptothecins for BCRP is for most compounds significantly higher than for P-gp. Usually, low cross-resistance is found with classical P-gp substrate drugs as paclitaxel and docetaxel (7). However, the affinity for BCRP can vary substantially among the different derivatives of camptothecin (6). Topotecan and irinotecan, but especially SN38, the pharmacologically active metabolite of irinotecan, have high affinity for BCRP.

They also have moderate affinity for P-gp. We previously reported that the camptothecin derivatives, which are substituted at the 7 position of the planar aromatic five-ring structure resulting in a more lipophilic molecule [such as lurtotecan (GI147211, NX211) and exatecan mesylate (DX-8951f)], have less affinity for BCRP than topotecan and SN38 (6). It was suggested by others that the 7-oxyiminomethyl-substituted lipophilic camptothecin derivative gimatecan (ST1481, LBQ707) is not a substrate for BCRP (8,9). However, based on our experience with a range of other camptothecin derivatives, we hypothesized that gimatecan might also be a substrate for BCRP. We tested this hypothesis in a panel of well-defined *in vitro* models, including the BCRP-overexpressing human ovarian cancer cell line T8 (10), the Madin-Darby canine kidney II (MDCKII) epithelial cells stably expressing mouse Bcrp1 (11) and Sf9-BCRP membrane vesicles. We used elacridar and pantoprazole as inhibitors of BCRP and topotecan as positive control.
Moreover, we investigated whether gimatecan is a substrate of MRP2 and P-glycoprotein (P-gp, MDR1) *in vitro*.
Affinity of gimatecan for BCRP could be clinically relevant as oral bioavailability may be reduced by BCRP as shown for topotecan (12) and especially after oral administration drug-drug interactions with other BCRP substrate drugs may take place.

MATERIALS AND METHODS

In vitro Studies

Chemicals and Reagents

[^{3}H]-inulin (0.78 Ci/mmol), [^{14}C]-inulin carboxylic acid (54 mCi/mmol), [^{14}C]-topotecan (SK&F 104864, 48 mCi/mmol), and [^{3}H]-methotrexate (5.9 Ci/mmol) were purchased from Amersham Biosciences (Little Chalfont, UK). Topotecan (Hycamtin®) was obtained from GlaxoSmithKline (GSK) Pharmaceuticals (King of Prussia, PA). Gimatecan (STI1481; LBQ707) and [^{3}H]-gimatecan (40 µCi/mg) were provided by Novartis Pharmaceuticals Inc. (East Hanover, NJ). Pantoprazole (Pantozol® 40 mg, Altana Pharma, Zwanenburg, The Netherlands) was obtained from the pharmacy of the Netherlands Cancer Institute. Elacridar (GF120918) was kindly provided by GSK (Research Triangle Park, NC) and zosuquidar (LY335979) was a generous gift from Dr. P. Multani (Kanisa Pharmaceuticals, San Diego, CA). All other chemicals and reagents were from Sigma (St Louis, MO) and of analytical grade or better.

Cell Lines

Polarized MDCKII (Madin-Darby canine kidney II) cells wild-type (WT) and transfected subclones stably expressing human MRP2 (ABCC2), human MDR1 (P-gp, ABCB1), or mouse Bcrp1 (Abcg2) cDNA were kindly provided by Dr. A.H. Schinkel (The Netherlands Cancer Institute) and were described previously (11,13). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with Glutamax (Life Technologies, Inc.) supplemented with 100 IU/ml penicillin G, 100 µg/mL streptomycin sulphate and 10% fetal calf serum (MP Biochemicals, ICN Biomedicals Inc.). Bcrp1, MDR1 and MRP2 expression in the various transfected MDCKII sublines was checked by Western Blot.
The IGROV1 human ovarian adenocarcinoma and the IGROV1-derived resistant T8 cell lines were cultured in RPMI 1640 Medium supplemented with 25 mM HEPES, L-Glutamine, 10% fetal calf serum, 100 IU/mL penicillin and 100 µg/mL streptomycin. T8 cells were

exposed to 950 nM concentration of topotecan weekly for 1 h, which keeps the resistance level in T8 constant for at least 25 weeks (10,14).
The polarized porcine kidney epithelial cells LLCPK-WT and LLCPK-MDR1, which were a generous gift from Dr. P. Borst (The Netherlands Cancer Institute), were described previously (15). They were cultured in M199 medium with L-glutamine (Life Technologies, Inc.) and supplemented with penicillin G (100 IU/mL), streptomycin (100 µg/mL) and 10% (v/v) fetal bovine serum (MP Biochemicals, ICN Biomedicals Inc.).
All cell lines were grown at 37°C with 5% CO_2 under humidifying conditions.

Cytotoxicity Assays
Exponentially growing-cells were plated (1000 cells/200 µL per well for the MDCKII-WT, MDCKII-Bcrp1, and MDCKII-MRP2 cells; 1500 cells/200 µL per well for the MDCKII-MDR1 cells; 5000 cells/200 µL per well for the IGROV1 and T8 cells) in 96-well microplates (Costar Corp.) and allowed to attach for 24 h at 37°C under 5% CO_2. After this attachment period, 100 µL of drug solution (diluted in culture medium), were added to the wells, and cells were incubated for 72 h at 37°C under 5% CO_2. Subsequently, the cytotoxicity was determined using the sulforhodamine B method as described previously (16).
In the combination experiments, elacridar (used as inhibitor of BCRP; however it is also known as a P-gp inhibitor) (17) was added 30 min before adding gimatecan or topotecan to obtain a final concentration of 500 nM and 2 µM in the MDCKII and IGROV1/T8 cell lines, respectively. The concentration of elacridar was lower than that in the transport experiments (5 µM) to circumvent toxicity, but sufficient to inhibit BCRP- and P-gp-mediated transport.
Each agent (and combination) was tested in quadruplicate in at least three independent experiments.

Transport across MDCKII and LLCPK Monolayers
Transepithelial transport assays were performed in Costar transwell plates with 3-µm pore membranes (Transwell 3414, Costar) as described previously (18,19). In brief, cells (MDCKII-WT, MDCKII-Bcrp1, MDCKII-MRP2, MDCKII-MDR1, LLCPK-WT and LLCPK-MDR1) were seeded at a density of 1 x 10^6 in 2 mL of complete medium. Cells were grown for three days and allowed to form tight monolayers, with medium replacement every day. Two hours before the start of the experiment, complete medium at both sides of the monolayer (apical and basolateral compartments) was replaced by 2.5 mL of (serum-free) Opti-MEM medium (Life Technologies) containing the appropriate concentration of transport modulator (5 µM zosuquidar to inhibit endogenous P-gp levels and/or 500 µM of pantoprazole or 5 µM of elacridar to inhibit endogenous P-gp and BCRP).
At $t = 0$, 2.5 mL of transport medium supplemented with zosuquidar (5 µM) and without (control) or with elacridar (5 µM) or pantoprazole (500 µM) were applied at both sides of the monolayers, whereas radiolabeled drug ([^{3}H]-gimatecan, 1 µM; [^{14}C]-topotecan, 5 µM) and radiolabeled inulin ([^{14}C]-inulin or [^{3}H]-inulin; to check the integrity of the monolayer), were added to the apical or basal side of the monolayer in different wells. After 1 and 4 h samples of 500 µL were taken and the amount of [^{3}H]-gimatecan or [^{14}C]-topotecan appearing in the compartment (apical or basal) opposite to which the labeled drug was added, was measured by liquid scintillation counting (Tri-Carb 2100 CA Liquid Scintillation Analyzer, Canberra Packard). Transepithelial transport of the drug and paracellular inulin

flux through the monolayer was expressed as percentage of total radioactivity added at the beginning of the experiment. Inulin leakage was tolerated up to 2% of the total radioactivity over 4 h.

Accumulation and Efflux Studies

Intracellular accumulation and efflux of gimatecan were measured in MDCKII-WT and MDCKII-Bcrp1 cell lines. Cells were seeded at a density of 1 x10^6 in cell culturing plates (ø 4.8 cm, Costar) in 5 mL of complete medium and grown to about 80% to 90% confluence. Then, plates were incubated for 30 min at 37°C with 5 mL of complete medium buffered with HEPES (25 mM), adjusted to pH 7.0 and containing 0, 1, 1.5, and 2 μM of [^{3}H]-gimatecan. After incubation, cells were washed twice with ice-cold PBS, scraped immediately, collected in plastic tubes and centrifuged (2 min, 1300 rpm, 0°C). Subsequently, the cells were resuspended in 1 mL of acetic acid 0.1%, to lyse the cells. Protein concentrations were determined using the Bio-Rad assay based on the Bradford method (20). The concentration of gimatecan in the samples was determined by scintillation counting.

For efflux studies, MDCKII-WT and MDCKII-Bcrp1 cells were loaded with 1.5 μM and 2 μM of [^{3}H]-gimatecan, respectively, for 30 min at 37°C to obtain approximately equal intracellular concentrations of the drug. After loading the cells, medium was removed and replaced by fresh medium. Directly after incubation and at several following time points intracellular concentrations of gimatecan were determined.

Efflux experiments were also performed in the presence of elacridar (5 μM).

Accumulation and efflux of gimatecan were determined in at least three independent experiments.

Preparation of Membrane Vesicles and Competition Experiments

Inside-out membrane vesicles from *Spodoptera frugiperda* (Sf9) cells were prepared as described previously (18). Using Sf9-WT and Sf9-BCRP membrane vesicles, we evaluated the effect of gimatecan on the transport of 0.31 μM methotrexate, a well-known BCRP substrate, in the presence of 4 mM ATP. Sf9-WT and Sf9-BCRP membrane vesicles were incubated with 0.31 μM [^{3}H]-methotrexate for 5 min at 37°C in the presence or absence of different concentrations (0.01, 0.1 and 2 μM) of gimatecan. The ATP-dependent transport is plotted as percentage of the control value. Of note, all the experiments were done in the presence and absence of ATP.

Statistical Analysis

Statistical analysis was performed using Student's *t*-test (two tailed, unpaired). Differences between two sets of data were considered statistically significant at $P<0.05$.

RESULTS

Reduced Cytotoxicity of Gimatecan by BCRP Expression

A significant difference in IC_{50} of gimatecan was found between MDCKII-WT and MDCKII-Bcrp1 cells, with a resistance index (RI) of 8.4 ($P<0.005$). A significant difference in IC_{50} with a RI of 10.4 ($P<0.005$) was also seen in the same assay using the IGROV1 and T8 cell lines, indicating that BCRP expression resulted in resistance to gimatecan. Topotecan was

chosen as reference drug (9,10). The RI of topotecan in MDCKII-Bcrp1 was 83 and in the T8 cell line 148, in line with previous publications (10), and substantially higher than the RI of gimatecan in these cell lines. In the applied cell lines gimatecan showed a markedly higher cytotoxicity than topotecan (Table 1).
To further show the role of BCRP/Bcrp1 in the resistance to gimatecan, the cytotoxicity assays were repeated in the presence of elacridar, an inhibitor of BCRP as well as of P-gp (6,17). The cytotoxicity of gimatecan in the MDCKII-WT and IGROV1 was not significantly (P>0.05) affected by coincubation with a non-toxic dose of elacridar (500 nM and 2 μM respectively). In contrast, coincubation with elacridar resulted in a partial reversal of resistance of gimatecan in the MDCKII-Bcrp1 and T8 cell lines, yielding an IC_{50} ratio without/with elacridar of 6.5 and 3, respectively (P<0.05, Table 1).

Cytotoxicity of Gimatecan is Not Affected by P-gp and MRP2
In contrast to the results obtained in BCRP/Bcrp1-over-expressing cells, no significant difference in IC_{50} of gimatecan was found between MDCKII-WT, MDCKII-MDR1, and MDCKII-MRP2 cell lines (P>0.05, Table1).

Transport of Gimatecan across MDCKII Monolayers
Transport of gimatecan by Bcrp1 was studied in MDCKII-WT and MDCKII-Bcrp1 cell monolayers. To exclude any contribution of P-gp, the P-gp inhibitor zosuquidar (LY335979, 5 μM) was added. An increased transport of gimatecan (1 μM) from the basolateral to the apical compartments (BA) compared with the transport from the apical to the basolateral compartments (AB) [i.e. active transport (BA/AB is 3.1 ± 0.46)] was observed in MDCKII-Bcrp1 compared to the WT cell line (BA/AB is 0.94 ± 0.08) (Fig. 1). Moreover, gimatecan transport was completely abolished in MDCKII-Bcrp1 monolayers in the presence of the BCRP/P-gp inhibitors elacridar (5 μM) or pantoprazole (500 μM) (6,18,21)(Fig. 1).
Transwell experiments using topotecan as control drug have also been performed: the results were in line with previous publications (11) and showed active transport of topotecan (data not shown). The magnitude of topotecan transport was of the same order as of gimatecan.
In contrast, no transport was found for gimatecan in transwell experiments performed with MDCKII-MRP2 and MDCKII-MDR1, LLCPK-WT and LLCPK-MDR1 monolayers (data not shown).

Accumulation and Efflux of Gimatecan in Bcrp1-Overexpressing Cell Lines
To further elucidate the effect of BCRP/Bcrp1 overexpression on cellular transport of gimatecan we performed accumulation and efflux experiments in MDCKII-WT and –Bcrp1 cell lines. The accumulation and efflux of gimatecan could not be tested at concentrations higher than 2 μM due to limited drug solubility.
Accumulation of gimatecan was ~1.5-fold reduced in the MDCKII-Bcrp1 compared with WT cell line (data not shown).
In efflux studies, a significantly increased initial efflux rate of gimatecan was observed in the MDCKII-Bcrp1 cells (~90%) within 1 min compared with WT cells (~30%) (Fig. 2).
Coincubation of the cells with elacridar (5 μM) completely restored the intracellular accumulation and efflux of gimatecan in MDCKII-Bcrp1 cells to the intracellular levels observed in the WT cell line. Efflux of gimatecan was not affected by co-incubation with elacridar in MDCKII-WT cells (Fig. 2).

Table 1. Cytotoxicity of gimatecan in IGROV1, T8 and MDCKII cell lines ± elacridar

	IGROV1		T8		MDCKII-WT		MDCKII-Bcrp1		MDCKII-MDR1		MDCKII-MRP2	
	IC_{50} (nM)[a]	RI[b]	IC_{50} (nM)[a]	RI[b]	IC_{50} (nM)[a]	RI[b]	IC_{50} (nM)[a]	RI[b]	IC_{50} (nM)[a]	RI[b]	IC_{50} (nM)[a]	RI[b]
Topotecan	33 ± 5	NA	4867 ± 337	148* [c]	122 ± 11	NA	10135 ± 1736	83* [c]	251 ± 23	2*	122 ± 7	1*
Gimatecan	3 ± 1	NA	33 ± 4	10.4* [c]	8.6 ± 3.6	NA	72 ± 19	8.4* [c]	7 ± 4	0.8*	10 ± 4	1.2*
Topotecan + elacridar	42 ± 11	0.8#	152 ± 21	32# [c]	143 ± 43	0.85#	177 ± 19	57# [c]				
Gimatecan + elacridar	3.8 ± 1.7	0.8#	10.9 ± 1	3# [c]	9.2 ± 2	0.9#	11 ± 0.5	6.5# [c]				

Abbreviation: NA, not applicable.
[a] Assessed by sulforhodamine B cytotoxicity assay after 72 h of drug exposure. Values are the mean ± SD of at least three experiments.
[b] RI, resistance index: ratio between the IC_{50} values of the resistant and WT cell lines (*) or ratio between the IC_{50} values in absence and presence of elacridar (#).
[c] Significant difference ($P<0.05$).

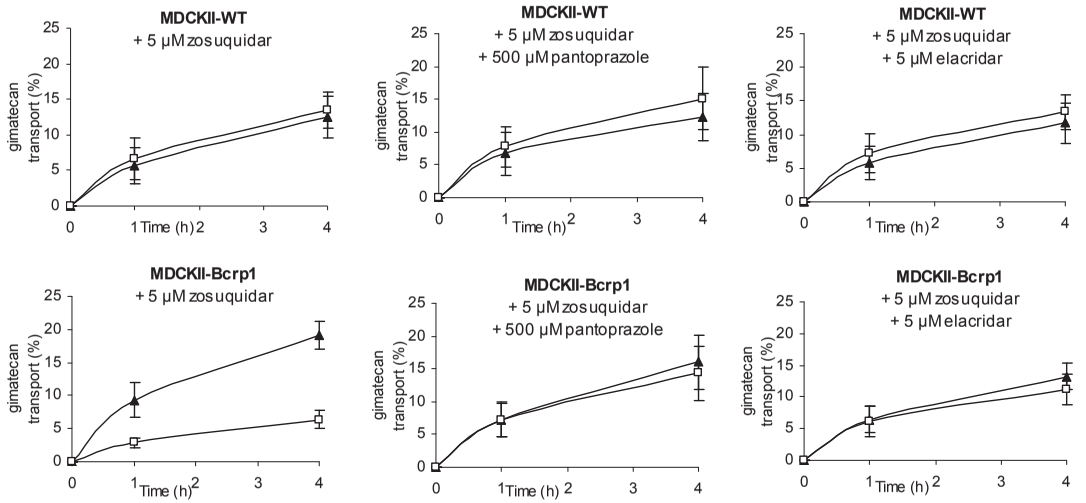

Figure 1. Transport of [^{3}H]-gimatecan (1 μM) across MDCKII-WT and MDCKII-Bcrp1 cell monolayers in the absence or presence of pantoprazole (500 μM) or elacridar (5 μM). Active transport of gimatecan is evidenced by an overall increased appearance of the drug in the apical compartment, as a result of an increased transport from the basolateral to the apical compartment and, as a consequence, a reduced translocation of the drug from the apical to the basolateral compartment. (▲)Translocation from basal to apical compartments; (□) translocation from apical to basolateral compartments. *Points*, mean of at least three experiments; *bars*, SD.

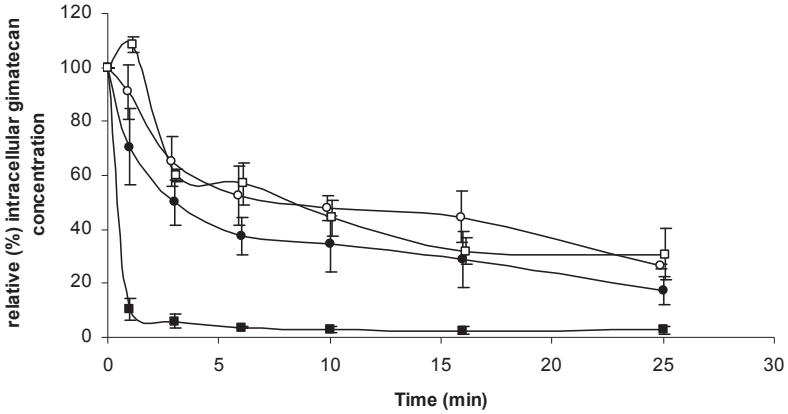

Figure 2. Efflux of gimatecan from MDCKII-WT (●) and MDCKII-Bcrp1(■) cells with (○ and □, respectively) or without (● and ■, respectively) 2-h pre-incubation with elacridar (5 μM).
MDCKII-WT and MDCKII-Bcrp1 cells were loaded for 30 min at 37°C with 1.5 and 2 μM [^{3}H]-gimatecan, respectively. Subsequently, efflux of gimatecan from the cells was determined. *Points*, mean of three independent experiments; *bars*, SD.

Effect of Gimatecan on BCRP-mediated Methotrexate Transport in Sf9 Membrane Vesicles

Using Sf9-BCRP and Sf9-WT membrane vesicles we studied the effect of different concentrations of gimatecan on the transport of 0.31 μM of [^{3}H]-methotrexate. The ATP-dependent transport of methotrexate by human BCRP was inhibited by gimatecan in a concentration-dependent manner, demonstrating competition between gimatecan and methotrexate for BCRP-mediated transport (Fig. 3). Control experiments have been performed in Sf9-WT vesicles as well as in Sf9-BCRP and WT vesicles in the presence of pantoprazole, a competitive BCRP transport inhibitor; the results observed were in line with previous publications (18) and further supported the competition of gimatecan for BCRP-mediated transport of methotrexate (data not shown).

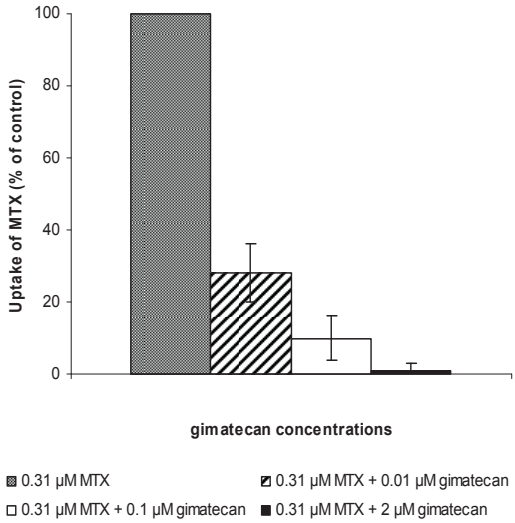

Figure 3. Effect of gimatecan on ATP-dependent transport of methotrexate by BCRP. Sf9-BCRP membrane vesicles were incubated with [^{3}H]-methotrexate (0.31 µM) for 5 min at 37°C in the absence or presence of increasing concentrations of gimatecan (0, 0.1, 0.01, and 2 µM). The ATP-dependent transport of methotrexate is plotted as percentage of the control value. *Columns,* mean of three independent experiments; *bars*, SD.

DISCUSSION

We tested the hypothesis that gimatecan is a substrate drug for BCRP/Bcrp1, P-gp, and MRP2 *in vitro*.

The first indication for affinity of BCRP/Bcrp1 for gimatecan was obtained in the cell survival studies using T8 and MDCKII-Bcrp1 cells. Compared with their parental counterparts, the BCRP expressing cells showed 8.4-fold (T8) and 10.4-fold (MDCKII-Bcrp1) resistance to gimatecan. This resistance index is clearly lower than the resistance factor for topotecan of 148 in T8 and 83 in MDCKII-Bcrp1, respectively. Furthermore, coincubation with a nontoxic concentration of elacridar resulted in a partial reversal of the resistance to gimatecan. This suggests that BCRP/Bcrp1 is involved in the resistance to gimatecan in the two cell systems.

Results obtained in the transport studies with MDCKII-Bcrp1 versus WT cells reveal that there is active Bcrp1-mediated transport of gimatecan. The magnitude of the difference in basolateral to apical versus apical to basolateral transport of gimatecan was in the order of topotecan, which was used as control substrate drug for BCRP. This shows that the difference in the level of resistance to gimatecan and topotecan in the cell survival studies is not the same as the difference in the level of active transport in the MDCKII monolayer experiments. Further proof of active Bcrp1-mediated transport was obtained in the transport studies by co-incubation with elacridar or pantoprazole, which collapsed the basolateral to apical/apical to basolateral curves completely.

Similar experiments conducted with LLCPK-MDR1, MDCKII-MDR1 and MDCKII-MRP2 showed that MDR1 and MRP2 do not mediate transport of gimatecan at detectable levels.

In addition, we determined the rate of efflux of gimatecan from loaded MDCKII-Bcrp1 and MDCKII-WT cells. The results support that Bcrp1 mediates the efflux of gimatecan. The Bcrp1-expressing cells extruded gimatecan significantly faster than the parental cells. Finally, we tested the affinity of gimatecan for human BCRP in competition experiments using Sf9-BCRP vesicles. We could not test gimatecan itself in transport experiments in vesicles, because gimatecan is a highly lipophilic drug. Consequently, it sticks to the applied filters in the assay. In competition experiments we tested the ability of gimatecan to compete with methotrexate for transport mediated by BCRP. Our results showed that gimatecan inhibited the ATP-mediated transport of methotrexate by BCRP in a concentration-dependent manner. Therefore, the applied *in vitro* assays revealed that BCRP is involved in resistance to and transport of gimatecan.

It is of interest that others have not found that gimatecan is transported by BCRP (8). However, in this previous study another cell system was employed, consisting of a human colon carcinoma cell line (HT29/MIT), selected by exposure of the parental (HT29) cell line to increasing concentrations of mitoxantrone, a well-known BCRP substrate. Although the selected HT29/MIT subline was checked for expression, along of BCRP, also of MDR1 and MRP1, expression of other ABC transporters and other mechanism of resistance could have been induced as well. An overlap in substrate specificities between different ABC transporters induced by mitoxantrone may potentially have affected the reported resistance of the HT29/MIT cell line. For instance, MRP2 and MRP4 (not identified yet at the time that the previous experiments were done) have recently been reported to transport mitoxantrone and several campthothecins (in particular topotecan, irinotecan and its metabolite SN38), respectively (22,23). Moreover, in the previous studies, control experiments with BCRP inhibitors to reverse resistance and/or drug transport, have not been done. This is relevant considering that recently it has been reported that inhibition of BCRP was not able to restore mitoxantrone sensitivity in irinotecan-selected human leukemia CPT-K5 cells (24). These findings support the hypothesis that induction of other transporters or other mechanisms besides the up-regulation of BCRP may contribute to the multidrug resistance phenotype of resistant cell sublines selected by increased exposure to substrate drugs. In our experiments, we used subclones of MDCKII cell stably transfected with the c-DNA of Bcrp1, MDR1, and MRP2, respectively, making the expression of other transporters unlikely. Moreover, we have performed control experiments using elacridar and/or pantoprazole as BCRP inhibitors: in the cytotoxicty (applying MDCKII-Bcrp1 and T8 cells) and transwell (in MDCKII-Bcrp1 monolayers) assays, the BCRP inhibitors (elacridar and pantoprazole) were able to reverse the resistance and transport of gimatecan, respectively.

Moreover, the parental cells in the earlier experiments (8) appear to be much less sensitive to gimatecan than those used in our study and this could explain why in the previous studies the overexpression of BCRP had relatively little effect. Another reason for the discrepancy between our results and the results of other authors can be that the expression level of BCRP in the cell systems used was different. This hypothesis is supported by the relatively higher resistance index of topotecan observed in our cytotoxicity experiments in Bcrp1/BCRP-overexpressing cells (RI in MDCKII-Bcrp1 cells: 83; RI in T8 cells: 148) compared with the previous study for topotecan (RI in HT29/MIT:13.2) (8). A lower BCRP expression in the HT29/MIT cells compared with our cell systems may have contributed to the different results. Finally, the authors in the previous study did not explore the efflux kinetics, which

might have shown a significant difference between the resistant and parental cells, nor have they studied transport in detail in monolayers of stably BCRP/Bcrp1 overexpressing cells as the MDCKII cells that we developed and used.
In a subsequent article Croce et al. (9) evaluated accumulation and efflux of gimatecan from parental and BCRP overexpressing cells, but the experiments have been conducted at a high concentration (22 μM) that most likely has resulted in precipitation of the drug. Moreover, the authors did not mention at which pH the experiments where done: this is relevant as the transport activity of BCRP has been recently reported to be affected by the pH (25). In our accumulation and efflux experiments, the medium with drug solution used was buffered with HEPES and the pH was adjusted. Finally, as hypothesized also for the other previous studies, a difference in expression of BCRP between the cell systems used may have also contributed to the discrepancy in results.

CONCLUSIONS

Our results reveal that *in vitro* gimatecan is a moderate substrate drug for mouse Bcrp1 as well as for human BCRP. The affinity for BCRP/Bcrp1 appears to be less than for topotecan that was used as control substrate drug.
Implications of BCRP expression in the gut for the oral development of gimatecan may be limited but need to be explored. The interaction between oral gimatecan and other BCRP substrate drugs and/or inhibitors warrants further clinical investigation.

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6

In vivo implications of BCRP/P-gp deletion on the pharmacokinetics of gimatecan (7-t-butoxyiminomethylcamptothecin)

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ABSTRACT

We previously reported that the 7-t-butoxyiminomethyl substituted lipophilic camptothecin derivative gimatecan is a substrate for BCRP *in vitro*. In order to assess the potential *in vivo* implications of such transport, we tested the pharmacokinetics and tissue distribution of gimatecan in WT and Bcrp1/Mdr1a/1b$^{-/-}$ mice, and the effect of the co-administration of elacridar and pantoprazole, well known BCRP/P-gp inhibitors.
Methods: Oral and i.v. pharmacokinetics and tissue accumulation were studied in WT and in Bcrp1/Mdr1a/1b$^{-/-}$ mice, in presence or absence of pantoprazole or elacridar.
Results: Systemic exposure to gimatecan after oral administration in Bcrp1/Mdr1a/1b$^{-/-}$ mice was 1.4-fold higher than in WT mice ($p<0.01$). Elacridar significantly increased systemic exposure to oral gimatecan in WT, but also in Bcrp1/Mdr1a/1b$^{-/-}$ mice, whereas pantoprazole did not significantly affect the pharmacokinetics of gimatecan. Conclusions: *In vivo*, absence of Bcrp1/Mdr1a/1b resulted in increased systemic exposure to gimatecan. The *in vivo* interaction between elacridar and gimatecan is partly mediated by other drug transporters than BCRP/Bcrp1.

INTRODUCTION

Gimatecan (ST1481; 7-[(E)-tert-butyloxyminomethyl]-camptothecin) is a new oral camptothecin analogue selected for clinical development on the basis of a promising preclinical antitumor activity and a favorable pharmacological profile. Actually gimatecan is tested in clinical phase I/II and an orphan designation has recently been granted by the European Commission for gimatecan for the treatment of glioma (EMEA/COMP 1536/03 Rev1).
Previously, we reported that gimatecan is transported *in vitro* by human BCRP (Breast Cancer Resistance Protein, ABCG2) and by the murine homologous Bcrp1 (Abcg2), but not by human P-gp (P-glycoprotein, MDR1, ABCB1) and MRP2 (Multidrug Resistance Protein 2, ABCC2) (Marchetti et al., 2007). BCRP, P-gp and MRP2 are ATP-binding cassette (ABC) drug efflux transporters originally involved in tumor resistance against anticancer drugs (Borst et al., 2002; Kruh et al., 2003). The localization of these transporters in tissues important for absorption (e.g., gut), metabolism and elimination (liver and kidney) of xenobiotics and in tissues involved in maintaining the barrier function of sanctuary site (e.g., blood-brain barrier, blood-cerebral spinal fluid barrier, blood-testis barrier and the maternal-fetal barrier or placenta) suggests for these transporters a physiological protective role for the body against xenotoxins. Similarly, they are increasingly recognized for their ability to modulate the absorption, distribution, metabolism, excretion, and toxicity of substrate drugs (Schinkel et al., 2003).
The camptothecin derived topoisomerase I inhibitors have been reported as substrates for BCRP and P-gp, although their affinity for such transporters vary substantially between the different derivatives (Maliepaard et al., 2001). In particular topotecan, irinotecan and its active metabolite SN38 have high affinity for BCRP and moderate affinity for P-gp (Maliepaard et al., 1999; Schellens et al., 2000). BCRP expression/activity has been shown to affect the bioavailability after oral administration and the brain penetration of topotecan (Schellens et al., 2000; Jonker et al., 2000; Kruijtzer et al., 2002; de Vries et al., 2007) and

genotype variants of BCRP have recently been suggested to affect the pharmacokinetics of topotecan (Sparreboom et al., 2005; de Jong et al., 2004).
Although it has been suggested that the 7-substituted camptothecin derivatives (i.e., lurtotecan and exatecan mesylate), have less affinity for BCRP than other analogues (Maliepaard et al., 2001), we previously demonstrated that gimatecan (7-[(E)-tert-butyloxyminomethyl]-camptothecin) is transported *in vitro* by BCRP (Marchetti et al., 2007). In order to test the potential *in vivo* implications of such transport for the clinical development of the drug, we evaluated the pharmacokinetics of gimatecan in WT and in Bcrp1/Mdr1a/1b$^{-/-}$ mice, obtained by cross breading of the Bcrp1$^{-/-}$ (Bcrp1 knockout) (van Herwaarden et al., 2003) and Mdr1a/1b$^{-/-}$ (P-gp knockout) mice (Schinkel at al., 1994). Although in our previous *in vitro* experiments no transport of gimatecan by MDR1 was detected, we chose this triple knockout mouse model because it was readily available at our institute and because some camptothecin derived top I inhibitors have low affinity for P-gp (Maliepaard et al., 2001). Moreover, we used elacridar and pantoprazole as BCRP/P-gp inhibitors (Evers et al., 2000, Breedveld et al., 2004; de Bruin et al., 1999; Pauli-Magnus et al., 2001).
Affinity of gimatecan for BCRP could be clinically relevant as, similar to the camptothecin analogue topotecan (Kruijtzer et al., 2002; Kuppens et al., 2005; Breedveld et al., 2006), the oral bioavailability and the brain penetration of the drug might be significantly affected by BCRP. Moreover, clinically relevant drug-drug interactions with other BCRP substrate drugs and/or inhibitors may take place.

MATERIALS AND METHODS

Chemicals and reagents

Gimatecan (STI1481; LBQ707) and [^{3}H]-gimatecan (40 µCi/mg) were provided by Novartis Pharmaceuticals Inc. (East Hanover, NJ). Pantoprazole (Pantozol® 40 mg, Altana Pharma, Zwanenburg, The Netherlands) was obtained from the pharmacy of the Netherlands Cancer Institute. Elacridar (GF120918) was kindly provided by GSK (Research Triangle Park, NC).

Animals

Animals used in this study were female WT and Bcrp1/Mdr1a/1b$^{-/-}$ mice, all with a >99% FVB genetic background between 10 and 14 weeks of age. Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Animals were kept in a temperature-controlled environment with a 12-hours light/12-hours dark cycle and received a standard diet (AM-II; Hope Farms, Woerden, the Netherlands) and acidified water *ad libitum*.

Drug preparation, administration and plasma analysis

For intravenous (i.v.) administration, gimatecan was dissolved in DMSO (2 mg/ml). For oral (p.o.) administration, gimatecan was dissolved in a microemulsion solution, provided by Novartis Pharmaceuticals Inc. (East Hanover, NJ) at 0.5 mg/ml. The microemulsion was stored in the darkness at 0°C and equilibrated before use for at least 1 hr at room temperature in the darkness.

Elacridar was suspended at 10 mg/ml in a mixture of hydroxypropylmethylcellulose (10 g/L)/2% Tween 80/H_2O (0.5:1:98.5 [v/v/v] for p.o. administration.
A vial of pantoprazole (Pantozol® 40 mg) was diluted with NaCl 0.9% to a final concentration of 4 mg/ml.
WT and Bcrp1/Mdr1a/1b$^{-/-}$ mice received gimatecan either by i.v. (in the tail vein) or p.o. administration at a dose of 2 mg/kg with or without co-administration of one oral dose of elacridar (25 mg/kg) 2 hours and 20 minutes before i.v. and p.o. gimatecan, respectively, or pantoprazole (40 mg/kg) 30 minutes before i.v. and p.o. gimatecan. To minimize variation in absorption, mice were fasted for 4 hours before gimatecan was administered orally. Multiple blood samples (~30μl each) were collected from the tail vein at 5 minutes and at 0.5, 2, 4, 8, 24 and 48 hours after i.v. administration, or at 0.5, 1, 2, 4, 8, 24 and 48 hours after p.o. administration of gimatecan using heparinized capillary tubes (Oxford Labware, St. Louis, MO). The plasma fraction of the blood samples was collected after centrifugation at 3,000 x *g* for 10 minutes at 4°C, and stored at –20°C until analysis according to a validated high performance liquid chromatography (HPLC) method as described below.
Brain penetration of the drug was also studied. Results are reported and discussed in **Chapter 7** of this thesis.

HPLC analysis
Amounts of gimatecan, as the ring-open carboxylate form, were determined in small mouse plasma and tissue samples by using an HPLC fluorimetric method. The HPLC system consisted of a model 300 isocratic pump (Gyncotek 300c, Germering, Germany), a Basic Marathon autosampler (Spark, The Netherlands), provided with a 50 μl sample loop and a Model FP-920 fluorescence detector (Jasco, Hachioju City, Japan) operating at excitation and emission wavelengths of 380 and 527 nm, respectively. Chromatographic separations were carried out using a narrow bore stainless steel column (2.1 x 150 mm I.D.) packed with 3.5 μm Symmetry C_{18} material (Waters, Milford, MA, USA). The mobile phase consisted of acetonitrile-50 mM ammonium acetate buffer pH 6.8 (30:70, v/v) and the flow rate was set at 0.2 ml/min. Chromatographic data acquisition and reprocessing was performed using Chromeleon version 6.60 (Dionex Corp. Sunnyvale, CA, USA). Volumes of 50 μl of calibration and quality control samples in human plasma sample or 5 to 20 μl of mouse plasma sample supplemented with blank human plasma to a total volume of 100 μl were vortexed with 200 μl of methanol. After centrifugation (14000 rpm, 5 min., 4°C), 200 μl of supernatant was mixed with 300 μl 0.01 M Borax (di-Natriumtetraboratdecahydrat, Merck) solution.
The samples were vortexed and centrifuged again (14000 rpm, 5 min, 4°C) and 100 μl of the clear solution was injected into the HPLC system. Calibration samples ranged from 2 to 2000 ng/ml and were prepared in drug free human plasma. QC samples containing 2, 10 and 1000 ng/ml prepared in blank human plasma showed that the accuracy and precision were within the acceptable ranges of 85% to 115% and ± 15%, respectively.

Pharmacokinetic and statistical analysis
Pharmacokinetic parameters of gimatecan were calculated by the noncompartmental trapezoidal method using WinNonlin Professional (version 5.0, Pharsight, Mountain View, CA, USA). The pharmacokinetic parameters of gimatecan were determined as follows:
$AUC_{0\text{-}24}$ as area under the curve (AUC) from time 0 up to 24 h (ng*h/ml), using the linear

trapezoidal rule, $AUC_{0\text{-}inf}$ as AUC from time 0 extrapolated to infinity (ng*h/ml).
The apparent oral bioavailability (F) was calculated by the following formula: $F = AUC_{oral} / AUC_{iv} \times 100\%$.
Statistical analysis was performed using Student's *t*-test (2-tailed, unpaired). Differences between 2 sets of data were considered statistically significant at $p<0.05$.

RESULTS

In vivo plasma pharmacokinetics of gimatecan in WT and Bcrp1/Mdr1a/1b$^{-/-}$ mice

To assess whether the *in vitro* observed BCRP mediated transport of gimatecan is also relevant *in vivo*, we tested the oral uptake of gimatecan in WT and Bcrp1/Mdr1a/1b $^{-/-}$ mice. At a dose of 2 mg/kg, the $AUC_{0\text{-}inf}$ of gimatecan after p.o. administration was 1.4-fold higher in Bcrp1/Mdr1a/1b$^{-/-}$ compared with WT mice ($p<0.01$; table 1; figure 1a). The $AUC_{0\text{-}inf}$ of gimatecan after i.v. administration was not significantly different (1.03-fold) in Bcrp1/Mdr1a/1b$^{-/-}$ *versus* WT mice ($p>0.05$; table 1; figure 1b). Considering the high contribution of the extrapolated area to the $AUC_{0\text{-}inf}$ observed in several mice treated we calculated also the $AUC_{0\text{-}24h}$ using the linear trapezoidal rule, obtaining however analogous results.

The calculated apparent oral availability was 48 ± 4.4 % and 35 ± 2.9 % for Bcrp1/Mdr1a/1b$^{-/-}$ and WT mice respectively, *i.e.*, moderately but significantly ($p<0.05$) increased in Bcrp1/Mdr1a/1b$^{-/-}$ mice.
Also the mean (± SD) maximum plasma concentration (Cmax) of gimatecan after p.o. administration was significantly higher in Bcrp1/Mdr1a/1b$^{-/-}$ compared with WT mice (368 ± 173 vs. 205 ± 80 ng/ml; $p<0.05$), but not after i.v. administration (1323 ± 145 vs 1178 ± 137 ng/ml, $p>0.05$).

Table 1. Pharmacokinetic parameters of gimatecan after p.o. and i.v. administration in WT and Bcrp1/Mdr1a/1b$^{-/-}$ mice ± pantoprazole or elacridar

	p.o. administration			***i.v. administration***	
	$AUC_{0\text{-}24h}$[a] (ng*h/ml)	**$AUC_{0\text{-}inf}$[b]** (ng*h/ml)		**$AUC_{0\text{-}24h}$[a]** (ng*h/ml)	**$AUC_{0\text{-}inf}$[b]** (ng*h/ml)
WT[c]	2042 ± 464	2583 ± 405	*, #	7207 ± 1004	7346 ± 1018
WT[c]+pantoprazole	2556 ± 565	3087 ± 649		10172 ± 5171	10657 ± 5458
WT[c]+elacridar	3541 ± 1179	4714 ± 961		13155 ± 2477	13683 ± 2520
TKO[d]	3011 ± 608	3648 ± 464		7462 ± 1417	7599 ± 1420
TKO[d]+pantoprazole	2804 ± 854	3425 ± 903		9249 ± 1520	9469 ± 1512
TKO[d]+elacridar	4154 ± 343	5862 ± 836	$	16715 ± 3973	16890 ± 3935

[a] Area under the concentration-time curve from 0 to 24 hours.
[b] Area under the concentration-time curve from 0 to infinity.
[c] WT: wild-type mice.
[d] TKO: Bcrp1/Mdr1a/1b$^{-/-}$(triple knockout) mice.
* $p<0.01$ WT vs. WT + elacridar.
# $p<0.01$ WT vs. TKO.
$ $p<0.01$ TKO vs. TKO + elacridar.
Data are presented as mean ± SD.

Effect of elacridar or pantoprazole on the plasma pharmacokinetics of gimatecan in WT and Bcrp1/Mdr1a/1b$^{-/-}$ mice

We administered an oral or i.v. dose of gimatecan (2 mg/kg) to WT and Bcrp1/Mdr1a/1b$^{-/-}$ mice pretreated with p.o. elacridar or pantoprazole. As shown in table 1 co-administration of elacridar increased the $AUC_{0\text{-}inf}$ p.o. and the $AUC_{0\text{-}inf}$ i.v. 1.8- and 1.9-fold, respectively, in WT and 1.6- and 2.2-fold, respectively, in Bcrp1/Mdr1a/1b$^{-/-}$ mice (p<0.001). The pharmacokinetic data of $AUC_{0\text{-}24}$ showed the same pattern as the $AUC_{0\text{-}inf}$ (table 1). These results suggest that co-administration of elacridar significantly affects the pharmacokinetics of p.o. and i.v. gimatecan, which could in part take place by inhibition of Bcrp1 and P-gp activity. However, the additional effect of elacridar on the $AUC_{0\text{-}inf}$ of gimatecan observed in Bcrp1/Mdr1a/1b$^{-/-}$ mice after p.o. and i.v. administration indicates that other mechanisms, such as interaction with other drug transporters or drug metabolizing enzymes by which elacridar could influence gimatecan absorption and disposition are involved.

In contrast, p.o. pantoprazole at the applied dosage of 40 mg/kg in this animal model did not significantly affect the p.o. and i.v. pharmacokinetics of gimatecan (table 1).

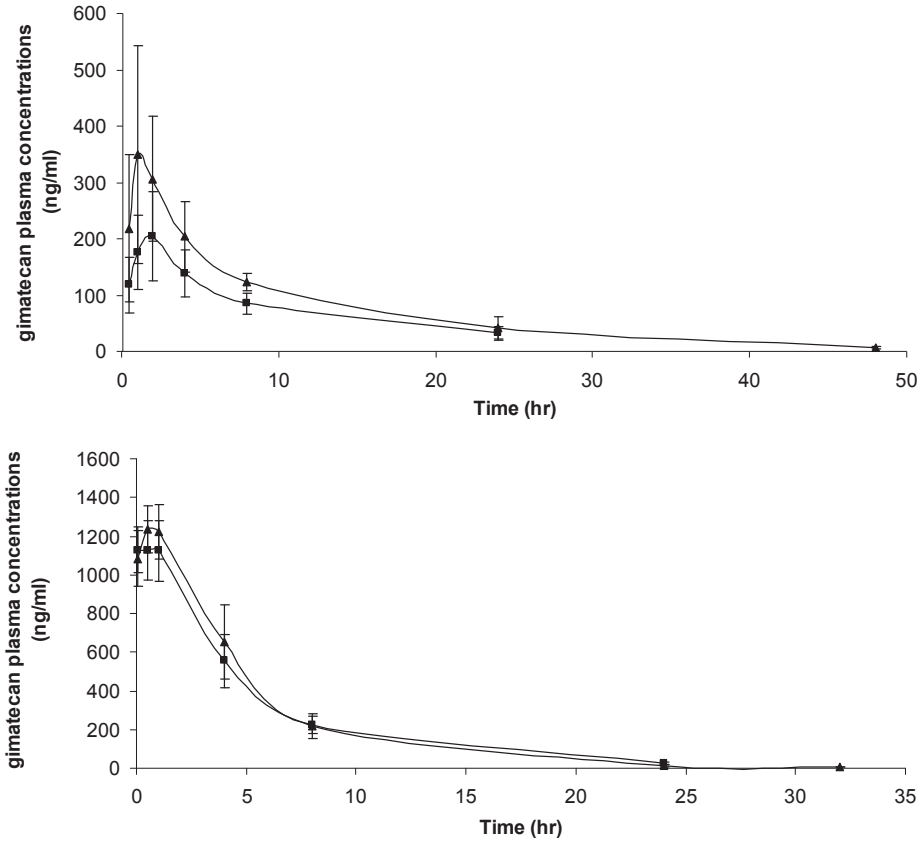

Figure 1. Plasma concentration time curves in WT(■) and Bcrp1/Mdr1a/1b$^{-/-}$ (▲) mice after oral (A) and i.v. (B) administration of gimatecan (2 mg/kg). At least 6 mice for each group were used. Points mean concentrations for oral and i.v. administration (n ≥ 6) ± SD.

DISCUSSION

We evaluated whether the absence of BCRP/Bcrp1 may affect the pharmacokinetics of gimatecan *in vivo*, employing WT and Bcrp1/Mdr1a/1b$^{-/-}$ mice. Brain penetration of gimatecan was also evaluated: results are presented and discussed in **Chapter 7** of this thesis. We chose the Bcrp1/Mdr1a/1b$^{-/-}$ (triple knockout) mouse model because it was readily available at our institute and because some camptothecin derived top I inhibitors have low affinity for P-gp (Maliepaard et al., 2001). No data are currently available on the affinity of gimatecan for mouse Mdr1a/Mdr1b; however, the results previously obtained by us and other authors with human MDR1 overexpressing cell lines support the evidence of a lack of affinity of gimatecan for MDR1 (Marchetti et al.; 2007; De Cesare et al., 2001). Therefore, the transport of gimatecan by murine Mdr1a/1b is unlikely and we believe that the effect of Mdr1a/1b gene deletion on gimatecan pharmacokinetics is negligible.

Results obtained after oral administration revealed a statistically significant effect of the absence of Bcrp1/Mdr1a/Mdr1b on the $AUC_{0\text{-}inf}$ as well as on the C_{max} of gimatecan. Considering the high contribution of the extrapolated area to the $AUC_{0\text{-}inf}$ observed in several mice treated we calculated also the $AUC_{0\text{-}24h}$ using the linear trapezoidal rule, which however did not lead to different results.

As expected, after i.v. administration no statistically significant difference was found between the curves of gimatecan in Bcrp1/Mdr1a/1b$^{-/-}$ versus WT mice.

The apparent bioavailability of gimatecan in WT mice was 35 ± 2.9% and in Bcrp1/Mdr1a/1b$^{-/-}$ mice 48 ± 4.4%, which difference is statistically significant (p<0.05) and indicates that BCRP expressed in the gut limits oral uptake of gimatecan.

In other experiments we tested the pharmacokinetics of p.o. and i.v. gimatecan when a P-gp and BCRP inhibitor (pantoprazole (Pauli-Magnus et al., 2001; Breedveld et al., 2004) or elacridar (Maliepaard et al., 2001; Evers et al., 2000)) was co-administered. The results revealed that pre-treatment with 40 mg/kg pantoprazole did not affect the pharmacokinetics of oral neither i.v. gimatecan in this mouse model. The lack of the pharmacokinetic interaction between pantoprazole (which was applied at a high dose-level in our experiments) and gimatecan indicates that a clinical interaction between these two drugs is unlikely.

In contrast, co-administration of elacridar significantly increased the $AUC_{0\text{-}inf}$ after oral and i.v. administration of the drug in WT as well as in the triple knockout mice. The additional effect on the AUC observed for elacridar in Bcrp1/Mdr1a/1b$^{-/-}$ mice suggests that besides BCRP/P-gp inhibition other mechanisms (such as interaction with other drug transporters or drug metabolizing enzymes) may contribute to this gimatecan-elacridar interaction and further experiments are warranted. Indeed, results obtained by Lee et al suggested that elacridar most likely inhibit one or more transporters distinct from BCRP/P-gp (Lee et al., 2005) and in *in vitro* experiments, recently we found that elacridar inhibits efficiently the transport mediated by organic anion-transporting polypeptide 1B1 (OATP1B1) and organic cation transporter T1 (OCT1)(data submitted). Currently, no data are available regarding the affinity of gimatecan for these drug efflux transporters. However, lately Nozawa et al. demonstrated that OATP1B1 transports SN-38, the active metabolite of the camptothecin irinotecan (Nozawa et al., 2005) and OATP1B1-polymorphisms have been reported to affect irinotecan-pharmacokinetics and clinical outcome of cancer patients (Han et al., 2008).

In the evaluation of our results, it cannot be excluded that as a consequence of P-gp and Bcrp1 gene deletion other transporters and/or drug metabolizing enzymes involved in absorption, metabolism, distribution and elimination of gimatecan are over-expressed in knockout mice thus reducing the impact of Bcrp1/P-gp deletion on gimatecan pharmacokinetics. Moreover, potential species differences in expression and localization of transporters should be taken into account in extrapolating our results from animal models to the human situation. Clearly, our results obtained in the mouse model need to be confirmed in the clinic.

CONCLUSIONS

In mice absence of Bcrp1/Mdr1a/1b significantly affected the oral pharmacokinetics of gimatecan, but had little effect on the i.v. pharmacokinetics. This means that most likely only the plasma pharmacokinetics of oral gimatecan is affected by endogenous epithelial BCRP in humans. Implications of BCRP expression in the gut for the oral development of gimatecan may be limited but need to be explored. Furthermore, a clinically relevant pharmacokinetic interaction between gimatecan and benzimidazole proton pump inhibitors is unlikely. In contrast, the *in vivo* interaction between elacridar and gimatecan appears to be partly mediated by other drug transporters than BCRP/Bcrp1. Further clinical investigations about the interaction between oral gimatecan and other BCRP substrate drugs and/or inhibitors are warranted.

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7

Abcc4 together with Abcb1 and Abcg2 form a robust cooperative drug efflux system that restricts the brain entry of camptothecin analogues

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Clin Cancer Res 2013; 19(8): 2084-95.

ABSTRACT

Background: Multidrug resistance-associated protein 4 (ABCC4) shares many features with P-glycoprotein (ABCB1) and breast cancer resistant protein (ABCG2), including broad substrate affinity and expression at the blood-brain barrier (BBB). However, the pharmacological relevance of ABCC4 at the BBB is difficult to evaluate, as most drugs are also substrates of ABCB1 and/or ABCG2.
Experimental Design: We have created a mouse strain in which all these alleles are inactivated to assess their impact on brain delivery of camptothecin analogues, an important class of antineoplastic agents and substrates of these transporters. Wilde-type (WT), Abcg2$^{-/-}$, Abcb1a/b$^{-/-}$, Abcc4$^{-/-}$, Abcb1a/b;Abcg2$^{-/-}$, Abcg2;Abcc4$^{-/-}$ and Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice received i.v. topotecan, irinotecan, SN-38 or gimatecan alone or with concomitant oral elacridar. Drug levels were analyzed by high-performance liquid chromatography (HPLC).
Results: We found that additional deficiency of Abcc4 in Abcb1;Abcg2$^{-/-}$ mice significantly increased the brain concentration of all camptothecin analogues by 1.2-fold (gimatecan) to 5.8-fold (SN-38). The presence of Abcb1 or Abcc4 alone was sufficient to reduce the brain concentration of SN38 to the level in WT mice. Strikingly, the brain distribution of gimatecan in brain of WT mice was more than 220-fold and 40-fold higher than that of SN-38 and topotecan, respectively.
Conclusion: Abcc4 limits the brain penetration of camptothecin analogues and teams up with Abcb1a/b and Abcg2 to form a robust cooperative drug efflux system. This concerted action limits the usefulness of selective ABC transport inhibitors to enhance drug entry for treatment of intracranial diseases. Our results also suggest that gimatecan might be a better candidate than irinotecan for clinical evaluation against intracranial tumors.

INTRODUCTION

The blood-brain barrier (BBB) is a complex but well-organized structure that serves to protect the brain by limiting the entry of most exogenous compounds into brain (1). On the other hand, it is seen as a major obstacle for many therapeutic agents which might otherwise be effective against brain diseases including brain cancer (2-4). The BBB is formed by the brain endothelial cells, which are closely linked to each other by tight junctions, lacking fenestrae and having low pinocytic activity. As a consequence, the brain entry of substances requires transendothelial passage, thus allowing strict regulation of brain entry by a range of uptake and efflux transporters (5). ATP-binding cassette (ABC) drug efflux transporters expressed at the blood brain barrier (BBB) restrict the entry of many compounds into the brain (6). The dramatic impact of P-glycoprotein (P-gp, ABCB1/Abcb1a) on the brain entry of substrate agents was first shown by Schinkel and colleagues (7) using Abcb1a-deficient mice and was later shown to be important for a plethora of agents. The cooperative action of breast cancer resistance protein (BCRP, ABCG2/Abcg2) in combination with Abcb1 became clear when we used compound Abcb1a/b;Abcg2-deficient mice (8). Until then, the functionality of Abcg2 at the BBB had not been convincingly shown by results obtained with single Abcg2 deficient mice because of the overlapping substrate affinities of Abcb1a/b and Abcg2 and because the presence of

Abcb1a/b alone is sufficient to reduce the concentration of dual ABCB1/ABCG2 substrates in the brain to the level of that in wild-type (WT) mice.

Camptothecin and its analogues are potent topoisomerase I inhibitors and represent an important class of antineoplastic agents with a wide spectrum of antitumor activity. Two camptothecin analogues, topotecan and irinotecan (CPT11), have already been approved for treatment of advanced ovarian cancer, small cell lung cancer, and colon cancer (9-11). Whether they could also be efficacious against brain malignancies is speculative, but CPT11 is receiving considerable attention with more than 10 ongoing or planned clinical trials involving brain cancer (source: http://clinicaltrials.gov). However, whether these drugs can cross the BBB in sufficient amounts to be active against intracranial tumors remains uncertain. *In vitro* studies have shown that CPT11 and its active metabolite SN-38 are also substrates of ABCB1 and/or ABCG2 (12-16), which may thus exclude these compounds from the brain, as was shown for topotecan (8). Gimatecan is another camptothecin analogue with different pharmaceutical properties. Relative to topotecan and CPT11/SN-38, it is a more lipophilic compound and a weaker substrate of ABCB1 (17, 18). It is also reported to have none or minimal affinity for ABCG2 (18, 19), although Marchetti and colleagues (17) reported that gimatecan is a substrate of Abcg2.
Besides ABCB1 and ABCG2, multidrug resistance protein 4 (MRP4, ABCC4/Abcc4) is also expressed at the BBB and the choroid plexus epithelium (20). ABCC4 transports a wide range of more polar endogenous molecules such as nucleotides, urate and folates, bile acids and glutathione conjugates but is also reported to transport antiviral, antibiotic, cardiovascular and anticancer agents including topotecan, CPT11/SN-38, and gimatecan (18, 20-24) *in vitro*. The initial claim (20) that the brain accumulation of topotecan is higher in single Abcc4-deficient mice could not be replicated, presumably because topotecan is also a substrate of Abcb1a/b and Abcg2, which dominate the restriction of its brain entry (8). More recently, however, it was shown that Abcc4-deficient mice accumulate more oseltamivir carboxylate (Ro64-0802) in their brain (25). Importantly, this relatively hydrophilic metabolite of oseltamivir is not also a substrate of Abcb1 (26), explaining why the substrate functionality of Abcc4 at the BBB could be shown in single Abcc4-deficient mice.
To allow more accurate assessment of the impact of Abcc4 on the BBB penetration without interference by Abcb1a/b an Abcg2, we conducted a comprehensive comparison between Abcb1a/b;Abcg2;Abcc4 *versus* Abcb1a/b;Abcg2-deficient mice and a range of other control strains. For this purpose, we used the camptothecin analogues topotecan, CPT11, SN-38, and gimatecan, which all differ to some extent in their lipophilicity and affinities towards these ABC-transporters. Our results clearly show the profound impact that Abcc4 can have on the brain penetration of substrate drugs.

MATERIALS AND METHODS

Chemicals and drugs

Topotecan and elacridar were kindly provided by GlaxoSmithKline. Irinotecan, (Campto®), was from Pfizer; SN-38 was from Sequoia Research Products; and gimatecan was provided by Novartis Pharmaceuticals Inc. Blank human plasma was obtained from healthy donors (Sanquin). All other chemicals were purchased from Merck.

Animals

Mice were housed and handled according to institutional guidelines complying with Dutch legislation. All experiments with animals were approved by the local animal experiment committee. The animals used in this study were WT, Abcg2$^{-/-}$, Abcb1a/b$^{-/-}$, Abcc4$^{-/-}$ (20), Abcb1a/b;Abcg2$^{-/-}$ (8), Abcg2;Abcc4$^{-/-}$ and Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice, all of a >99% FVB genetic background, between 8 and 14 weeks of age. The animals were kept in a temperature-controlled environment with a 12-hour dark cycle and received a standard diet (AM-II, Hope Farm B.B.) and acidified water *ad libitum*.

Plasma and brain pharmacokinetics

Topotecan and CPT11 were diluted to 0.5 mg/mL in 5% (w/v) glucose, whereas gimatecan and SN-38 were dissolved (2 mg/mL) in dimethyl sulfoxide (DMSO). They were administered i.v. at a dose of 2 mg/kg (for topotecan, SN-38 and gimatecan) or 5 mg/kg (for CPT11). Elacridar prepared as described earlier (8) was given p.o. at a dose of 100 mg/kg 2 hours before camptothecins. Continuous infusion of topotecan was achieved with Alzet minipumps model 1003D (Durect Corp.) filled with 2 or 10 mg/mL of topotecan in 5% (w/v) glucose. Pumps were placed in the peritoneal cavity under isoflurane anesthesia, and mice were sacrificed 28 to 30 hours after placement.

Mice were anesthetized by isoflurane. Blood was collected by cardiac puncture and kept on ice. The mice were sacrificed and brains were dissected. Plasma was separated by centrifugation at 10,000 *g* for 5 min at 4°C. Brains were homogenized in 3 mL 1% (w/v) bovine serum albumin (BSA). Both plasma and brain homogenates were stored at −20°C until analysis. The brain accumulation was corrected for the amount of drug in the brain vasculature (1.4%, ref. 27).

Drug analysis

Topotecan, CPT11, and SN-38 samples were analyzed by high-performance liquid chromatography (HPLC) as described previously (28, 29) but with minor modification. In brief, 100 µL of biological sample was mixed with 200 µL of ice-cold methanol and centrifuged at 4°C, 20.000 x *g* for 10 minutes. Next, 100 µL of the supernatant fraction was mixed with 200 µL of ice-cold perchloric acid (2% in water). After centrifugation at 4°C, 20,000 x *g* for 3 minutes, 100 µL of clear supernatant was analyzed by HPLC.

For gimatecan, 50 µL of sample was mixed with of ice-cold methanol and centrifuged at 4°C, 20,000x *g* for 10 minutes. A volume of 200 µL of supernatant fraction was mixed with 300 µL 0.01 mol/L sodium borate, centrifuged at 4°C, 20,000 x *g* for 5 minutes and 100 µL of clear solution was injected into the HPLC system.

HPLC was conducted using a DGP-3600A pump with SRD-3600 Solvent Rack, a model WPS-3000TSL autosampler (Dionex) and a model FP-1520 fluorescence detector (Jasco) operating at 380/527 nm (excitation/emission). Separation of topotecan, CPT11, and SN-38 was performed using a Zorbax SB-C_{18} column (75 × 4.6 mm i.d., Rockland Technologies Inc.) and for gimatecan a Symmetry C_{18} column (2.1 × 150 mm i.d. Waters). The mobile phase for topotecan consisted of methanol, 0.1 mol/L hexane-1-sulfonic acid, and 0.01 mol/L TEMED adjusted to pH 6.0 with phosphoric acid (25:10:65, v/v/v). The mobile phase for CPT11 and SN-38 consisted of 0.1 mol/L ammonium acetate buffer pH 6.4 containing 5 mmol/L tetrabutylammonium bromide, triethylamine, and acetonitrile (790:1:210, v/v/v). The mobile phase for gimatecan consisted of acetonitrile, 50 mmol/L ammonium acetate

buffer adjusted to pH 6.8 (30:70, v/v). Chromatographic data analysis was conducted using Chromeleon softare v6.8 (Dionex). The lower limit of quantitation was 0.05 ng/mL (plasma) and 0.5 ng/g (brain) for topotecan and SN38 and 0.2 ng/mL (plasma) and 2 ng/g (brain) for CPT11 and gimatecan.

Ex vivo carboxylesterase activity measurement

Carboxylesterase activity in mouse plasma was measured by monitoring *ex vivo* conversion of CPT11 into SN-38 using previously described methods with slight modification (30). In short, 20 µL 2.5 mmol/L CPT11 was mixed with 800 µL 20 mmol/L Tris-HCl buffer (pH 7.5) and incubated at 37°C for 30 minutes to reach equilibrium between the lactone and carboxylate forms of CPT11. Next, 200 µL fresh plasma collected from WT, Abcg2$^{-/-}$ or Abcb1a/1b$^{-/-}$;Abcg2$^{-/-}$ mice (n=3) was added (final CPT11 concentration is 50 µmol/L) and the mixtures were kept at 37°C with shaking. At 0 minute, 1, 2, 4 and 4 hours, 100 µL samples were collected for the determination of CPT11 and SN-38 concentrations.

RESULTS

Role of Abcc4 in topotecan brain delivery

The novel Abcg2;Abcc4$^{-/-}$ and Abcb1a/b;Abcg2;Abcc4$^{-/-}$ strains were obtained by cross-breeding of the Abcc4$^{-/-}$, Abcg2, and Abcb1a/bAbcg2$^{-/-}$ mice. These mice are viable, fertile, and also do not display any overt phenotype.

To investigate the role of Abcc4 in the brain penetration of topotecan without the interference by Abcg2 and Abcb1a/b, we determined the topotecan concentrations in brain and plasma from WT, Abcg2$^{-/-}$, Abcb1a/b;Abcg2$^{-/-}$ and Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice after i.v. administration of 2 mg/kg topotecan. In line with our previous results (8), the absence of Abcg2 alone caused a marked 5-fold increased plasma concentration of topotecan in comparison with Abcg2-proficient WT mice (Fig. 1A), whereas it caused only a small (2-fold) and not significant higher brain concentration. As a consequence, the brain-to-plasma ratio was lower in Abcg2$^{-/-}$ mice relative to WT controls. To understand this counterintuitive result, we also determined the brain and plasma levels at 5 minutes after drug administration and found that the differences between the strains were much smaller at this very early time point (Fig. 1D), implicating that the absence of Abcg2 alone has little effect on the distribution to the brain. At 1 hour, the plasma levels were reduced by about 20- and 6-fold in WT and Abcg2$^{-/-}$ mice, respectively, whereas the brain levels were reduced by only 5- and 3-fold. Thus, it appears that efflux from the brain cannot keep up with the much more rapid elimination from plasma and the reduced brain-to-plasma ratio in Abcg2$^{-/-}$ versus WT mice is a consequence of the more rapid decay in the plasma concentration in WT mice.

At both 1 and 4 hours, the plasma concentration of topotecan in Abcb1a/b;Abcg2$^{-/-}$ and Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice was similar, whereas the concentration in brains of Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice was significantly higher than in brains of Abcb1a/b;Abcg2$^{-/-}$ mice (Fig. 1B). Overall, this resulted in a 2.0-fold and 1.9-fold elevated brain-to-plasma ratio of topotecan at 1 and 4 hours, respectively, in Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice versus Abcb1a/b;Abcg2$^{-/-}$ mice (Fig. 1C).

Because topotecan is relatively good water soluble, we decided to carry out a similar experiment where topotecan (2 mg/kg/d) was delivered by Alzet minipumps to achieve steady-state plasma concentrations. The difference in systemic exposure in WT and Abcg2$^{-/-}$ strains at this dose was modest as steady-state plasma levels differed only by about 3-fold. The brain concentration was significantly higher in Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice than in Abcb1a/b;Abcg2$^{-/-}$ mice (Fig. 1E–G), confirming the impact of Abcc4. We also included a cohort of WT mice that received a 5-fold higher dose level (10 mg/kg/d) in an attempt to compensate for the higher clearance in WT mice; however, the plasma levels were not proportionally higher. We have no clear explanation for this finding.
Together these results show that Abcc4 restricts the brain penetration of topotecan in the absence of Abcb1a/b and Abcg2 but has no effect on the plasma level of topotecan.

The roles of Abcb1a/b, Abcg2, and Abcc4 in brain delivery of CPT11 and its active metabolite SN-38

Abcb1a/b;Abcg2$^{-/-}$ mice had significantly higher plasma levels of CPT11 and SN-38 than Abcg2$^{-/-}$ mice, whereas there was no difference between Abcb1a/b;Abcg2$^{-/-}$ and Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice receiving CPT11 (Fig. 2A). Interestingly, the CPT11 level in plasma of all Abcg2$^{-/-}$ mice was markedly lower than that of WT mice, whereas the plasma level of SN-38 was significantly higher than that of WT mice. Given the fact that the sum of concentrations (SUM[CPT11+SN-38]) in plasma of WT and Abcg2$^{-/-}$ mice were similar, the reduction in CPT11 levels are probably not due to elimination (efflux) by Abcg2. As carboxylesterase(s) are principally involved in the conversion of CPT11 to its active metabolite SN-38, an increased expression of carboxylesterase(s) in Abcg2 deficient mice might underlie this accelerated conversion of CPT11 into SN-38. We evaluated the *ex vivo* conversion rate of CPT11 into SN-38 using freshly collected plasma of WT, Abcg2$^{-/-}$, and Abcb1a/b;Abcg2$^{-/-}$ mice. Following the incubation of CPT11 in Abcg2$^{-/-}$ murine plasma for only 1 hour, more than 20% of the parent drug was already converted into SN-38, whereas only 1% of CPT11 was converted to SN-38 after 4 hr incubation in WT plasma. A similar conversion rate to that in Abcg2$^{-/-}$ mice was observed in plasma of Abcb1a/b;Abcg2$^{-/-}$ mice. The marked elevation of the CPT-11 to SN-38 conversion in all strains that are deficient in Abcg2 relative to Abcg2 proficient strains makes it more difficult to interpret the role of the ABC transporters on the brain penetration. It is clear, however, that Abcb1a/b plays a pivotal role in brain penetration of CPT11 given the 10.2-fold and 15.9-fold higher brain CPT11 levels at 1 an 4 hours, respectively, in Abcb1a/b;Abcg2$^{-/-}$ mice versus Abcg2$^{-/-}$ (Fig. 2C). Abcb1a/b also limits the brain penetration of SN-38, although the difference between Abcg2$^{-/-}$ and Abcb1a/b;Abcg2$^{-/-}$ mice was smaller (about 2.5-fold). Importantly, however, the additional deletion of Abcc4 in the absence of Abcb1a/b and Abcg2 resulted in a further 3.4-fold higher brain concentration of SN-38 at 1 hour. Similarly, the CPT11 levels were higher, but the difference was only 1.5-fold. Overall, the SUM[CPT-11+SN-38] in brain and the brain-to-plasma ratio of Abcb1a/b;Abcg2$^{-/-}$;Abcc4$^{-/-}$ mice were significantly higher than those of Abcb1a/b;Abcg2 mice (Fig. 2F and table 1)

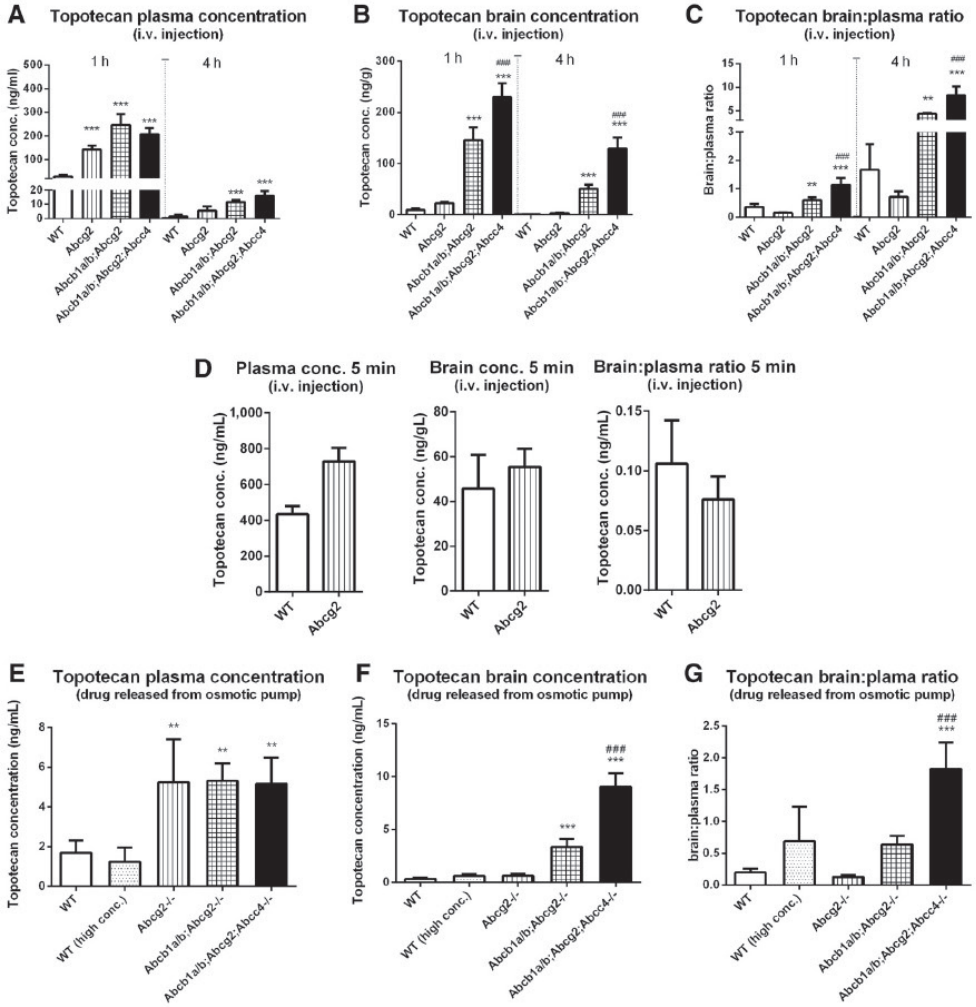

Figure 1. Topotecan brain and plasma pharmacokinetics. Plasma concentrations, brain concentrations, and brain-to-plasma ratios of topotecan in WT, Abcg2$^{-/-}$, Abcb1a/b;Abcg2$^{-/-}$, and Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice 1 and 4 hours (A-C) and 5 minutes (D) after i.v. administration of 2 mg/kg topotecan or 28 to 30 hours after implantation of an Alzet minipump delivering 2 or 10 (high-dose) mg/kg/d (E-G). Data are means ± SD. n=8, 5, 10, 5 (1 hour) and 5, 5, 5, 5 (4 hours) for WT, Abcg2$^{-/-}$,Abcb1a/b;Abcg2$^{-/-}$, and Abcb1a/b;Abcg2;Abcc4$^{-/-}$mice (A–C); n= 5 and 5 for WT and Abcg2$^{-/-}$ mice (D); n = 5, 4, 5, 5 and 5 for WT, WT (high-dose), Abcg2$^{-/-}$, Abcb1a/b;Abcg2$^{-/-}$, and Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice (E–G). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with WT mice. ###, $P < 0.001$, compared with Abcb1a/b;Abcg2$^{-/-}$ mice.

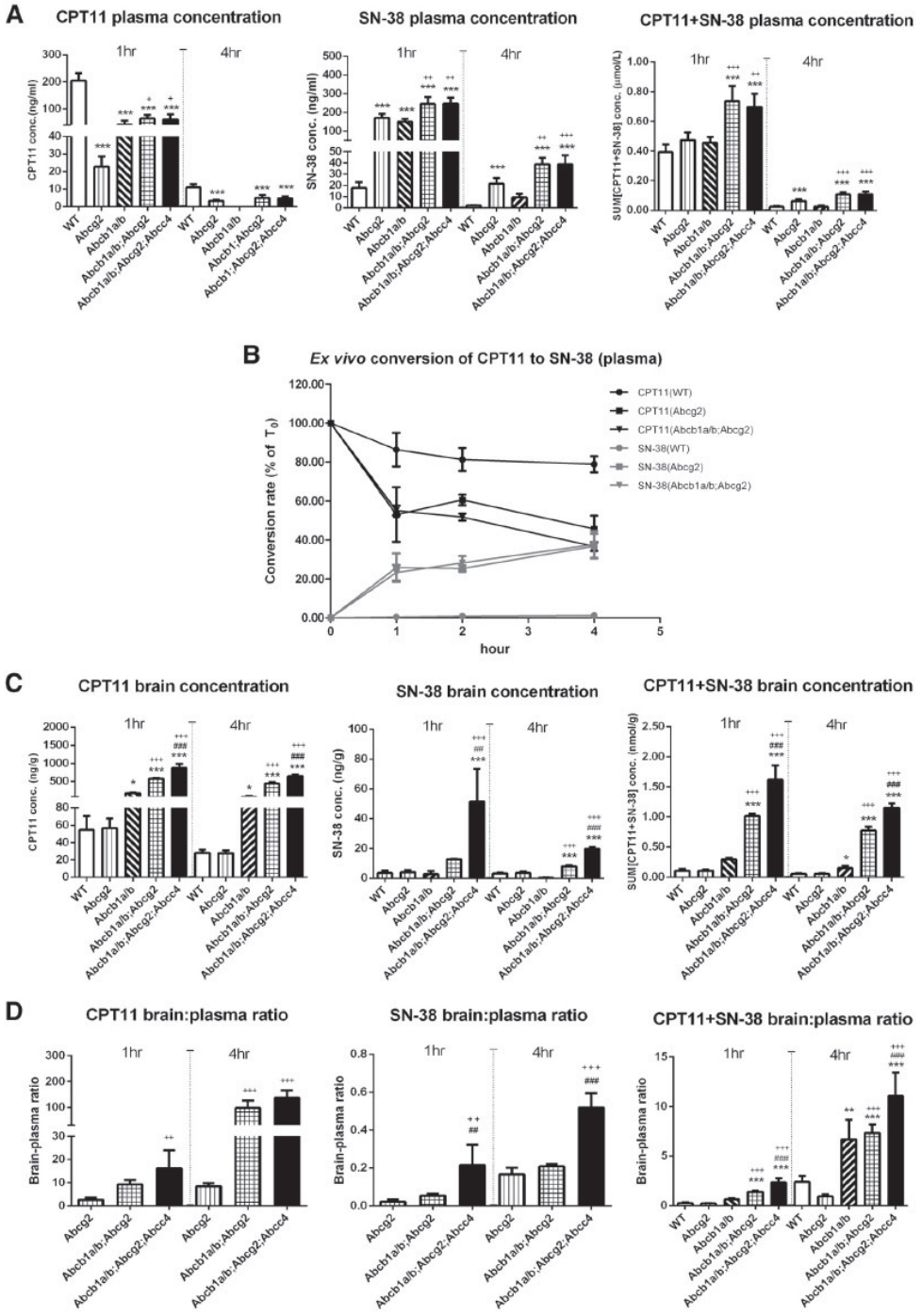

Figure 2. Irinotecan (CPT11) and SN-38 brain and plasma pharmacokinetics. Plasma concentrations (A), brain concentrations (C), brain-to-plasma ratios (D) of CPT11, SN-38 and SUM[CPT11+SN-38] (sum of molarities of CPT11 and SN-38) in WT, Abcg2$^{-/-}$, Abcb1a/b$^{-/-}$, Abcb1a/b;Abcg2$^{-/-}$, and Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice 1 and 4 hours after i.v. administration of 5 mg/kg CPT11. The conversion

rate of CPT11 to SN-38 during 4-hour *ex vivo* incubation of plasma from WT, Abcg2$^{-/-}$, and Abcb1a/b;Abcg2$^{-/-}$ mice with 50 µmol/L CPT11 in buffer (B). For A, C, and D, data are means ± SD. n = 5, 5, 5, 5, 6 (1 hour) and 5, 5, 5, 5, 5 (4 hours) for WT, Abcg2$^{-/-}$, Abcb1a/b$^{-/-}$, Abcb1a/b;Abcg2$^{-/-}$, and Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice (A, C, and D). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with WT mice. +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$, compared with Abcg2$^{-/-}$ mice. #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$, compared with Abcb1a/b;Abcg2$^{-/-}$ mice. Note break in *y*-axes of the CPT11 and SN-38 plasma concentrations. For B, data are means ± SD. n = 3 per strain.

Table 1. Increase (fold change) of the brain concentrations, brain-to-plasma ratios, and percentages of dose in brain after administration of topotecan, irinotecan (SUM[CPT11 and SN-38]), SN-38 and gimatecan caused by deficiency in Abcb1a/b, Abcg2, and/or Abcc4.

	Time point	Topotecan	SUM[CPT11+SN38]	SN-38	Gimatecan
Brain concentration (Abcg2;Abcb1a/b;Abcc4 *vs.* WT)	**1 h**	24-fold	15-fold	6.6-fold	4.1-fold
	4 h	94-fold	21-fold	N/A	4.4-fold
Brain concentration (Abcg2;Abcb1a/b *vs.*.Abcg2)	**1 h**	6.5-fold	9.1-fold	1.2-fold	2.1-fold
	4 h	14-fold	14-fold	N/A	2.4-fold
Brain concentration (Abcg2;Abcb1a/b;Abcc4 *vs.* Abcg2;Abcb1a/b)	**1 h**	1.6-fold	1.6-fold	5.8-fold	1.4-fold
	4 h	2.6-fold	1.5-fold	N/A	1.2-fold
Brain-plasma ratio (Abcg2;Abcb1a/b;Abcc4 *vs.* WT)	**1 h**	3.2-fold	8.8-fold	0.7-fold	3.4-fold
	4 h	4.2-fold	4.5-fold	N/A	3.8-fold
Brain-plasma ratio (Abcg2;Abcb1a/b *vs.* Abcg2)	**1 h**	3.8-fold	5.9-fold	1.3-fold	1.7-fold
	4 h	6.2-fold	7.6-fold	N/A	1.8-fold
Brain-plasma ratio (Abcg2;Abcb1a/b;Abcc4 *vs.* Abcg2;Abcb1a/b)	**1 h**	1.9-fold	1.7-fold	2.7-fold	1.3-fold
	4 h	1.9-fold	1.5-fold	N/A	1.5-fold
Percentage of the dose in brain (Abcg2;Abcb1a/b;Abcc4 *vs.* WT)	**1 h**	23-fold	16-fold	7.5-fold	3.8-fold
	4 h	81-fold	23-fold	N/A	3.9-fold
Percentage of dose in brain (Abcg2;Abcb1a/b *vs.* Abcg2)	**1 h**	6.1-fold	8.1-fold	1.1-fold	2.1-fold
	4 h	12.7-fold	13-fold	N/A	2.2-fold
Percentage of the dose in brain (Abcg2;Abcb1a/b;Abcc4 *vs.* Abcg2;Abcb1a/b)	**1 h**	1.5-fold	1.6-fold	5.6-fold	1.3-fold
	4 h	2.4-fold	1.5-fold	N/A	1.2-fold

NOTE: Comparison of Abcg2;Abcb1a/b;Abcc4$^{-/-}$ and WT mice shows the overall impact from Abcg2, Abcb1a/b, and Abcc4. Comparison of Abcg2;Abcb1a/b;Abcc4$^{-/-}$ and Abcg2;Abcb1a/b $^{-/-}$ mice shows the impact of Abcc4. Comparison of Abcg2;Abcb1a/b$^{-/-}$ and Abcg2$^{-/-}$ mice shows the impact of Abcb1a/b. Unit, fold. N/A, not assessed.

Roles of Abcb1a/b, Abcg2 and Abcc4 in brain delivery of gimatecan

Gimatecan is a relatively new camptothecin analogue and little is known about the impact of drug efflux transporters of this analogue *in vivo*. Therefore, we evaluated the roles of Abc1a/b, Abcg2, and Abcc4 in gimatecan plasma and brain pharmacokinetics using our knockout mice. Unlike topotecan and CPT11, the plasma concentration of gimatecan was not different across all strains on i.v. administration of 2 mg/kg gimatecan, except there was a 1.5-fold elevation of the gimatecan plasma level in Abcb1a/b;Abcg2$^{-/-}$ at 4 hours (Fig. 3A). Moreover, the plasma levels of all strains were much higher than those of topotecan or CPT11/SN-38.

Interestingly, there were also smaller differences in the brain concentration of gimatecan across all strains, relative to topotecan and CPT11/SN-38 (Fig. 3B). However, there were clear differences between Abcb1a/b-deficient strains (Abcb1a/b;Abcg2$^{-/-}$ and Abcb1a/b;Abcg2;Abcc4$^{-/-}$) and Abcb1 proficient strains (WT, Abcg2, Abcc4$^{-/-}$ and Abcg2;Abcc4$^{-/-}$). Deletion of Abcb1a/b alone caused a small but significant 1.9-fold increase relative to WT mice. Vice versa, the brain penetration in Abcb1a/b-proficient Abcg2;Abcc4$^{-/-}$ mice was similar to that in WT mice and much lower than in Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice. Consequently, Abcb1a/b appears to be the most important factor limiting the brain penetration of gimatecan. Although the difference in the brain concentration of gimatecan between Abcb1a/b;Abcg2$^{-/-}$ and Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice was small, the brain-to-plasma ratio in the latter was significantly higher at both 1 and 4 hours, indicating that Abcc4 also contributes to limiting the brain penetration of gimatecan. The same was seen for Abcg2 by comparing Abcb1a/b$^{-/-}$ versus Abcb1a/b;Abcg2$^{-/-}$ mice. Overall, however, the impact of these drug efflux transporters on gimatecan brain penetration is not as strong as for topotecan or CPT11.

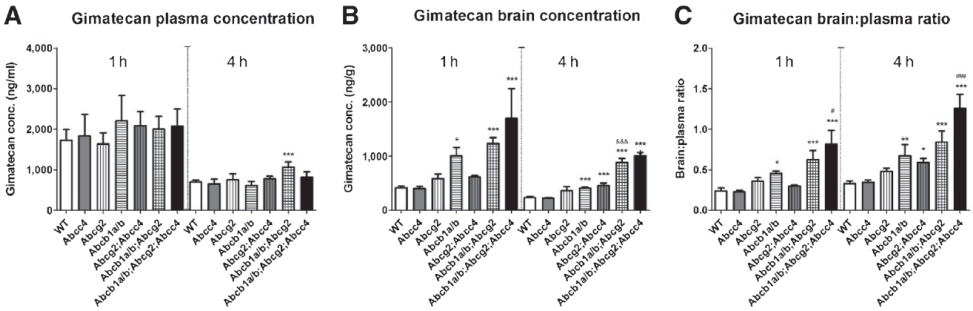

Figure 3. Gimatecan brain and plasma pharmacokinetics. Plasma concentrations (A), brain concentrations (B), and brain-to-plasma ratios (C) of gimatecan in WT, Abcc4$^{-/-}$, Abcg2$^{-/-}$, Abcb1a/b$^{-/-}$, Abcg2;Abcc4$^{-/-}$ Abcb1a/b;Abcg2$^{-/-}$, and Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice 1 and 4 hours after i.v. administration of 2 mg/kg gimatecan. Data are means ± SD. n = 5, 5, 5, 4, 6, 5, 5 (1 hour) and 5, 4, 5, 5, 5, 5, 5 (4 hours) for WT, Abcc4$^{-/-}$, Abcg2$^{-/-}$, Abcb1a/b$^{-/-}$, Abcg2;Abcc4$^{-/-}$, Abcb1a/b;Abcg2$^{-/-}$, and Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice (A–C). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, compared with WT mice. .&, $P < 0.05$; &&, $P < 0.01$; and &&&, $P < 0.001$, compared with Abcb1a/b$^{-/-}$ mice. #, $P < 0.05$; ##, $P < 0.01$; and ###, $P < 0.001$, compared with Abcb1a/b;Abcg2$^{-/-}$ mice.

Effect of the dual ABCB1 and ABCG2 inhibitor elacridar on brain penetration of SN-38 and gimatecan

We previously reported that co-administration of the dual ABCB1 and ABCG2 inhibitor elacridar together with topotecan markedly increased the brain penetration of topotecan (8). Because our present findings show that Abcc4 also impairs the brain penetration of camptothecin analogues, we investigated the effect of elacridar on the brain penetration of SN-38 in various Abcc4-deficient strains. SN-38 was used because the previous experiments clearly indicated that this compound was one of the best Abcc4 substrates. Co-administration of elacridar markedly enhanced the plasma SN-38 concentration across all strains, with the most dramatic increase in WT and Abcc4$^{-/-}$ mice (Fig. 4A). Presumably, the impact of elacridar on the plasma level of SN-38 is mainly due to the inhibition of Abcg2-mediated elimination because i) the plasma level of SN-38 in Abcg2$^{-/-}$ mice was

markedly higher than that of WT mice; ii), the plasma level of SN-38 was only moderately increase in Abcg2;Abcc4$^{-/-}$ mice upon concomitant elacridar; and iii) the SN-38 plasma level did not differ between Abcg2$^{-/-}$ and Abcb1a/b;Abcg2$^{-/-}$ mice or between Abcg2;Abcc4$^{-/-}$ and Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice, suggesting that Abcb1a/b is not actively involved in the elimination of SN-38. Intriguingly, elacridar also substantially increased the plasma level of SN-38 in Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice by a yet unknown cause.

Similar to what we found for topotecan, the brain-to-plasma ratio cannot be compared when the plasma elimination is very different, such as between Abcg2-proficient and -deficient strains.

Therefore, we focused on the actual brain concentration of SN-38. Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice lacking all 3 transporters had a 6.6-fold higher brain concentration of SN-38 relative to WT mice. Strikingly, there was no difference between the concentrations of SN-38 in brains of Abcb1a/b;Abcg2$^{-/-}$ and WT mice (Fig. 4B). In line with this result, administration of elacridar did not increase the SN-38 concentration in brain of WT mice as well. These results suggest that Abcc4 alone is sufficient to maintain a similar brain level of SN-38 as achieved in WT mice.

Similarly, the brain concentrations in Abcg2$^{-/-}$, Abcc4$^{-/-}$, and Abcg2;Abcc4$^{-/-}$ mice were also not different than in WT mice, suggesting that the presence of Abcb1a/b alone is also enough to reduce the brain concentration of SN-38 to the level of WT.

Because Abcb1a/b;Abcc4$^{-/-}$ mice do not exist, we could not evaluate the role of Abcg2 by a similar genetic analysis. However, with the help of elacridar to inhibit Abcb1a/b and (partially) Abcg2, we can make an estimation of the relative importance of Abcg2. The brains of Abcb1a/b;Abcg2$^{-/-}$ mice that receive elacridar accumulate to about 70% of the level observed in Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice, indicating that Abcb1a/b is substantially, albeit not completely, inhibited by elacridar (Fig. 4). When we compare the brain penetration of SN-38 in Abcc4$^{-/-}$ mice versus Abcc4$^{-/-}$ versus Abcc4;Abcg2$^{-/-}$ mice both receiving elacridar and assume that Abcb1a/b is inhibited to the same extent in both strains, the difference in brain penetration between these two strains will be mainly due to the activity of Abcg2.

We also investigated the effect of elacridar on gimatecan, as gimatecan appears to be a weaker substrate of Abcc4. Surprisingly, co-administration of elacridar did not increase the brain-to-plasma ratio of gimatecan. The brain concentration of gimatecan in mice receiving elacridar did not differ at 1 hour and was 1.7-fold increased at 4 hours (P<0.05) relative to mice not receiving elacridar. However, when corrected for the plasma concentration, the brain penetration of gimatecan was not significantly different in WT mice in the presence or absence of elacridar (Fig 4E and F), suggesting that the increased brain penetration at 4 hours is mainly a reflection of the higher gimatecan plasma levels. Also, no significant additional effect of elacridar was found on brain concentration of gimatecan in Abcb1a/b;Abcg2$^{-/-}$ mice. Similar to SN-38, elacridar increased the plasma levels in Abcb1a/b;Abcg2$^{-/-}$ mice by a yet unidentified mechanism.

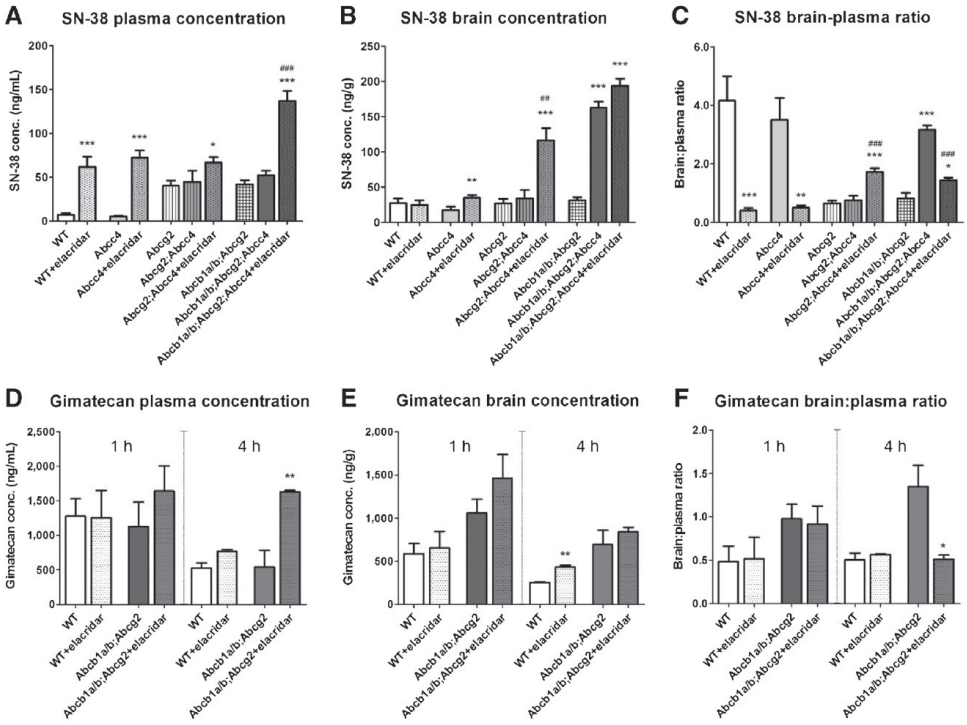

Figure 4. Effect of elacridar on the brain and plasma pharmacokinetics of SN-38 and gimatecan. Plasma concentrations (A), brain concentrations (B), and brain-to-plasma ratios (C) of SN-38 in WT, Abcg2$^{-/-}$, Abcc4$^{-/-}$, Abcg2$^{-/-}$, Abcg2;Abcc4$^{-/-}$ Abcb1a/b;Abcg2$^{-/-}$, and Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice 1 hour after i.v. administration of 2 mg/kg SN-38 with or without co-administration of elacridar (2 hours before the SN-38 administration, oral 100 mg/kg). Data are means ± SD. n = 5, 5, 4, 5, 5, 4, 5, 5, 4, and 5 for WT, WT + elacridar, Abcc4$^{-/-}$, Abcc4$^{-/-}$ + elacridar, Abcg2$^{-/-}$, Abcg2;Abcc4$^{-/-}$, Abcg2;Abcc4$^{-/-}$ + elacridar, Abcb1a/b;Abcg2$^{-/-}$, Abcb1a/b;Abcg2;Abcc4$^{-/-}$, Abcb1a/b;Abcg2;Abcc4$^{-/-}$ + elacridar groups (A–C). n = 3, 3, 3, 4 (1 hour) and 3, 3, 3, 3 (4 hours) for WT, WT + elacridar, Abcb1a/b;Abcg2$^{-/-}$, and Abcb1a/b;Abcg2$^{-/-}$+ elacridar groups (D–F). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$, compared with the first column of the same subgroup. #, $P < 0.05$; ##, $P < 0.01$; and ###, $P < 0.001$, compared with the second column of the same subgroup. Plasma concentrations (D), brain concentrations (E) and brain-to-plasma ratios (F) of gimatecan in WT, Abcg2$^{-/-}$, and Abcb1a/b;Abcg2$^{-/-}$ mice 1 and 4 hours after i.v. administration of 2 mg/kg gimatecan with or without co-administration of elacridar (2 hours before the SN-38 administration, oral 100 mg/kg). Data are means ± SD, n = 3. *, $P < 0.05$; **, $P < 0.01$; compared with the first column of the same subgroup.

Impact of Abcb1a/b, Abcg2, and Abcc4 on brain distributions of camptothecins

For all tested camptothecin analogues, their brain distributions (rendered as the percentage of total dose present in brain) were significantly increased when Abcb1a/b, Abcg2, and Abcc4 were simultaneously deleted. Loss of these transporters caused a profound increase of percentage of total dose of topotecan in brain (22- and 84-fold at 1 and 4 hours, respectively), slightly less profound for CPT11 (16- and 23-fold) and only a moderate increase for gimatecan (3.8- and 3.9-fold, Table 1), suggesting that the brain distributions of all camptothecins are affected by the drug efflux transporters but to differing extents depending on their unique structures.

We also compared the brain distributions of all camptothecins (Fig. 5). The brain distributions of the camptothecin analogues show a wide variation between WT and Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice. Strikingly, even in WT mice in which all these efflux transporters are present, up to 0.40% of total gimatecan dose was present in the brain at 1 hour and 0.21% at 4 hours. These values are more than 40- and 220-fold higher than those found for topotecan and for SN-38 at 1 hour, and 160- and 140-fold at 4 hour, respectively. In constrast, the percentage of CPT11 that was converted to SN-38 and detected in brains of Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice was exceptionally low (0.026% and 0.010% at 1 and 4 hours, respectively) and was even lower in WT mice (0.002% and 0.001% at 1 and 4 hours, respectively). At 1 hour after drug administration, this was only 9% of the total SN-38 and CPT11 level in brain.

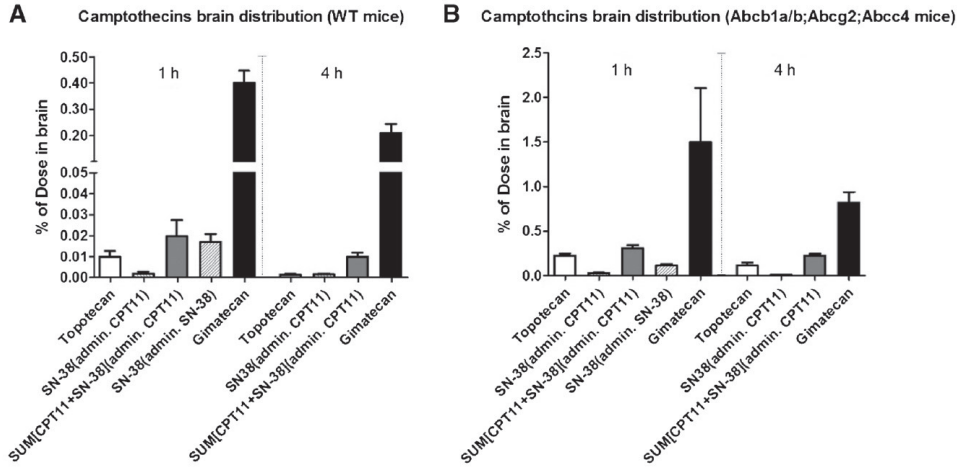

Figure 5. Brain distributions (represented by percentage of dose in brain) of topotecan, SN-38 after administration of CPT11 or SN-38, and gimatecan in WT and Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice 1 and 4 hours after i.v. administration of 2 mg/kg topotecan, 5 mg/kg CPT11, 2 mg/kg SN-38 (only with data of 1 hour), and 2 mg/kg gimatecan, respectively. Note break in *y*-axes of the percentage of dose in brain.

DISCUSSION

The present study shows that Abcc4 is an important factor limiting the brain penetration of the camptothecin analogues topotecan, CPT11, SN-38, and gimatecan. However, because of the overlapping affinities of Abcb1a/b and Abcg2 (2 other dominant drug efflux transporters at BBB), the actual contribution of Abcc4 to the elimination of camptothecins in brain can only be assessed when the other two others are absent. Together, this cooperative drug-efflux system of functionally overlapping transporters constructs a robust protective barrier against molecules entering the brain. Unfortunately, however, this protection may also lead to inadequate exposure of brain malignancies when treated with camptothecin analogues or other triple-substrate drugs. Because of the presence of Abcc4, coadministration of the dual ABCB1 and ABCG2 inhibitor elacridar is unable to

enhance the brain penetration of SN-38. In contrast to SN-38, the more lipophilic analogue gimatecan has itself much more favorable brain distribution properties and, from that perspective, may be a more useful candidate for treatment of intracranial tumors than any of the other camptothecin analogues.

Abcc4 is well known for its ability to transport a range of endogenous molecules and drugs including camptothecin analogs (18, 20-24). Abcc4 was first found by reverse transcriptase PCR in the microvessel-enriched fraction of bovine brain (31). Its presence in the apical membranes of mouse brain microvessels and in the basolateral membranes of choroid plexus epithelium was shown by Leggas et al (20) using Abcc4-knockout mice. The absence of Abcc4 in the choroid plexus in Abcc4$^{-/-}$ mice resulted in about 10-fold higher topotecan levels in the cerebrospinal fluid. Similarly, they observed a higher brain concentration of topotecan in Abcc4$^{-/-}$ mice versus Abcc4-proficient WT mice (20). This latter result, however, could not be confirmed when we compared Abcc4$^{-/-}$ and WT mice, even when looking into multiple strain backgrounds, gender, dose levels, and sampling times (8). The lack of an increased brain penetration of topotecan in the Abcc4$^{-/-}$ mice can now be explained by the presence of Abcb1a/b and Abcg2 at the BBB. The relative importance of each of the transporters at the BBB is shown using our Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice as a reference. In most earlier studies comparing WT and ABC transporter knockout mice, the WT has been taken as reference (e.g., stating that absence results in an x-fold increase of compound y). This makes sense when looking at single knockouts but becomes much more complex when analyzing compound knockouts. Instead, by taking the mouse model in which all of the studied transporters have been deleted as reference, we can now add in one of each ABC transporter at a time. Thus, by comparing Abcb1a/b;Abcg2;Abcc4$^{-/-}$ mice versus Abcb1a/b;Abcg2$^{-/-}$ mice, we can assess the role of Abcc4 and it turns out that Abcc4 alone is sufficient to reduce the SN38 levels (following SN38 administration) to those achieved in WT mice. It also follows that Abcc4 is an important factor for topotecan, gimatecan, and CPT11, albeit in a decreasing order (Table 1). A similar analysis was done for SN38 and Abcb1a/b by comparing Abcb1a/b;Abcg2;Abcc4$^{-/-}$ and Abcg2;Abcc4$^{-/-}$ mice, showing that also Abcb1a/b alone is also sufficient to reduce the brain level of SN38 to that of WT mice. Taking the most extensive combination knockout mouse as reference also provides a different view on what is frequently referred to as synergistic interaction between Abcb1a/b and Abcg2 in restricting the brain penetration of substances (32, 33). This claim of synergy is based on the finding that absence of both Abcb1a/b and Abcg2 together results in a much greater brain accumulation than the absence of only Abcb1a/b or Abcg2, as was first described for topotecan (8). However, synergy implicates that 2 or more factors (*e.g.,* drug transporters) together are more efficient in their action *(i.e.,* reducing brain entry) than each of these factors by themselves. Obviously, this is not the case, as the presence of Abcb1a/b or Abcc4 alone was sufficient to reduce the brain concentration of SN38 to the level in WT mice. True synergy would have implied that the presence/action of a single transporter would have had only a very minor effect on the brain accumulation, whereas only the combined presence would result in a profound reduction in the brain accumulation. Therefore, it is more appropriate to use the term cooperative drug efflux or concerted efflux, rather than synergy, to describe the interaction of these ABC transporters at the BBB. Following the SN-38 example, this expression of multiple transporters with overlapping affinities for substrates probably not only limits the entry of camptothecins in brain, but also serves as a general defense mechanism protecting

brain from potentially harmful substances. Inactivation or inhibition of one or even two transporters may challenge the transport capacity of the remaining transporter(s) but would not jeopardize the cooperative protection that is offered by the combination. This should be kept in mind when trying to modulate ABC transporter- mediated efflux at the BBB for pharmacologic purposes. Moreover, it is also important to realize that besides the drug efflux transporters ABCC4, ABCB1, and ABCG2 examined here, the relevance of other drug transporters (such as ABCC5 and ABBC10) still needs to be addressed.

Camptothecin analogues, in particular CPT11, are frequently applied in clinical trials in patients with glioma (34-37). Our work here calls into question whether CPT11 would be the most appropriate candidate. CPT11 is a prodrug that needs conversion into the active metabolite SN38, which is 100- to 1000-fold more potent. However, the brain accumulation of SN38 is the lowest of this panel of camptothecin analogues. The amount of SN-38 found in the brain was less than 0.002% of the total dose (after administration of CPT11) and this is only about 9% of SUM[CPT11+SN-38] in brain. Importantly, mice express the carboxyl-esterases that are responsible for the CPT11 to SN38 conversion more abundantly than humans (38, 39). As a result, humans have even lower plasma levels of SN38, which may further diminish its brain accumulation.

On the other hand, gimatecan has a much more favorable brain penetration. A key difference between gimatecan and topotecan and CPT11 is the substitution of a lipophilic chain in position 7 of the planar aromatic 5-ring structure, making this compound more lipophilic and therefore probably more cell membrane permeable (18, 19). Gimatecan has shown significant efficacy in a number of experimental tumor models, including orthotopic brain tumors (40). Moreover, it was shown that the *in vitro* cytotoxicity to gimatecan was not affected by the overexpression of ABCB1 or ABCG2, although transwell experiments showed that this compound is a substrate of Abcg2 (17, 18). The negligible transport by ABCB1 in the *in vitro* transwell assays is not in line with the significant effects of Abcb1a/b on the brain penetration. This may be due to species differences in substrate affinity, or the ABC transporter knockout model may be a more stringent test than the *in vitro* assays to establish whether a compound is an ABCB1/Abcb1a/b substrate. Taking Abcb1a/b;Abcg2;Abcc4$^{-/-}$ as reference (Fig. 3), Abcb1a/b alone appears to be capable of reducing the brain levels of gimatecan almost to those found inWT mice, whereas Abcg2 and Abcc4 together were less efficient. Importantly, however, together these 3 ABC transporters cooperatively reduce the brain penetration of gimatecan by about 4-fold (Table 1).

Elacridar is an inhibitor of ABCB1 and ABCG2 and has been successfully used to increase the brain penetration of many substrate drugs, for example gefitinib and sunitinib (41, 42), and also to some extent topotecan (8). Unfortunately, co-administration of elacridar to WT mice did not improve the brain penetration of SN-38 or gimatecan. The lack of effect on SN-38 brain penetration can be explained by the fact that Abcc4 alone was already sufficient to achieve low brain levels similar to those in WT mice and that elacridar does not inhibit Abcc4. Because of the concerted action by these 3 ABC transporters, it will be a challenging task to increase the brain penetration of SN-38 by modulation of ABC transporter activity at the BBB.

On the other hand, the BBB penetration of gimatecan was much less affected by the ABC transporters, although also in this case, elacridar did not result in any improvement. A possible explanation could be that gimatecan is in fact a good substrate of Abcb1a/b and/

or Abcg2, but the high cell membrane permeability of the gimatecan masks this property thus making the partial inhibition of Abcb1a/b and Abcg2 by elacridar unnoticeable.
In conclusion, using a collection of compound ABC transporter knockout mice, we have shown that the cooperative action by Abcc4 with Abcb1a/b and Abcg2 at the BBB restricts the brain penetration of triple-substrate drugs. This work underscores the importance of preclinical models, as such information cannot be obtained from clinical studies in patients. Importantly, patients may benefit from these animal models as they may assist in selecting the most appropriate BBB-penetrable candidates of drugs that have to act inside the brain for clinical trial.

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8

Effect of the drug transporters ABCG2, Abcg2, ABCB1 and ABCC2 on the disposition, brain accumulation and myelotoxicity of the aurora kinase B inhibitor barasertib and its more active form barasertib-hydroxy-QPA

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Invest New Drugs 2013; 31(5): 1125-35.

ABSTRACT

We explored whether barasertib (AZD1152), a selective Aurora B kinase inhibitor, is a substrate for P-glycoprotein (Pgp, MDR1), breast cancer resistance protein (BCRP), and multidrug resistance protein 2 (MRP2) *in vitro*. Cell survival, drug transport, and competition experiments with barasertib pro-drug and the more active form of the drug (barasertib-hQPA) were performed using MDCKII (wild type, MDR1, BCRP, and MRP2) and LLCPK (wild type and MDR1) cells and monolayers, and Sf9-BCRP membrane vesicles. Moreover we tested whether P-gp and BCRP affect the oral pharmacokinetics, tissue distribution, and myelotoxicity of barasertib *in vivo* using Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ (triple knockout) and wild type mice.

Results: In cell survival experiments expression of BCRP and MDR1 resulted in significant resistance to barasertib. In transwell experiments, barasertib-hQPA was transported by BCRP and MDR1 efficiently. In Sf9-BCRP membrane vesicles, both barasertib and barasertib-hQPA significantly inhibited the BCRP-mediated transport of methotrexate. In contrast, no active transport of barasertib by MRP2 was observed, and overexpression of MRP2 did not affect cytotoxicity of barasertib.

In vivo, systemic exposure as well as bioavailability, brain penetration, kidney and liver distribution and myelotoxicity of barasertib-hQPA were statistically significantly increased in Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ compared with wild type mice ($p<0.001$).

Conclusion: Barasertib is transported efficiently by P-gp and BCRP/Bcrp1 *in vitro*. *In vivo*, genetic deletion of P-gp and BCRP in mice significantly affected pharmacokinetics, tissue distribution and myelotoxicity of barasertib-hQPA. Possible clinical consequences for the observed affinity of barasertib for P-gp and BCRP need to be explored.

INTRODUCTION

Barasertib (AZD1152) is an acetanilide-substituted pyrazole-aminoquinazoline prodrug that is rapidly converted to the more active drug barasertib hydroxy-QPA (barasertib-hQPA) in human plasma. Barasertib-hQPA is a selective Aurora B kinase inhibitor (IC50 of 0.37 nM), with minor activity against Aurora A (IC50 1368 nM) and more than 50 other serine-threonine and tyrosine kinases, including FLT3, JAK2 and Abl (1). The Aurora family of serine/threonine kinases (Aurora A, B and C) plays an important role in chromosome alignment, segregation, and cytokinesis during mitosis (2-5). Recently, preclinical studies have suggested that Aurora kinases A and B may play a critical role in both tumorigenesis and tumor growth. Aberrant expression of Aurora kinases has been reported in several solid tumors, including colon (6,7), prostate (8,9), pancreas (10), breast (11,12), lung (13), and thyroid cancers (14), as well as in hematologic malignant cells from acute and chronic myeloid leukemia (15,16), multiple myeloma (17) and Non-Hodgkin lymphoma (18). Increased levels of Aurora kinases correlated with advanced clinical stage in patients with prostate (9) and head and neck cancers (19). Aurora B overexpression has been recently found as a molecular predictor for tumor invasiveness and poor prognosis in hepatocellular carcinoma (20). Following such findings several Aurora kinase inhibitors have been developed and are currently being tested in the clinic. Clinical studies with barasertib, a selective Aurora B kinase inhibitor, are ongoing in patients with haematological malignancies (21,22).

P-glycoprotein (P-gp, MDR1, ABCB1), Breast Cancer Resistance Protein (BCRP, ABCG2) and Multidrug Resistance Protein 2 (MRP2, ABCC2) are drug efflux transporters belonging to the ATP binding cassette (ABC) family. They are located in apical membranes of epithelial cells (i.e., intestine, blood brain barrier, liver, kidney, placenta syncythiotrophoblast) where they can actively extrude a variety of structurally diverse endogenous and exogenous compounds. Due to their strategic location, they exert a physiological protective role for the body by reducing/preventing intestinal absorption, brain and foetal penetration against toxic compounds, and by facilitating/mediating excretion of substrate compounds via the liver, kidney and intestine. As a consequence, they can substantially affect the pharmacokinetics, oral availability, tissue distribution and toxicity of substrates drugs (23). Overexpression of ABC drug efflux transporters in tumor cells has also been associated with resistance to cancer chemotherapy (24). Inhibition of P-gp, BCRP and MRP2 might be a useful strategy to overcome drug resistance, to improve the oral bioavailability and penetration of anticancer agents to primary or metastatic brain tumors (25-27). Moreover, evaluation of affinity for BCRP, P-gp, MRP2 is of increasing clinical relevance because clinically relevant drug-drug interactions between drug substrates and/or inhibitors of these ABC drug efflux transporters have been increasingly described (23,28).

We have explored whether barasertib (pro-drug) and barasertib-hQPA (more active form) are a substrate for BCRP, Pgp, and MRP2 in several *in vitro* models. We performed cell survival experiments and transport studies using MDCKII cells stably overexpressing human BCRP or its murine homologue Bcrp1, MDR1 or MRP2. In addition, we tested affinity of barasertib for BCRP in vesicles in competition experiments with methotrexate. Finally, we explored the influence of Pgp and BCRP on the oral bioavailability, pharmacokinetics, tissue distribution and myelotoxicity of barasertib *in vivo* using wild type and Bcrp1$^{-/-}$/ Mdr1a/1b $^{-/-}$ (triple knockout) mice.

MATERIALS AND METHODS

Chemicals and reagents

Barasertib dihydrogen phosphate pyrazoloquinazoline prodrug trihydrate (AZD1152, pro-drug), barasertib-hydroxyquinazoline pyrazol anilide (barasertib-hQPA) and their ^{14}C-labeled forms were a generous gift from Astrazeneca Pharmaceuticals (Macclesfield, UK) (Fig.1). [^{3}H] inulin (0.78 Ci/mmol), inulin[^{14}C]carboxylic acid (54 mCi/mmol) and [^{14}C] topotecan (SK&F104864, 48 mCi/mmol) were purchased from Amersham Biosciences (Little Chalfont, UK). Topotecan (Hycamtin®) was obtained from GlaxoSmithKline (GSK) Pharmaceuticals (King of Prussia, PA). Pantoprazole (Pantozol® 40 mg, Altana Pharma, Zwanenburg, The Netherlands) and methotrexate (Emthexate®, MTX) were obtained from the pharmacy of the Slotervaart hospital, Amsterdam, the Netherlands. GF120918 (elacridar) was kindly provided by GSK (Research Triangle Park, NC) and LY335979 (zosuquidar), was a generous gift from Dr. P. Multani (Kanisa Pharmaceuticals, San Diego, CA).

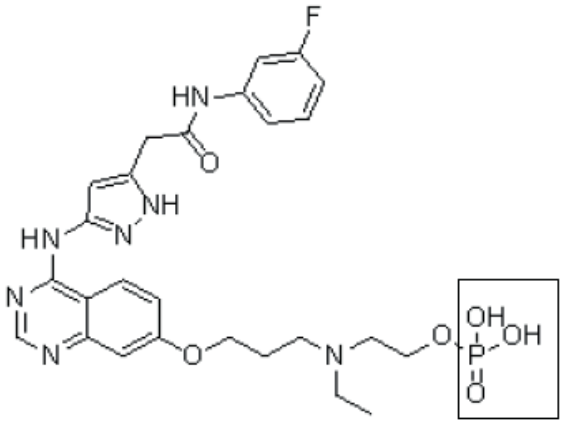


Fig. 1 Chemical structure of barasertib (pro-drug) and barasertib-hydroxy-QPA (more active form). In the box the chemical group responsible of the conversion of the pro-drug to the more active form of the drug.

Cell lines – culture conditions

Polarized MDCKII (Madin-Darby canine kidney) cells stably expressing human MRP2 (ABCC2), human MDR1 (ABCB1), human BCRP (ABCG2) or mouse Bcrp1 (Abcg2) cDNA were provided by Dr. A.H. Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands). Polarized pig kidney epithelial cell line LLC-PK wild type and MDR1 transfected subclones, were provided by Dr. P. Borst (the Netherlands Cancer Institute). All cell lines were cultured as described previously (29).

Cytotoxicity Assays - Clonogenic Survival Assay

Exponentially growing MDCKII cells were trypsinized and plated into Costar six well plates (3.8 cm Ø well, 100 cells/well) and allowed to attach for 20-24 h at 37°C under 5% CO_2. After this attachment period, barasertib pro-drug or barasertib-hQPA was added at different concentrations. Cells were allowed to form colonies for 8 days. Subsequently, they were fixed and stained by 0.4% crystal violet/2.5 glutardialdehyde. The number of colonies containing at least 50 cells was visually counted under a light microscope. Cell survival was expressed as a percentage of the control-cloning efficiency. In each experiment, two replicates at each concentration of barasertib pro-drug or barasertib-hQPA were evaluated; at least three independent experiments with each cell line were performed.

Elacridar (GF120918) was used as inhibitor of BCRP, however the drug is also known as a Pgp inhibitor (30). In the experiments elacridar was added 30 min prior to adding barasertib pro-drug or barasertib-hQPA to obtain a final concentration of 350 nM. The concentration of elacridar was lower than that in the transport experiments (5 μM), to circumvent toxicity, but sufficient to inhibit BCRP- and P-gp-mediated transport. Similarly, in some experiments zosuquidar (LY335979) was added at nontoxic concentrations (150 nM) in order to specifically inhibit P-gp mediated transport (31).

Transport across MDCKII monolayer

Transepithelial transport assays were performed in Costar Trans-well plates with 3-μm-pore membranes (Transwell 3414, Costar, Corning, NY) using MDCKII wild type, hBCRP,

Bcrp1, MRP2, MDR1, LLCPK wild type and MDR1 cell lines, as described previously (32). Trans-epithelial transport of [^{14}C]- barasertib pro-drug (2 μM) or [^{14}C]- barasertib-hQPA (1.6 μM) was evaluated. [^{14}C]-topotecan (5 μM) or [^{3}H]-digoxin (5 μM) were used as control substrates for BCRP and P-gp, respectively. Transport modulators were also added to inhibit endogenous Pgp levels (zosuquidar, 5 μM) and/or Pgp and BCRP (500 μM pantoprazole or 5 μM elacridar). Radiolabeled inulin was used to check the integrity of the monolayer. Inulin leakage was tolerated up to 3% of the total radioactivity over 4 hours. At least three independent experiments for each cell line and/or combination were done.

Preparation of membrane vesicles and competition experiments

Inside-out membrane vesicles from *Spodoptera frugiperda* (Sf9) cells were obtained after infection with a human BCRP cDNA containing baculovirus and were prepared as described previously (32). Using Sf9-BCRP and Sf9-Wild type membrane vesicles, we evaluated the effect of barasertib pro-drug and barasertib-hQPA on the transport of 0.31 μM methotrexate (MTX), a well known BCRP substrate, in the presence of 4 mM ATP. Sf9-BCRP and Wild type membrane vesicles were incubated with 1 μM [^{3}H]MTX for 5 minutes at 37°C in the presence or absence of different concentrations of barasertib (1, 10, 20 100 and 250 μM) or barasertib-hQPA (5, 10, 20 100 and 250 μM). The ATP-dependent transport was plotted as percentage of the control value. In each experiment pantoprazol (250 μM) was used as reference competitor for BCRP transport, in accordance with previously published experiments (32). All the experiments were done in presence and absence of ATP.

Animals

Animals used in this study were Bcrp1$^{-/-}$/Mdr1a/1b $^{-/-}$ (triple knockout), cross bread using Bcrp1$^{-/-}$ and Mdr1a/1b $^{-/-}$ mice, which were previously developed at our institute (33,34) and wild type mice of a comparable genetic background between 10 and 14 weeks of age. They were housed and handled according to institutional guidelines complying with Dutch legislation. Mice were kept in a temperature-controlled environment with a 12-h light/12-h dark cycle, and received a standard diet (AM-II; Hope Farms, Woerden, The Netherlands) and acidified water *ad libitum*.

Drug preparation and administration

Barasertib pro-drug and barasertib-hQPA were stored at –20°C until preparation of the intra-peritoneal (i.p.) or oral (p.o.) solution. For p.o. and i.p. administration of barasertib pro-drug at 100 mg/kg dose we dissolved 10 mg barasertib pro-drug in a mixture containing 0.9% NaCl and 35 mM $Na_3PO_4.12H_2O$ (pH 9). For i.p. administration barasertib-hQPA was dissolved in 10 mg/ml DMSO due to its very low water solubility. For p.o. administration of barasertib-hQPA at 10 mg/kg dose we prepared a vehicle containing 0.5% Tween-20 and 0.25% carboxymethylcellulose (CMC) in MQ by heating to 80-100°C until complete gelatination of the CMC was observed. Subsequently, a 1 mg/ml barasertib-hQPA was prepared by suspending 10% (v,v%) 10 mg/ml barasertib-hQPA in DMSO in the vehicle at room temperature. Each mouse received 250μl/25g barasertib-hQPA in vehicle.

In pharmacokinetic experiments, Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ and wild type mice were treated i.p. or p.o. at 100 mg/kg barasertib pro-drug dose. Whole blood samples (75 µl) were collected at 0.5, 1, 2, 4, 6, 8, and 24 hours after drug administration from the tail vein in heparinized capillaries. Mice treated i.p. were divided in three groups, which were sampled at t=0.5, 1 and 2 h (group 1), at t=2, 4 and 6 h (group 2), and at t=6, 8, 24 h (group 3) respectively. After the last blood sampling at time points 2, 6 and 24 hours, respectively, mice were anesthetized with methoxyflurane and sacrificed by cervical dislocation in order to collect and analyze brain, liver, and both kidneys. At least 9 mice for each group were treated.
In a second series of experiments, the pharmacokinetics of barasertib-hQPA have been evaluated after p.o. and i.p. administration of 10 mg/kg barasertib-hQPA in wild-type and Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ mice. Plasma concentrations of barasertib-hQPA were measured 0.5, 1, 2, and 4 hours after drug administration. At least 4 mice for each group were treated.
Processing of blood and tissue samples was performed as reported previously (35).

HPLC analysis
High-performance liquid chromatography (HPLC) was performed according to a validated method as described previously (35).

Myelotoxicity experiments
Myelotoxicity of i.p. barasertib has been evaluated at three different dose levels (25 mg/kg, 50 mg/kg and 75 mg/kg) in Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ and wild type mice. White Blood Cells (WBC) and platelet counts, as well as hemoglobin (Hb) determinations were performed 3 days before and 4, 7, 11, 15 and 21 days after drug administration. Hb level, WBC and platelet counts were determined in heparinized blood using a Beckman coulter AcT differ (Beckman Coulter, Woerden, the Netherlands). Mice weight was also monitored during the experiment. At least 9 mice for each group were evaluated.

Statistics and pharmacokinetic analysis
Statistical analysis was performed using Student's *t*-test (2-tailed, unpaired). Differences between 2 sets of data were considered statistically significant at $p<0.05$.
WinNonlin Professional (version 5.0, Pharsight, Mountain View, CA, USA) was used for all pharmacokinetic analyses. A non-compartmental analysis was performed with bolus injection for i.p. (intraperitoneal) or extravascular dose for oral administration of barasertib.

RESULTS

Reduced cytotoxicity of barasertib pro-drug and barasertib-hQPA by BCRP/Bcrp1 or MDR1 expression
In cytotoxicity assays a significant difference in IC50s for both barasertib pro-drug and barasertib-hQPA was observed between MDCKII-wild type and mouse MDCKII-Bcrp1 cells, with a RI (resistance index) of 52 ($p < 0.001$) and 97 ($p < 0.001$), respectively (table 1). To further demonstrate the role of BCRP in this resistance, the cytotoxicity assays were repeated in the presence of elacridar, an inhibitor of BCRP as well as of P-gp. The cytotoxicity of barasertib pro-drug and barasertib-hQPA in the MDCKII wild type cells was not significantly affected by co-incubation with a nontoxic dose of elacridar (350

nM) (p>0.05). In contrast, co-incubation with elacridar resulted in a partial reversal of resistance for barasertib pro-drug and barasertib-hQPA in the MDCKII-Bcrp1 cell line (IC_{50} ratio without/with elacridar: 33 with barasertib pro-drug and 66 with barasertib-hQPA, respectively; data not shown).
A significant difference in IC50s for both barasertib pro-drug and barasertib-hQPA was found between MDCKII wild type and MDCKII-MDR1 cells, with a resistance index (RI) of 7.8 and 12.5, respectively (p < 0.001) (table 1). Of note, co-incubation of the cells with a nontoxic dose of zosuquidar (150 nM), a selective MDR1 inhibitor, resulted in a reversal of resistance for both drugs in MDCKII-MDR1 cells, whereas it did not affect the cytotoxicity of the drugs in parental cells (data not shown).

Table1. Cytotoxicity of barasertib (pro-drug) and barasertib-hQPA in MDCKII cell lines

	Barasertib			**Barasertib-hQPA**		
	IC_{50} (nM)[a]	RI[b]	p[c]	**IC_{50} (nM)**[a]	RI[b]	p[c]
MDCKII-Wild type	91 ± 7			65 ± 5		
MDCKII-Bcrp1	4742 ± 536	52	<0.001	6290 ± 691	96.7	<0.001
MDCKII-MDR1	711 ± 33	7.8	<0.001	815 ± 60	12.5	<0.001
MDCKII-MRP2	128 ± 50	1.4	>0.05	79 ± 13	1.2	>0.05

[a] Assessed by Colony Forming Assay after 8 days of drug exposure. Values are the mean (± SD) of at least three experiments.
[b] RI, resistance index: ratio between the IC_{50} values of the resistant and parental cell lines.
[c] p-value, level of statistical significance.

Transport of barasertib-hQPA by BCRP and P-gp
Transport of barasertib-hQPA by human BCRP (hBCRP) and by the murine homologue Bcrp1 was studied using epithelial monolayers of MDCKII-Bcrp1 and MDCKII-hBCRP, as well as wild type cells as controls. Bcrp1 and human BCRP transported barasertib-hQPA efficiently, as can be seen by the increased transport after 4 h from the basolateral to the apical side and decreased transport from the apical to the basolateral side, which was more than two fold increased in MDCKII-Bcrp1 (ratio of basolateral to the apical side to apical to the basolateral side [BA/AB]: 2.8) and in MDCKII-hBCRP (ratio BA/AB: 2.49) compared with the wild type monolayer (ratio BA/AB: 1.08) (Fig. 2). Furthermore, we showed that the observed active transport of barasertib-hQPA was completely inhibited in MDCKII-Bcrp1 and hBCRP monolayers in the presence of the BCRP/P-gp inhibitor pantoprazole (500 µmol/L) or elacridar (10 µmol/L; Fig. 2).
Active transport of barasertib-hQPA was found in LLCPK-MDR1 cell monolayers: the BA/AB ratio for barasertib-hQPA was 1.8-fold increased in the LLCPK-MDR1 compared with the wild type cell line (data not shown). Incubation with zosuquidar was able to block the transport. LLCPK cells were used due to low endogenous P-gp expression in MDCKII wild type cells.
Topotecan and digoxin were used as reference drugs, as they are well-known substrates for BCRP and P-gp, respectively (36-38). Results obtained in control experiments were in line with previous publications (39, 40)(data not shown).

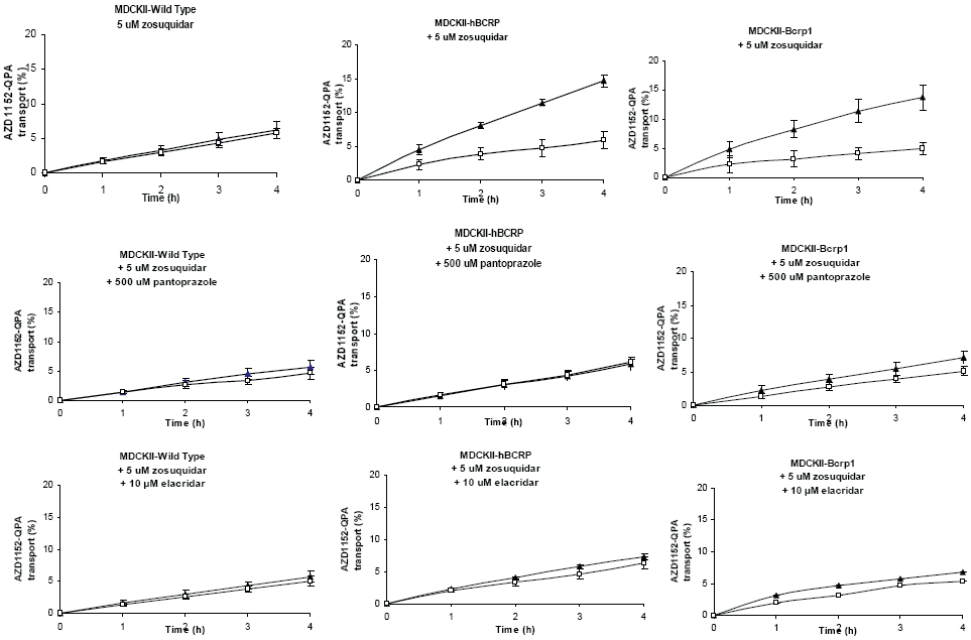


Fig. 2 Transport of [^{14}C] barasertib-hQPA (AZD1152-QPA, 1.6 µmol/L) across MDCKII-WT, -humanBCRP and -Bcrp1 cell monolayers in the absence or presence of pantoprazole (500 µmol/L) or elacridar (10 µmol/L). Zosuquidar (5 µM) was added in order to inhibit endogenous P-gp. Active transport of barasertib-hQPA (AZD1152-QPA) is evidenced by an overall increased appearance of the drug in the apical compartment, as a result of an increased transport from the basolateral to the apical compartment and, as a consequence, a reduced translocation of the drug from the apical to the basolateral compartment. ▲, translocation from basal to apical compartments; □ , translocation from apical to basolateral compartments. Points, mean of at least three experiments; bars, SD.

Cytotoxicity and Transport of barasertib-hQPA is not affected by MRP2

In cytotoxicity experiments no significant difference in IC50s for both barasertib pro-drug and barasertib-hQPA was found between MDCKII-wild type and MDCKII-MRP2 cell lines (p>0.05, Table 1). Accordingly, no active transport of barasertib-hQPA was found in transwell experiments performed with MDCKII-MRP2 cell monolayers (ratio BA/AB: 1.28) (data not shown).

Inhibition of BCRP-mediated MTX transport in Sf9 membrane vesicles

Using Sf9-BCRP membrane vesicles we studied the effect of barasertib pro-drug and barasertib-hQPA on the transport of 0.31 µM of methotrexate (MTX), a well known BCRP substrate (32). The ATP-dependent transport of MTX by human BCRP was inhibited by both barasertib pro-drug and barasertib-hQPA in a concentration-dependent manner, suggesting that the two compounds could compete with MTX for BCRP-mediated transport (Fig. 3). Results of control experiments employing pantoprazole (250 µM), a competitive inhibitor of BCRP, were in line with previous publications (32) (data not shown).

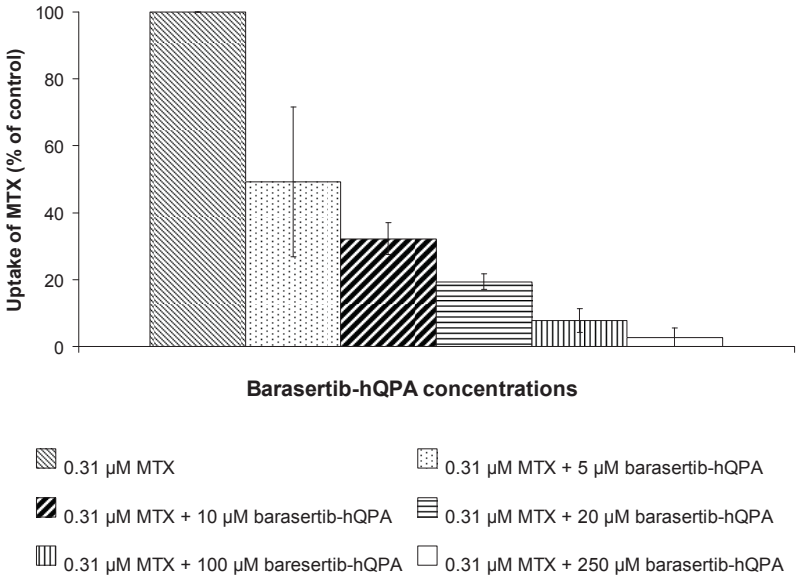


Fig. 3 Effect of barasertib-hQPA on ATP-dependent transport of MTX by BCRP. Sf9-BCRP membrane vesicles were incubated with [^{3}H]MTX (0.31 µM) for 5 min at 37°C in the absence or presence of increasing concentrations of barasertib-hQPA (5, 10, 20, 100, 250 µM). The ATP-dependent transport is plotted as percentage of the control value. Columns, means of each experiment in triplicate; bars, SD.

In vivo plasma pharmacokinetics of barasertib in Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ and wild type mice

After p.o. administration of 100 mg/kg barasertib pro-drug, the $AUC_{0\text{-inf}}$ of barasertib-hQPA was around 27-fold higher in Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ compared with wild type mice (7856 ± 437*versus* 295 ± 5 h*ng/ml) (p<0.001, Table 2). Of note in wild type mice plasma concentrations of barasertib-hQPA 2 hours after oral administration were already too low to be detected by the HPLC method employed. Analogously, mean Cmax after oral administration was significantly higher in Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ compared with wild type mice (2264 ± 220 versus 101 ± 8 ng/ml, p<0.001). In contrast, the $AUC_{0\text{-inf}}$ of barasertib-hQPA after i.p. administration of barasertib pro-drug at 100 mg/kg dose was not significantly different between Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ compared with wild type mice (122624 ± 5155 *versus* 133134 ± 4146 ng*h/ml, p>0.05, Table 2). The calculated apparent oral availability of barasertib-hQPA was around 6.4% for Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ mice and negligible (around 0.2%) in wild type mice.

In order to assess whether the observed effect of genetic BCRP/Bcrp1 and Pgp deletion could be ascribed to the pro-drug formulation, pharmacokinetics of barasertib-hQPA was evaluated also after p.o. and i.p. administration of barasertib-hQPA at 10 mg/kg dose in wild type and Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ mice. The pharmacokinetic analysis showed similar results to the ones observed after p.o. or i.v. administration of barasertib pro-drug (data not shown).

These results clearly suggest a significant effect of Bcrp1 and Mdr1a/1b on the pharmacokinetics of barasertib-hQPA.

Table 2. Pharmacokinetic parameters of barasertib-hQPA after p.o. and i.p. administration (100 mg/kg) in wild type and Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ mice

	p.o. administration			i.p. administration		
	Wild type	**Bcrp1$^{-/-}$/ Mdr1a/1b$^{-/-}$**	***P*[c]**	**Wild type**	**Bcrp1$^{-/-}$/ Mdr1a/1b$^{-/-}$**	***P*[c]**
AUC $_{0\text{-inf}}$ (ng*h/ml)[a]	295 ± 5	7856 ± 437	<0.001	133134 ± 4146	122624 ± 5155	>0.05
C_{max} (ng/ml)[b]	101 ± 8	2264 ± 220	<0.001	45626 ± 1834	39865 ± 2365	>0.05

[a]Area under the concentration-time curve from 0 up to infinity.
[b]Maximal plasma concentration.
[c]p-value, level of statistical significance.
Data are presented as mean ± SE.

CNS, liver and kidney accumulation of barasertib-hQPA in Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ and wild-type mice

We studied brain penetration as well as liver and kidney distribution of barasertib-hQPA in Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ and wild type mice 2, 6 and 24 hours after i.p. administration of barasertib pro-drug at 100 mg/kg. Tissue penetration of the drug was calculated by determining the barasertib-hQPA tissue concentration at t = 2, 6 and 24 hours relative to the plasma concentration at the same time points.

As shown in figure 4, brain, liver and kidney concentrations of barasertib-hQPA at t=2 and 6 hours (absolute and corrected for plasma values) were significantly higher in Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ compared with wild type mice (p<0.001).

At t=24h, absolute barasertib-hQPA brain, liver and kidney concentrations were significantly increased in Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ compared with control mice (p<0.05). However, when corrected for the plasma concentrations, the results were not significantly different between the two groups of mice (p>0.05), probably due to the somewhat higher variability at this time point.

These results indicate that genetic deletion of Bcrp1 and P-gp affects the distribution of barasertib-hQPA by significantly increasing the CNS penetration and the liver and kidney concentration of the drug.

Myelotoxicity studies

Substantial P-gp and BCRP levels have been recently found in hematopoietic stem cells, and in several more differentiated hematological subclasses (41-43). It has therefore been hypothesized that expression of these transporters in the bone marrow cells could affect the myelotoxicity of substrate drugs. To test this hypothesis, we evaluated the myelotoxicity of i.p. barasertib at three different dose levels (25 mg/kg, 50 mg/kg and 75 mg/kg) in Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ and wild type mice. Mice weight was also monitored.

No significant differences in baseline blood counts and weight were observed between Bcrp1/Mdr1a/1b knockout and wild type mice (p>0.05).

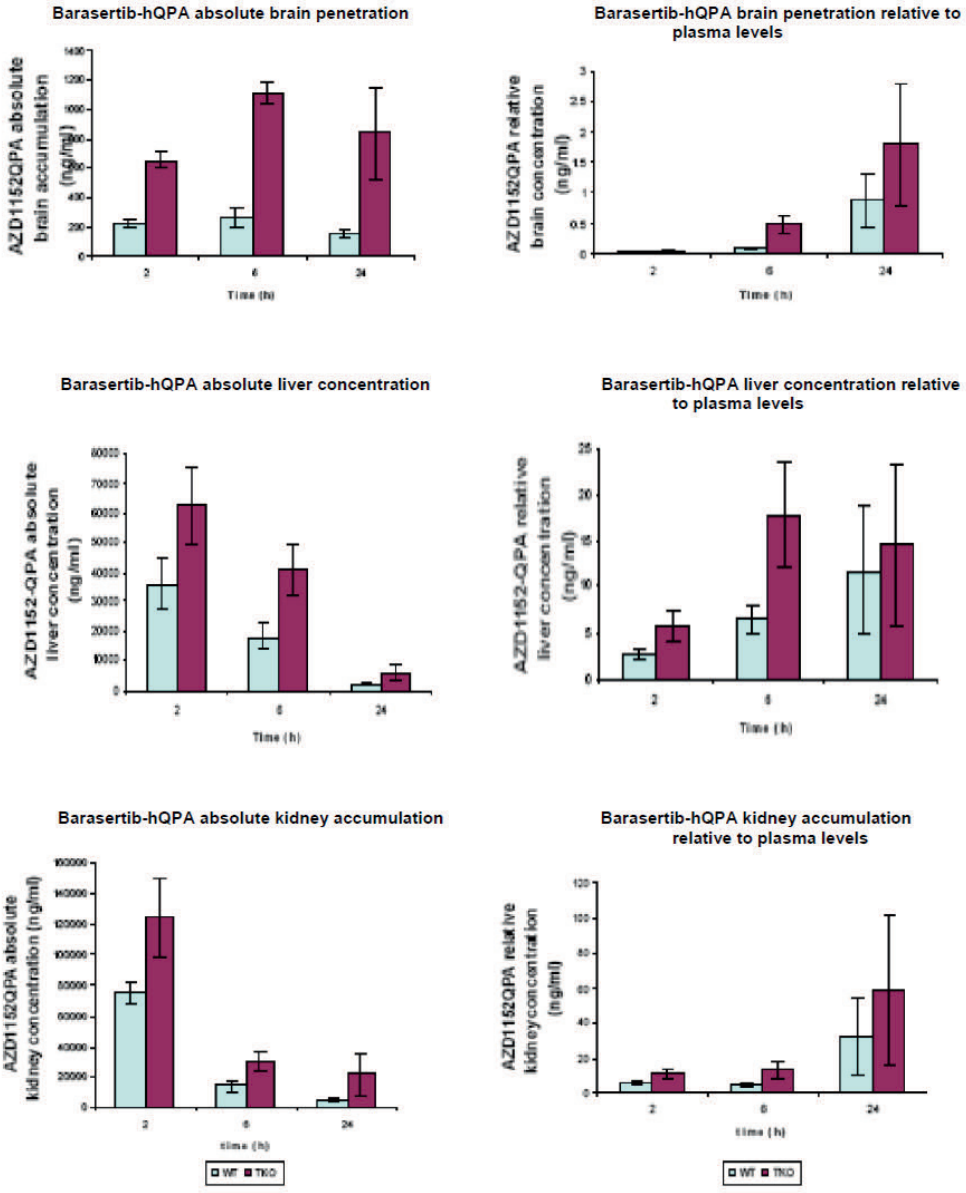
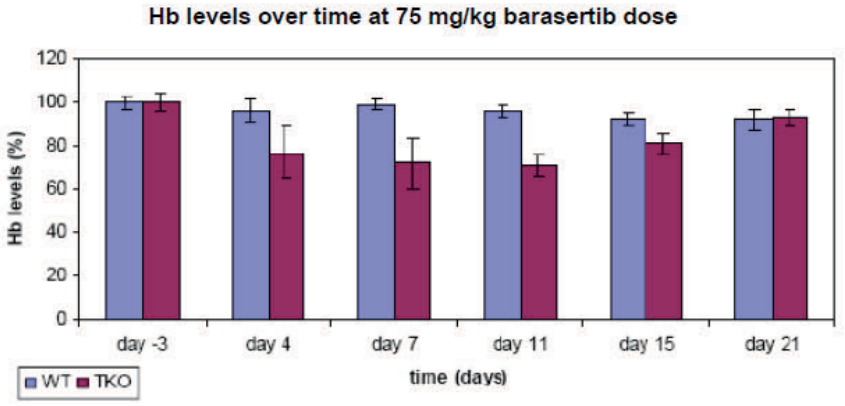
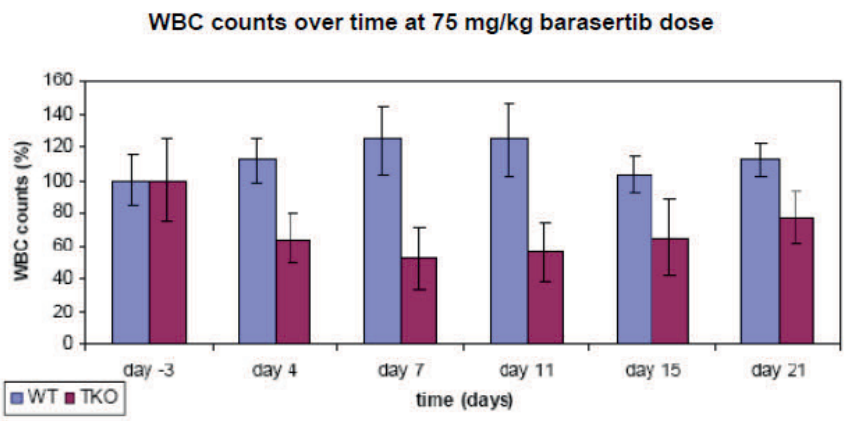


Fig. 4 Tissue distribution of barasertib-hQPA (AZD1152-QPA) in brain, liver and kidney 2, 6 and 24 hours after administration of i.p. barasertib pro-drug 100 mg/kg to wild type (WT) or triple knockout mice (TKO). Tissue concentrations have been reported as absolute values and after correction for the plasma concentrations at the same time point. At least four mice for each group were used. Columns, mean of barasertib-hQPA (AZD1152-QPA) tissue concentration; bars, standard deviation.





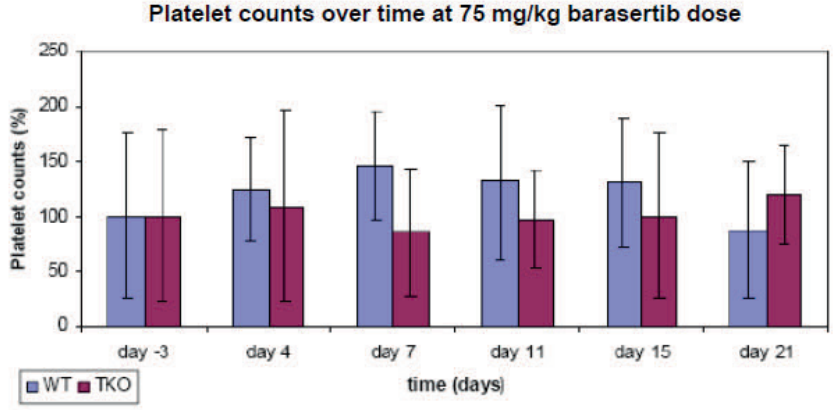


Fig. 5 Myelotoxicity of barasertib at 75 mg/kg i.p. dose in wild type (WT) and Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ (triple knockout, TKO) mice. Hb levels WBC and platelet counts are expressed as percentage of the value observed at baseline. Columns, mean of value; bars, standard deviation.

In wild type mice, no statistically significant reductions in Hb values, WBC and platelet counts were observed at all three doses tested and at all time points examined (p>0.05), with the exception of Hb values at the barasertib 75 mg/kg dose. Indeed, at such dose, a significant reduction in Hb level was observed 15 days after drug administration. However, the severity of this reduction was far less than in triple knockout mice (Fig.5). In faith, in Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ mice a significant reduction in Hb levels was observed over time at all doses employed (p<0.05). This reduction was significantly more pronounced at 75 mg/kg barasertib dose (p< 0.001), with nadir on day 11 and only partial recovery on day 21 (Fig. 5).
In Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ mice no significant difference in WBC counts was observed at 25 and 50 mg/kg doses over time (p>0.05, data not shown). However, at 75 mg/kg dose a statistically significant reduction in WBC counts compared with baseline was observed on day=4, 7, and 11 (p<0.001), with nadir on day 7 and partial recovery on day 21 (Fig.5).
No statistically significant differences in platelet counts have been reported at all dose levels and at all time points examined in wild type and triple knockout mice. However, interpretation of the results is jeopardized by the high intra- and inter- mouse data variability.
At all dose levels employed no statistically significant reductions in mouse weight have been observed during the entire experiment.
Overall these results suggest that myelotoxicity of barasertib is dose dependent and affected by Pgp and BCRP/Bcrp1 expression. However, due to the absence of Pgp and BCRP/Bcrp1 higher drug levels were achieved in plasma (refer to above mentioned results).

DISCUSSION

Our *in vitro* results indicate that barasertib-hQPA is a substrate of P-gp and BCRP but not of MRP2. Moreover, data obtained *in vivo* support affinity for P-gp/Bcrp1, as the combined genetic deletion of P-gp and Bcrp1 in the triple knockout (Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$) model resulted in a significantly increased systemic exposure and bioavailability of the drug. Brain penetration and myelotoxicity were also increased in triple knockout mice.
It should be noted that affinity of barasertib-hQPA for BCRP could have been predicted also by quantitative structure–activity relationship (QSAR) models. These approaches have become more widely applied to assess interactions between drug-like molecules and transporters, in particular P-gp and BCRP (44). However, when the drug (like barasertib) has already been chosen for further clinical development, confirmation of affinity of the drug for transporters in *in vitro* and *in vivo* experiments is usually still needed, also in order to evaluate whether clinical drug-drug interaction studies are required (45).
In our models, transport of barasertib prodrug and barasertib-hQPA by BCRP and P-gp was firstly suggested by the cell survival studies employing Bcrp1 and MDR1 overexpressing cells. Compared with their parental counterparts, Bcrp1 overexpressing cells showed 52-fold and 96-fold resistance to barasertib pro-drug and barasertib-hQPA, respectively. Cytotoxicity of barasertib pro-drug and barasertib-hQPA was also 7.8-fold and 12.5-fold higher, respectively, in MDCKII-MDR1 compared with wild type cells. Furthermore, co-incubation with non-toxic concentrations of the BCRP and Pgp inhibitor elacridar (in MDCKII-Bcrp1 cells) and of the selective P-gp inhibitor zosuquidar (in MDCKII-MDR1

cells) resulted in complete reversal of the resistance to both barasertib formulations. This suggests that BCRP and to a lesser extent P-gp are involved in resistance to barasertib in the cell systems applied.
Transwell experiments clearly showed a significant active transport of barasertib-hQPA by Bcrp1 and MDR1 in MDCKII and LLCPK cell monolayers. LLCPK cells were employed due to the low level of endogenous P-gp expression in MDCKII cells. The magnitude of the transport of barasertib-hQPA observed was of the same order as topotecan, a well-known BCRP substrate used as control. Active transport of barasertib-hQPA by P-gp was also observed, although at a lower extent than the control P-gp substrate digoxin, thus supporting the results of our cytotoxicity experiments. Of note, co-incubation with the BCRP inhibitors elacridar or pantoprazole, or the selective P-gp inhibitor zosuquidar, reversed the transport completely, further supporting the active transport of barasertib-hQPA by BCRP/Bcrp1 and P-gp.
Finally, in competition experiments performed using Sf9-BCRP vesicles, bothbarasertib pro-drug and barasertib-hQPA inhibited the ATP-mediated transport of methotrexate by BCRP in a concentration-dependent manner.
Therefore, the applied *in vitro* assays suggested that BCRP, and to less extent P-gp, are involved in resistance to and transport of barasertib-hQPA. In contrast, the results suggested that barasertib-hQPA is not a substrate for MRP2, as in our *in vitro* models overexpression of MRP2 did not reduce cytotoxicity neither mediate transport of barasertib-hQPA at detectable levels.
In order to evaluate whether the BCRP and P-gp mediated transport of barasertib observed *in vitro* was also relevant *in vivo*, we explored the pharmacokinetics, tissue distribution, myelotoxicity and excretion of the drug after p.o. and i.p. administration in Bcrp1/Mdr1a/1b knockout and wild type mice.
Pharmacokinetic results obtained after oral administration of the barasertib pro-drug (100 mg/kg) revealed a statistically significant increase in plasma exposure (AUC and Cmax) of barasertib-hQPA in triple knockout compared with wild-type mice (Table 2). Plasma concentrations 2 hours after p.o. administration in wild type mice were already too low to be detected by our HPLC analysis. In contrast, measurable concentrations of barasertib-hQPA over time were observed in Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$mice at the same doses. Results were further confirmed by the pharmacokinetic analysis performed after p.o. and i.p. administration of the activated form of the drug (barasertib-hQPA, 10 mg/kg). Clearly, genetic deletion of Bcrp1 and P-gp was able to affect oral absorption of barasertib. These data support our *in vitro* results.
Of note, bioavailability of barasertib after p.o. administration, although significantly increased in triple knockout compared with wild type mice, remained low (around 6.5%). As the oral bioavailability of barasertib-hQPA was similar after administration of the pro-drug and of the more active form of the drug, it can be concluded that the poor bio-availability of barasertib-hQPA is mostly due to low intestinal absorption of the activated form of the drug rather than to incomplete conversion from the pro-drug in the gastrointestinal tract. This is not unexpected, as the chemical structure of both barasertib and barasertib-hQPA confer low permeability to the compounds. Clinically, barasertib is being developed intravenously.
The evaluation of the tissue distribution of barasertib suggests that BCRP and P-gp limit the brain penetration of the drug. Indeed, brain accumulation (both absolute and

corrected for plasma values) of barasertib-hQPA was significantly increased in triple knockout compared with wild type mice.
Finally, the evaluation of myelotoxicity of barasertib at three different doses (25, 50 and 75 mg/kg) suggested that, as expected, myelotoxicity of barasertib was dose-dependent and affected by BCRP/Pgp expression. Indeed, a statistically significant reduction in WBC counts and Hb levels was measured at the 75 mg/kg barasertib dose in triple knockout over time compared with baseline, whereas only a minor effect on Hb level was observed in wild type mice. The hypothesis of a possible influence of BCRP/P-gp expression on myelotoxicity of substrate drugs has been formulated in view of recent publications reporting substantial P-gp and BCRP levels in hematopoietic stem cells and in several more differentiated haematological subclasses (41-43). In haematological malignancies, where the target is malignant bone marrow stem cells, such expression could lead to resistance to drugs, such as barasertib, that are BRCP/Pgp substrates.
Interestingly, in our *in vivo* model no thrombocytopenia has been observed after treatment with barasertib. This is in line with preliminary results of phase I clinical studies, showing no dose-limiting thrombocytopenia in patients treated with Aurora kinase inhibitors. Down-regulation of Aurora kinases during maturation of megakariocytes, the thrombocyte-producing bone marrow cells has been recently reported in preclinical experiments (46) and could provide an intriguing explanation of such finding.

CONCLUSIONS

This is the first report regarding affinity of barasertib for ABC drug efflux transporters. Our experiments indicate affinity of barasertib for BCRP/Bcrp1 and to a lesser extent for P-gp. In contrast, barasertib does not seem to be transported by MRP2. In mice, a significantly increased systemic exposure and brain penetration of barasertib after p.o. and i.p. administration, respectively, in Bcrp1$^{-/-}$/Mdr1a/1b$^{-/-}$ compared with wild type mice has been observed. Myelotoxicity of the drug was also clearly affected by Bcrp1/Mdr1a/1b gene expression.
The results are of potential clinical relevance. Inhibition of BCRP and/or P-gp could be an important strategy in order to improve brain penetration of barasertib and to reduce cancer-resistance mediated by BCRP and/or P-gp. On the other hand, as BCRP mediated transport of the drug *in vitro* appears to be substantial (efflux ratio greater than 2 in transport experiments, with complete blockade of the transport by addition of a selective BCRP inhibitor), according to recent guidelines (45), careful evaluation of preclinical and clinical information is necessary in order to determine whether a clinical drug-drug interaction study is warranted. In the case of barasertib, considering the molecular characteristics and the pharmacokinetics of the drug (rapidly converted to barasertib-hQPA by cleavage of the phosphate group and subsequently extensively distributed to tissues and eliminated via the liver through the hepatic metabolic route) (47), as well as the fact that genetic deletion of BCRP and P-gp in mice resulted in significantly increased myelotoxicity in our *in vivo* experiments, the potential for clinically relevant drug-drug interactions between barasertib and other clinically used BCRP/Pgp substrates or inhibitors warrants further clinical investigations.

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9

"Effect of the drug transporters ABCB1, ABCC2, and ABCG2 on the disposition and brain accumulation of the taxane analog BMS-275183"

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ABSTRACT

BMS-275183 is a novel oral C-4 methyl carbonate analogue of paclitaxel containing modifications to the side chain. Recently, a drug-drug interaction between BMS-275183 and the benzimidazole proton pump inhibitors (PPIs) was suggested in clinical trials resulting in elevated drug exposure and toxicity. We explored whether the interaction takes place at the level of P-glycoprotein (Pgp, MDR1, ABCB1), Breast Cancer Resistance Protein (BCRP, ABCG2) and MRP2 (ABCC2) using *in vitro* and *in vivo* models. *In vitro* cell survival, drug accumulation, efflux and transport studies with BMS-275183 were performed employing MDCKII (wild-type, MDR1, BCRP, MRP2) and LLCPK (wild-type and MDR1) cells and monolayers. *In vivo* the pharmacokinetics and tissue distribution of BMS-275183 after p.o. and i.v. administration were explored in Mdr1a/1b$^{-/-}$ and wild-type mice, in presence or absence of the PPI pantoprazole.

Results: *In vitro*, BMS-275183 was found to be a good substrate drug for MDR1, a moderate substrate for MRP2 and not a substrate for BCRP. Active Pgp transport was completely inhibited by pantoprazole (1.25 mM; MDR1 and BCRP inhibitor), or zosuquidar (5 μM; MDR1 inhibitor). *In vivo*, oral bioavailability, plasma $AUC_{0\text{-}6h}$ and brain concentrations were significantly 1.5-, 4-, and 2-fold increased, respectively, in Mdr1a/1b$^{-/-}$ compared with wild-type mice ($p<0.001$). However, oral co-administration of pantoprazole (40 mg/kg) did not alter the pharmacokinetics of BMS-275183 in wild-type mice.

Conclusions: BMS-275183 is efficiently transported by Pgp and to a lesser extent by MRP2 *in vitro*. Genetic deletion of Pgp significantly altered the pharmacokinetics and brain distribution of p.o. and i.v. administered BMS-275183 in Mdr1a/1b-/- compared to wild-type mice. Oral co-administration of BMS-275183 with pantoprazole did not affect the pharmacokinetics of BMS-275183 in wild-type mice, suggesting no interaction with PPI at the dose employed. This indicates that in mice different mechanisms, other than modulation of Pgp, are involved in the reported interaction with PPIs. Clinical studies in humans are warranted to further explore this potentially clinically relevant interaction.

INTRODUCTION

BMS-275183 (3'-tert-Butyl-3'-N-tert-butyloxycarbonyl-4-deacetyl-3'-dephenyl-3'-N-debenzoyl-4-O-methoxy-paclitaxel) is a C-4 methyl carbonate analogue of paclitaxel that contains additional modifications on the side chain where two phenyl groups have been replaced by *tert*-butyl groups (Mastalerz H 2003). Like paclitaxel, it exerts its antitumor activity by stabilization of tubulin polymerization in cancer cells. Paclitaxel is a cytotoxic anticancer drug with demonstrated activity against several types of cancer, including non-small cell lung cancer, breast and ovarian cancer, and AIDS-related Kaposi's sarcoma. Paclitaxel is administered intravenously in clinical practice due to its poor oral bioavailability, mostly related to its affinity for P-glycoprotein (Pgp, ABCB1) expressed at the intestinal barrier, together with poor passive diffusion.(Sparreboom 1997) This is considered a relevant limitation as in preclinical models the antitumor activity of paclitaxel is schedule-dependent and improved by prolonged tumor exposure times. (Huizing MT 1997) Due to its relatively good oral bioavailability in animals and humans (~24%), an antitumor activity comparable with that of intravenous paclitaxel

in preclinical models, and less affinity for Pgp compared with paclitaxel in preliminary *in vitro* experiments, BMS-275183 has been developed in the clinic for oral administration (Rose WC 2001). Oral drug administration is usually preferred over the intravenous route as it allows chronic continuous dosing, is often less costly and is preferred by the majority of patients (Liu G 1997). Moreover, as formulation of BMS-275183 does not require the vehicle Cremophor EL, no risk of hypersensitivity reactions is expected. To date BMS-275183 has been evaluated in cancer patients in phase I studies where 3 different dosing schedules where evaluated (weekly, bi-weekly and daily, respectively) showing promising antitumor activity. (Broker LE 2007, Broker LE 2006, Heath EI 2011) However, relatively high interpatient variability in exposure to BMS-275183, i.e., from 53% to 94% in the different dose schedules analyzed, has been observed, which is approximately 2-fold higher when compared with historical i.v. paclitaxel data. (Schiller JH 1994) Moreover, a significant correlation between drug exposure and toxicity has been documented in the studies performed to date. This translates into unpredictable high exposure to the drug in several patients leading to life-threatening and even fatal events (Heath EI 2011). In particular in a clinical study where the daily dose schedule of BMS-275283 was explored, one patient experienced an unexpected and very high exposure to BMS-275183 with severe toxicity. An analysis of the case did not reveal any concomitant medication potentially interacting with CYP3A4 metabolizing enzymes that could explain such enhanced toxicity, as BMS-275183 is extensively metabolized by CYP3A4 enzymes (Ly T 2009, Zhang D 2008). As the patient was under treatment with a benzimidadole proton pump inhibitor, it has been hypothesized that the high plasma levels observed in the patient could be related to a drug-drug interaction between BMS-275183 and proton pump inhibitors.

Benzimidazoles (e.g., pantoprazole, omeprazole) are a class of drugs that inhibit the gastric hydrogen-potassium adenosine triphosphatase (H+, K+-ATPase) and are frequently used in the treatment of peptic ulcers, pyrosis, and gastroesophageal reflux disease. Several drug-drug interactions have been reported in the literature between benzimidazole proton pump inhibitors and several classes of drugs, resulting in increased toxicity of co-administered drugs or, conversely, leading to reduction of therapeutic efficacy. One of the mechanisms involved in some of the reported clinically relevant interactions is the inhibition of P-glycoprotein (Pgp, MDR1) or Breast Cancer Resistance Protein (BCRP, ABCG2) mediated by benzimidazoles (Pauli Magnus 2001, Breedveld 2004). Pgp and BCRP, together with various multidrug resistance proteins (MRP1-9, ABCC1-9) are members of the ATP-binding cassette (ABC) transporter superfamily, a class of transporters that function as ATP-dependent efflux pumps for small molecules through the plasma membrane. Due to their expression in organs like intestine, liver, kidney, blood-brain barrier and placenta they play an important physiological role in protection of the body against toxins and xenobiotics. Transporters have demonstrated to affect the absorption, distribution, efficacy and toxicity of several classes of drugs. As such, clinically relevant drug-drug interactions mediated by inhibition or induction of these transporters have been reported in the literature. (Marchetti S 2007)

We explored whether the potential interaction between BMS-275183 and benzimidazoles proton pump inhibitors could be mediated by the ABC-drug efflux transporters Pgp, BCRP and MRP2. First we tested whether BMS-275183 is a substrate of Pgp, BCRP or MRP *in vitro* in cytotoxicity and transwell experiments employing Madin-Darby canine kidney II (MDCKII) cells stably expressing human Pgp (MDR1), human MRP2 and human BCRP and

mouse Bcrp1, as well as polarized porcine kidney epithelial cell line (LLCPK) transfected with human MDR1. We employed elacridar and pantoprazole as MDR1 and BCRP inhibitors and paclitaxel as positive control. Furthermore, we explored the effect of Pgp on the p.o. and i.v. pharmacokinetics of BMS-275183 using Mdr1a/1b$^{-/-}$ mice in absence and presence of the proton pump inhibitor pantoprazole.

MATERIALS AND METHODS

Chemicals and reagents

BMS-275183 and [14C]-BMS-275183 (40 µCi/mg) were provided by Bristol Meyers Squibb (Wallingford, CT). For cytotoxicity experiments BMS-275183 was dissolved in DMSO (10 mg/ml). For transport experiments [^{14}C]-BMS-275183 was dissolved in ethanol (70 mg/ml); labeled and unlabeled drugs were stored at –20°C in the darkness until use. [3H]-inulin (0.78 Ci/mmol) and inulin[^{14}C]carboxylic acid (54 mCi/mmol) were purchased from Amersham Biosciences (Little Chalfont, UK). Paclitaxel was purchased from Sequoia Research Products Ltd (Pangbourne, UK) and MK-571 from Enzo Life Sciences (Farmingdale, NY, USA). Pantoprazole (Pantozol® 40 mg, Altana Pharma, Zwanenburg, The Netherlands) and ranitidine (Zantac® 25 mg/ml, GlaxoSmith Kline) were obtained from the pharmacy of the Netherlands Cancer Institute. GF120918 (elacridar) was kindly provided by GSK (Research Triangle Park, NC) and LY335979 (zosuquidar), was a generous gift from Dr. P. Multani (Kanisa Pharmaceuticals, San Diego, CA). All other chemicals and reagents were from Sigma (St Louis, MO) and of analytical grade or better.

Cell lines – culture conditions

Generation of polarized MDCKII (Madin-Darby canine kidney) cells stably expressing human MRP2 (ABCC2), or human MDR1 (ABCB1), or human BCRP (ABCG2) or mouse Bcrp1 (Abcg2) cDNA has been described previously (Horio M 1989, Evers R 1998, Pavek P 2005). They were a generous gift from Dr AH Schinkel (Netherlands Cancer Institute, Amsterdam, The Netherlands). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with Glutamax (Life Technologies, Breda, The Netherlands) supplemented with 100 IU/ml penicillin G, 100 µg/ml streptomycin sulphate and 10% fetal calf serum (MP Biochemicals, ICN Biomedicals Inc.).
The polarized pig kidney epithelial cell line LLC-PK WT and -MDR1 transfected subclones, which were kindly provided by Dr P Borst at the Netherlands Cancer Institute, were cultured in M199 medium supplied with L-glutamine (Life Technologies, Inc., Breda, The Netherlands) and supplemented with penicillin G (100 IU/ml), streptomycin (100 µg/ml) and 10% (v/v) fetal calf serum (MP Biochemicals, ICN Biomedicals Inc.).
All cell lines were cultured at 37°C with 5% CO_2 under humidifying conditions.

Cytotoxicity assays

Exponentially growing-cells were trypsinized and plated (1000 cells/200 µl per well for the MDCKII-Wild-type (WT), MDCKII-Bcrp1 and MDCKII-MRP2 cells; 1500 cells/200 µl per well for the MDCKII-MDR1 cells) in 96-well microplates (Costar Corporation, Cambridge, Mass., USA) and allowed to attach for 24 h at 37°C under 5% CO_2. Subsequently, 100 µl of drug solution (diluted with DMEM containing Glutamax) were serially diluted and added to the

wells on day 2, and cells were incubated for 72 h at 37°C under 5% CO_2. After a 72 hours exposure period the cytotoxicity was measured using the sulforhodamine B method (SRB) as described previously (Mistry P 1991). The selective Pgp inhibitor zosuquidar (LY335979) was added, in the combination experiments, 30 min prior to adding BMS-275183 or paclitaxel to obtain a final concentration of 500 nM. The concentration of zosuquidar was lower than that in the transport experiments (5 µM) to avoid toxicity but sufficient to inhibit Pgp-mediated transport. Each agent (and combination) was tested at least in quadruplicate in at least three independent experiments.

Accumulation and efflux of BMS-275183

Intracellular accumulation and efflux of BMS-275183 were measured in LLCPK-WT and -MDR1 cell lines. LLCPK cells were seeded at a density of 1.5 x10^6 in cell culturing plates (ø 4.8 cm, Costar Corning, NY) in 5 ml of complete medium and cultured to about 80-90 % confluency (approximately 2-3 x 10^6 cells per plate). Plates were incubated for 30 min at 37°C in a humidified CO_2 incubator with 5 ml of complete medium containing 0, 1, 10, 20 µM of [^{14}C]-BMS-275183. After incubation, cells were washed twice with ice-cold PBS, scraped immediately, collected in plastic tubes and centrifuged (2 min, 1300 rpm, 0°C). Subsequently, the cells were resuspended in 1 ml of acetic acid 0.1 %) to lyse the cells. Protein concentrations were determined using the Bio-Rad assay based on the Bradford method (21). The concentration of [^{14}C]-BMS-275183 in the samples was determined by scintillation counting.

For efflux studies, LLCPK-WT and -MDR1 cells were loaded with 1 µM and 10 µM of [^{14}C]-BMS-275183, respectively, for 30 min at 37°C to obtain approximately equal intracellular concentrations of the drug. After loading the cells, medium was removed and replaced by fresh medium. Directly after incubation and at several time points after ending the incubation, intracellular concentrations of BMS-275183 were determined. Accumulation and efflux of BMS-275183 were determined in at least three independent experiments.

Transport across MDCKII and LLCPK monolayers

Transepithelial transport assays were performed in Costar trans-well plates with 3-µm-pore membranes (Transwell 3414, Costar, Corning, NY) as described previously (Breedveld 2004). Briefly, cells (MDCKII-WT, -Bcrp1, -MRP2, -MDR1; LLCPK-WT and -MDR1) were seeded at a density of 1 x 10^6 in 2 ml of complete medium. Cells were allowed to culture for 72 hours to form tight monolayers, with daily medium replacement. Two hours before the start of the experiment, complete medium on both sides of the monolayer (in the apical and basolateral compartments) was replaced with 2 ml of (serum-free) Optimem medium (Life Technologies, Inc. Ltd., Paisley, Scotland) containing the appropriate concentration of transport modulator (from 500 µM up to 1250 mM of pantoprazole; from 1 up to 3 mM of ranitidine; 5 µM zosuquidar to inhibit P-gp; 50 µM of MK571 to inhibit MRP2). At t=0 the experiment was initiated by replacing the pre-incubation medium from both compartments (apical and basolateral) with 2 ml of the similar medium also containing the appropriate concentration of the radiolabeled drug ([^{14}C]-BMS-275183, 2 µM) and radiolabeled inulin ([3H]-inulin). Inulin was added to check the integrity of the monolayer. The plates were incubated at 37°C in 5% CO_2. Every hour up to 4 hours aliquots of 200 µl were taken from the basal and apical compartments and the radioactivity was measured by addition of 4 ml of scintillation fluid (Ultima-gold; Packard, Meriden, CT) and subsequent liquid scintillation counting (tri-Carb 2100 CA Liquid Scintillation Analyzer;

Canberra Packard, Groningen, The Netherlands). Trans-epithelial transport of the drug and paracellular inulin flux through the monolayer was expressed as percentage of total radioactivity added at the beginning of the experiment. Inuline leakage was allowed up to 3% of the total radioactivity over 4 hours. At least 3 independent experiments for each cell line and/or combination were performed.

Animals

Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Animals used in this study were female Mdr1a/1b$^{-/-}$ (Pgp knockout), which were previously developed at our institute (Schinkel 1997), and wild-type (WT) mice of a comparable genetic background (> 99% FVB) between 10 and 14 weeks of age. Mice were kept in a temperature-controlled environment with a 12-h light/12h dark cycle, and received a standard diet (AM-II; Hope Farms, Woerden, The Netherlands) and acidified water ad libitum. In pharmacokinetic and tissue distribution studies at least 3 mice for each time point sampling were treated. To minimize variation in absorption due to food interaction, mice were fasted for 4 hours before BMS-275183 was administered and kept in cages to prevent coprofagy.

Drug preparation and administration

BMS-275183 was stored at –20°C until use. For oral (p.o.) and intravenous (i.v.) administration BMS-275183 was formulated in 50/50 Tween-80/ethanol mixture (10 mg/ml) and diluted before administration with NaCl 0.9% to a concentration of 1 mg/ml. Pantoprazole (Pantozol®, 40 mg) was diluted with NaCl 0.9% to a final concentration of 8 mg/ml and administered orally, 30 min prior to BMS-275183 (10 mg/kg), at a dose of 40 mg/kg.

Wild type and Mdr1a/1b$^{-/-}$ mice received BMS-275183 either by i.v. (in the tail vein) or p.o. administration at a dose of 10 mg/kg with or without co-administration of single oral dose of pantoprazole (40 mg/kg) 30 minutes before i.v. or p.o. BMS-275183. After BMS-275183 (10 mg/kg) ± pantoprazole (40 mg/kg) administration, at least 3 mice for each time point (5 and 30 minutes and 1, 4 and 6 hours) were anesthetized with methoxyflurane, their blood was collected by cardiac puncture and organs were removed after sacrifice by cervical dislocation. The blood samples were immediately centrifuged and plasma was collected, whereas tissue samples were homogenized in 1% bovine serum albumin by a polytron PT1200 (kinematica AG, Littau, Switzerland). Plasma and tissue homogenates were stably stored at –20°C until HPLC analysis, according to a validated high performance liquid chromatography (HPLC) method with UV-detection at 230 nm. The lower limit of quantification (LLQ) of BMS-275183 was 25 ng/ml.

Pharmacokinetic and statistical analysis

In general, statistical analysis was performed using Student's t-test (2-tailed, unpaired). Differences between 2 sets of data were considered statistically significant at $p < 0.05$.

Pharmacokinetic variables after p.o. and i.v. administration of BMS-275183 were calculated using WinNonLin Professional (version 5.0, Pharsight). The area under the curve in plasma (AUC) was calculated by employing the linear trapezoidal rule up to the last sampling time point (i.e. 6 hours after BMS administration). For each sampling time point mean and standard error calculated from all mice sacrificed at the same time point were employed for final AUC calculation.

RESULTS

Reduced cytotoxicity of BMS-275183 by Pgp (MDR1) expression

In cytotoxicity experiments a significant difference in IC_{50} was observed between MDCKII-WT and MDCKII-MDR1 cells, with a RF (resistance factor) of 14 ($p<0.001$), indicating that MDR1 expression led to resistance to BMS-275183 (Table 1). However the RF of BMS-275183 (RF=14) was significantly lower than the RF of paclitaxel (RF= 39), employed as reference drug. This is in line with previous publications suggesting less affinity of BMS-275183 for MDR1 compared with paclitaxel (Rose 2011). To further demonstrate the role of MDR1 in resistance to BMS-275183 the cytotoxicity assays were repeated in the presence of zosuquidar (LY335979), a selective Pgp inhibitor (Tang R 2008). The cytotoxicity of BMS-275183 in the MDCKII-WT cells was not significantly ($p>0.05$) affected by co-incubation with a non-toxic dose of zosuquidar (500 nM), yielding an IC_{50} ratio without/with zosuquidar of 1. In contrast, co-incubation with zosuquidar resulted in a partial reversal of resistance to BMS-275183 in the MDCKII-MDR1 cells (IC_{50} ratio without/with zosuquidar: 8.7, table 2).

Table 1. Cytotoxicity of BMS-275183 in MDCKII cell lines

	Wildtype	MDCKII-MDR1		MDCKII-MRP2		MDCKII-Bcrp1	
	IC_{50}(nM)[a]	IC_{50} (nM)[a]	RF[b]	IC_{50} (nM)[a]	RF[b]	IC_{50} (nM)[a]	RF[b]
BMS-275,183	2.0 ± 1.5	28 ± 3.4	14[c]	1.8 ± 0.2	0.9	1.2 ± 0.4	0.6
Paclitaxel	15 ± 10	585 ± 88	39[c]	20 ± 11	1.3	19 ± 14	1.3

[a] Assessed by cytotoxicity SRB assay after 72 hours of drug exposure. Values are the mean (± SD) of at least three independent experiments.
[b] RF, resistance factor: ratio between the IC_{50} value of the resistant and parental cell lines
[c] $p<0.01$.

Table 2. Cytotoxicity of BMS-275183 in MDCKII -WT and -MDR1 cells ± zosuquidar

	MDCKII-WT +	zosuquidar	MDCKII-MDR1+	zosuquidar
	IC_{50} (nM)[a]	IC_{50}ratio[b]	IC_{50} (nM)[a]	IC_{50}ratio[b]
BMS-275,183	2 ± 0.9	1[c]	3.2 ± 2.3	8.75[d]
Paclitaxel	1.9 ± 0.7	7.9[d]	4 ± 1.5	146[d]

[a] IC_{50} (nM) assessed by SRB cytotoxicity assay after 72 h of drug exposure in presence of zosuquidar (500 nM). Values are the mean (± SD) of at least three experiments.
[b] IC_{50}ratio: ratio between the IC_{50} values in absence and presence of zosuquidar.
[c] No significant difference ± zosuquidar ($p>0.05$).
[d] Significant difference ± zosuquidar ($p<0.005$).

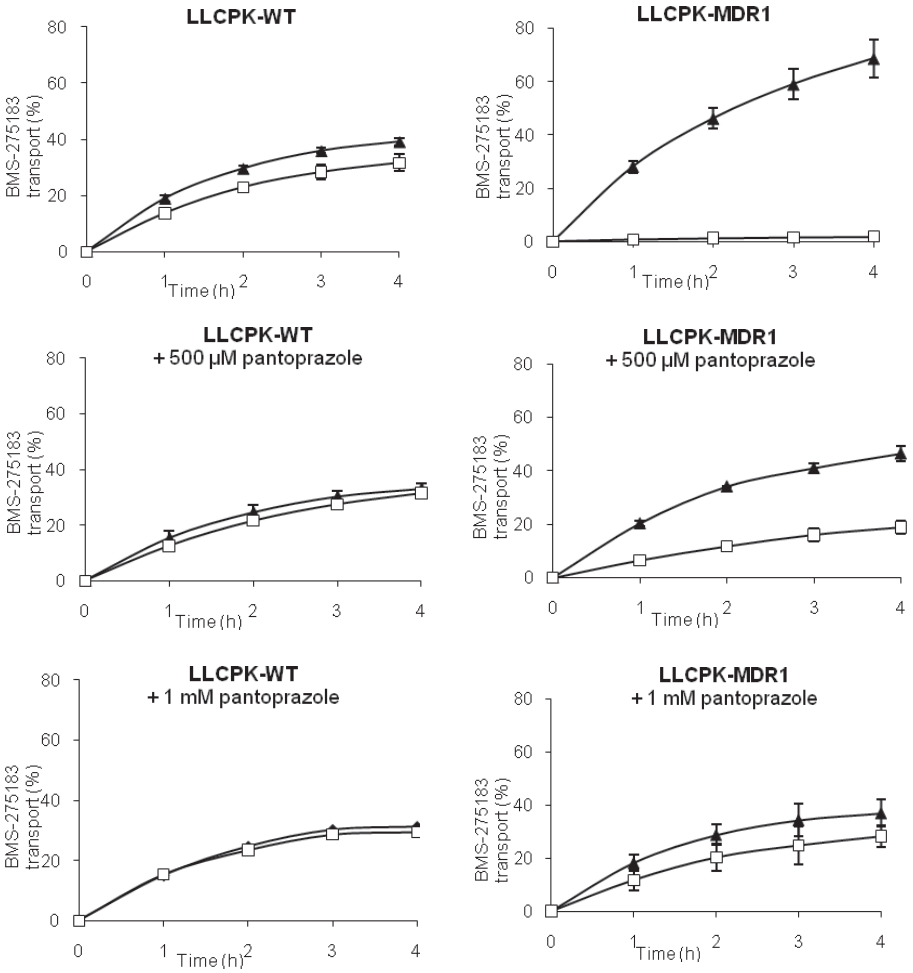
Cytotoxicity of BMS-275183 is not affected by BCRP and MRP2

No significant difference in IC_{50} of BMS-275183 was found between MDCKII-WT, -human BCRP, murine and -MRP2 cell lines ($p>0.05$, table1).

Transport of BMS-275183 across MDCKII and LLCPK monolayers

To further investigate the role of MDR1 in the resistance to BMS-275183, we have utilized MDCKII and LLCPK epithelial monolayer cells stably transfected with human MDR1, as well as WT MDCKII and LLCPK monolayers as controls. MDR1 transported BMS-275183

efficiently, as can be seen by the >30 –fold increased transport to the apical direction and decreased transport to the basolateral direction in both LLCPK-MDR1 (BA/AB: 36) and MDCKII-MDR1 (BA/AB: 32) cells, compared with the LLCPK (MDR1/WT: 30) and MDCKII-WT (MDR1/WT: 13) cell lines (figure 1). Of note, the relative transport (MDR1/WT) of BMS-275183 in MDCKII appears to be lower than in LLCPK monolayers, as some transport of the drug was observed also in MDCKII wild type cells (BA/AB: 2.4), probably due to some endogenous expression of Pgp. Moreover, transport of BMS-275183 was completely abolished in the MDR1 overexpressing monolayers (MDCKII and LLCPK) in the presence of the selective P-gp inhibitor zosuquidar (5 μM) and progressively blocked by increasing concentrations of the BCRP/MDR1 inhibitor pantoprazol (from 500 μM up to 1250 mM), achieving a complete inhibition of the transport at concentrations of 1250 mM (figure 1). In contrast, MDR1-mediated transport of [14C]-BMS-275183 was not affected by co-incubation with ranitidine (1 and 2 mM), a histamine H2-antagonist that effectively suppresses gastric acid secretion (data not shown).



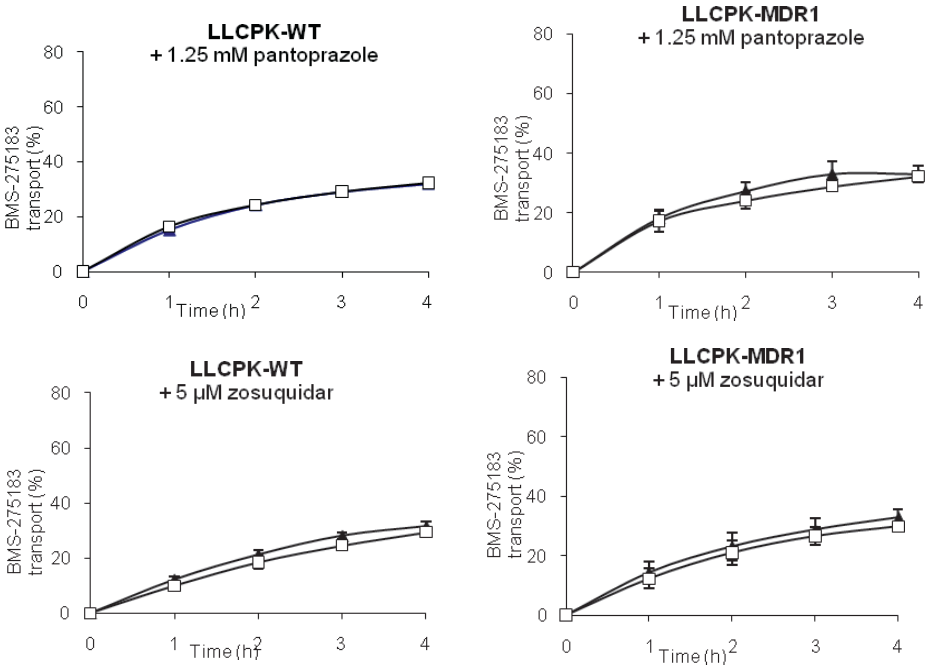


Fig. 1 Transepithelial transport of [^{14}C]-BMS-275183 (2 µM) across LLCPK-Wild-type and LLCPK-MDR1 cell monolayers in absence or presence of pantoprazole (500 µM, 1 mM, 1.25 mM) or the selective Pgp inhibitor zosuquidar (5 µM). Transport of BMS-275183 is evidenced by an increased appearance of the drug in the apical compartment, due to translocation from the basolateral to the apical compartment. (▲)Translocation from basal to apical compartments; (□) translocation from apical to basolateral compartments. Points, means of at least three experiments; bars, SD.

In MDCKII-MRP2 monolayers transport of BMS-275183 was found at relatively low rate: the transport to the apical direction was 1.6-fold increased compared with the transport to the basolateral direction (figure 2). Co-incubation with the selective MRP inhibitor MK571 (50 µM) completely inhibited the transport.
These results are in line with those reported in literature for the class analogue paclitaxel, that has been reported to be actively transported by MDR1 and MRP2 (Huisman MT 2005, Lagas JS 2006, Hendrikx JJ 2013).
In contrast, no transport was found for BMS-275183 in human and murine BCRP-expressing monolayers (data not shown).

Accumulation and efflux of BMS-275183 in MDR1-overexpressing cell lines
To further explore the effect of MDR1 overexpression on cellular transport of BMS-275183 we performed accumulation and efflux experiments in LLCPK-WT and –MDR1 cell lines. Accumulation of BMS-275183 was significantly reduced (2-fold) in the LLCPK-MDR1 compared with WT cell lines (data not shown). Furthermore in efflux studies a significantly increased initial efflux rate of BMS-275183 was observed in the LLCPK-MDR1 cells as compared with WT cells: ~59% of the intracellular BMS-275183 pool was transported out of the LLCPK-MDR1 cells within 1 minute vs ~24% in WT cells (figure 3).

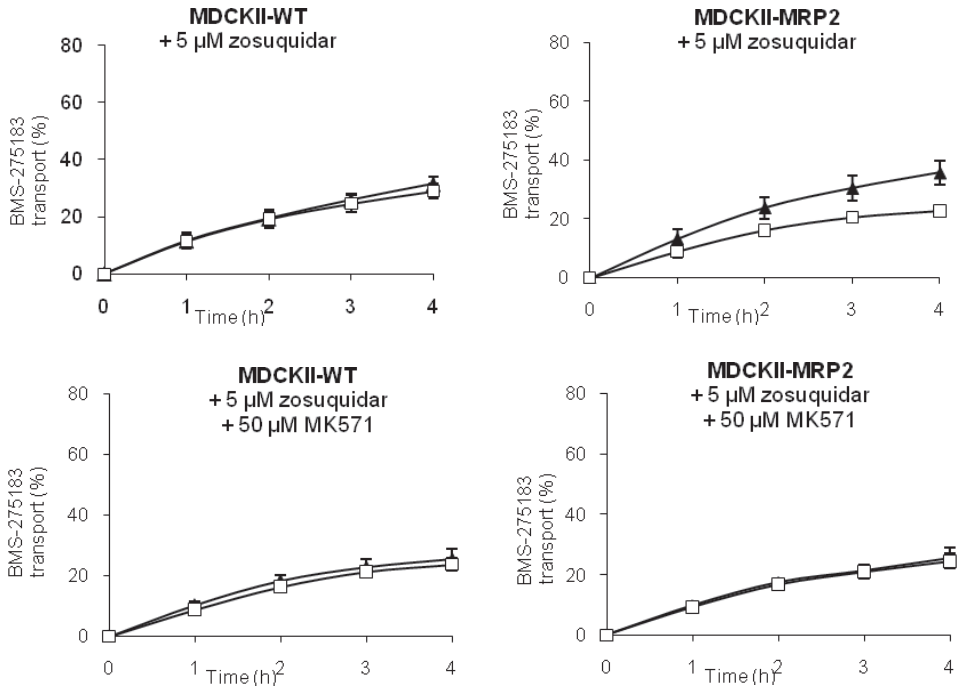


Fig. 2 Transepithelial transport of [^{14}C]-BMS-275183 (2 µM) across MDCKII-Wild-type and MDCKII-MRP2 cell monolayers in absence or presence of the selective MRP inhibitor MK571 (50 µM). Zosuquidar (5 µM) was added to inhibit endogenous Pgp. (▲)Translocation from basal to apical compartments; (□) translocation from apical to basolateral compartments. Points, means of at least three experiments; bars, SD.

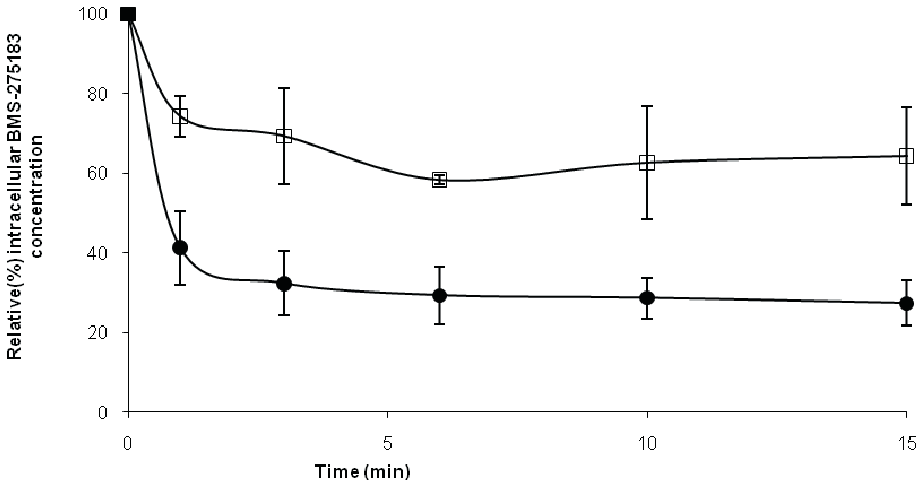


Fig. 3 Efflux of BMS-275183 from LLCPK-WT (□) and MDR1-overexpressing (●) cells. LLCPK-WT and MDR1 cells were loaded for 30 minutes with 1 and 10 µM [14C]-BMS-275183. Subsequently, efflux of the drug from the cells was measured. Points, mean of three independent experiments; bars, SD.

In vivo pharmacokinetics of BMS-275183 in Mdr1a/1b$^{-/-}$ and WT mice

To assess whether the observed MDR1 mediated transport of BMS-275183 *in vitro* is also relevant *in vivo*, we tested the oral up-take of BMS275183 in WT and Mdr1a/1b$^{-/-}$ mice. At a dose of 10 mg/kg, the $AUC_{0\text{-}6h}$ of BMS-275183 after p.o. administration was 4-fold higher in Mdr1a/1b$^{-/-}$ compared with WT mice (12020 ± 842 vs 3036 ± 336 ng*h/ml, respectively, $p<0.01$; table 3, figure 4). Maximum plasma concentration (C_{max}) measured was 2.6-fold higher in Mdr1a/1b$^{-/-}$ compared with WT mice (3058 ± 399 vs 1173 ± 54 ng/ml, respectively, $p<0.05$). The $AUC_{0\text{-}6h}$ and C_{max} of BMS-275183 after i.v. administration were also significantly (2.6- and 1.5-fold, respectively) higher in Mdr1a/1b$^{-/-}$ versus WT mice (12388 ± 368 vs 4842 ± 257 ng*h/ml, and 9589 ± 454 vs 6454 ± 327, respectively $p<0.05$; table 3, figure 4). The calculated apparent oral bioavailability was 97 ± 7.4% and 63 ± 7.7% for Mdr1a/1b$^{-/-}$ and WT mice respectively, i.e., significantly ($p<0.01$) increased in Mdr1a/1b$^{-/-}$ mice.

Table 3 Pharmacokinetic variables of BMS-275183 after p.o. and i.v. administration in WT and Mdr1a/1b$^{-/-}$ mice ± pantoprazole

		WT	WT + Pantoprazole	P^a	Mdr1a/1b$^{-/-}$	Mdr1a/1b$^{-/-}$+ Pantoprazole	P^a	P^b
p.o.$^{\$}$	**$AUC_{0\text{-}6h}$** (ng*h/ml)*	3036 ± 336	2080 ± 143	>0.05	12020 ± 842	13750 ± 695	>0.05	<0.05
	C_{max} (ng/ml)§	1173 ± 54	1137 ± 58	>0.05	3058 ± 399	3842 ± 115	>0.05	<0.05
i.v.$^{\&}$	**$AUC_{0\text{-}6h}$** (ng*h/ml)*	4842 ± 257	5066 ± 299	>0.05	12388 ± 368	15233 ± 506	>0.05	<0.05
	C_{max} (ng/ml)§	6454 ± 327	7553 ± 491	>0.05	9589 ± 454	9408 ± 291	>0.05	<0.05

Values are means ± SE of at least three mice.
$ Data obtained after oral administration (p.o.) of BMS-275183 (10 mg/kg)
& Data obtained after intravenous administration (i.v.) of BMS-275183 (10 mg/kg)
*AUC up to 6 hours after administration of BMS-275183
§ Maximum plasma concentrations after BMS-275183 administration
a) Level of statistical significance (p value) between WT and Mdr1a/1b$^{-/-}$ mice in absence or presence of co-treatment with pantoprazole (40 mg/kg)
b) Level of statistical significance (p value) between WT and Mdr1a/1b$^{-/-}$ mice

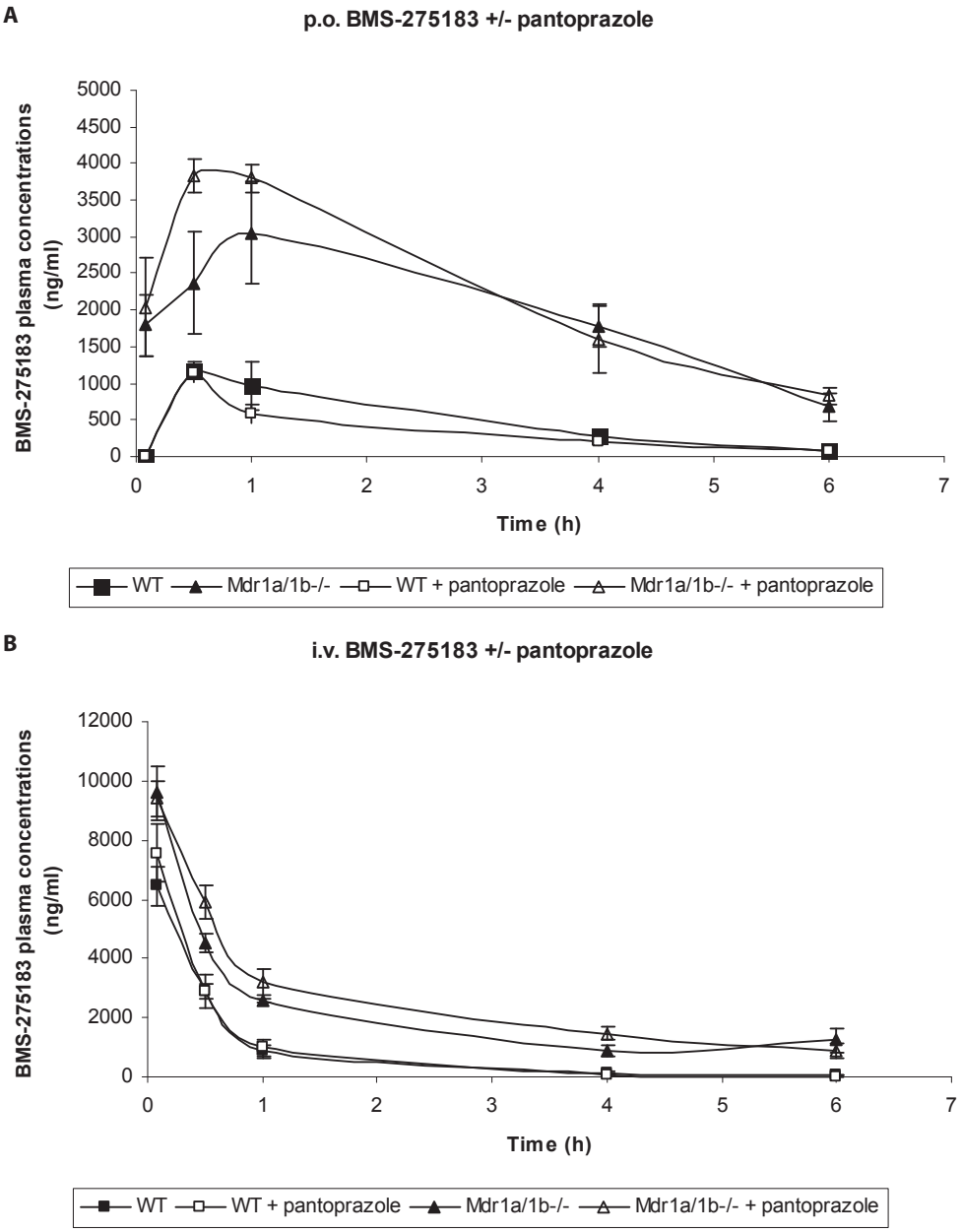


Fig. 4 Plasma concentration time curves in WT(■, □) and Mdr1a/1b-/- (▲,Δ) mice after oral (A) and i.v. (B) administration of BMS-275183 (10 mg/kg) with (□, Δ) or without (■, ▲) pre-treatment with pantoprazole (40 mg/kg). Points, mean concentrations for oral and i.v. administration ($n \geq 3$) ± SD.

CNS and other tissues accumulation of BMS-275183 in Mdr1a/1b$^{-/-}$ and WT mice

The CNS penetration of BMS-275183 was evaluated by determining the absolute BMS-275183 brain concentration (ng/g tissue) at t = 0.5, 1, 4 and 6 hours after p.o. and i.v. administration and by calculating the BMS-275183 brain concentration relative to the plasma concentration at the same time points. As shown in figure 5 after p.o. administration CNS concentrations of BMS-275183 in WT mice were below the lower limit of quantification of the assay, whereas they were 706 $\pm$ 145, 1852 $\pm$ 678, 1819 $\pm$ 523 and 1359 $\pm$ 160 ng/gr tissue 0.5, 1, 4 and 6 hours respectively after oral administration of BMS-275183 in the Mdr1a/1b$^{-/-}$ mice. After i.v. administration absolute brain concentrations of BMS-275183 in Mdr1a/1b$^{-/-}$ mice were significantly (8, 7-, 7- and 16-fold) increased 0.5, 1, 4 and 6 hours after administration, respectively, compared with control mice. When corrected for the plasma levels, CNS concentrations of BMS-275183 were 5- and 2-fold higher in the knockout mice at 0.5 and 1 hour after treatment, respectively, compared with WT mice (figure 5).

In contrast, no significant differences were found in BMS-275183 levels in kidney, lung, heart, and spleen between WT and Mdr1a/1b$^{-/-}$ mice after p.o. and i.v. administration, in particular when corrected for the plasma concentrations at the same time points.

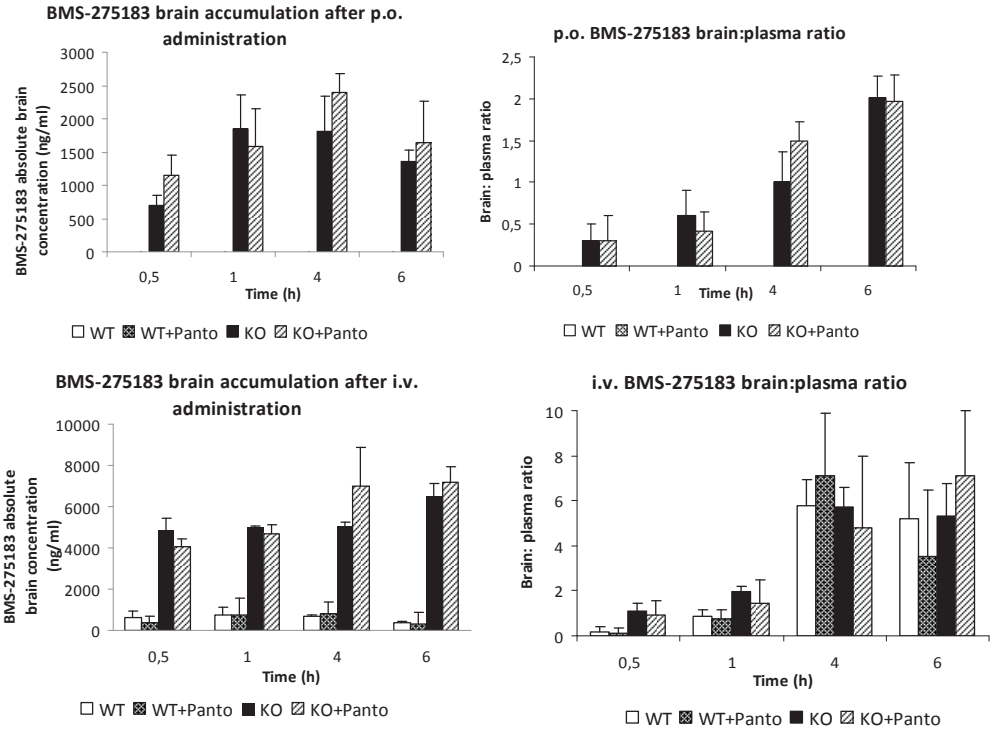


Fig. 5 Brain accumulation of BMS-275183 in wild-type and Mdr1a/1b$^{-/-}$ mice after p.o. (A) or i.v (B). administration of BMS-275183.Tissue concentrations have been reported as absolute values. At least 3 mice for each group were treated at each time point. Columns, mean of BMS-275183 brain tissue concentration; bars, SD.

Effect of pantoprazole on the pharmacokinetics and tissue distribution of BMS-275183 in WT and Mdr1a/1b$^{-/-}$ mice

We administered p.o. pantoprazole (40 mg/kg) 30 minutes before a p.o. or i.v. dose of BMS-275183 (10 mg/kg) to WT and Mdr1a/1b$^{-/-}$. As shown in table 3 co-administration of pantoprazole did not significantly affect the pharmacokinetics of BMS-275183. Indeed, after p.o. and i.v. administration of BMS-275183 to WT mice pre-treated with pantoprazole the $AUC_{0\text{-}6}$ and C_{max} were not significantly increased compared with control mice treated with BMS-275183 alone ($p>0.05$) (table 3, figure 4). Similarly, pantoprazole did not significantly affect CNS concentrations of oral BMS-275183 in WT and Mdr1a/1b$^{-/-}$ mice. Moreover, no significant difference was found in BMS-275183 levels in kidney, lung, heart, brain and spleen after p.o. and i.v. administration of BMS-275183 in WT mice when pantoprazole was co-administered (data not shown). These results suggest that oral pantoprazole at the applied dosage of 40 mg/kg in this animal model does not significantly affect p.o. and i.v. pharmacokinetics as well as tissue distribution of BMS-275183.

DISCUSSION

Our *in vitro* data indicate that BMS-275183 is a good substrate of Pgp, a moderate substrate for MRP2, but it is not transported by BCRP. The affinity of BMS-275183 for Pgp is further supported by our *in vivo* data as the deletion of the Mdr gene in the double-knockout (Mdr1a/1b$^{-/-}$) resulted in a significantly increased bioavailability, systemic exposure and brain accumulation after oral administration of BMS-275183. However, although pantoprazole was able to reverse the Pgp mediated transport of BMS-275183 in transwell experiments, co-administration of pantoprazole did not significantly alter the pharmacokinetics of BMS-275183 in wild type mice, suggesting that the drug-drug interaction observed in the clinic cannot be explained by pantoprazole-mediated modulation of Pgp activity.

The first indication of affinity of BMS-275183 for Pgp was obtained in *in vitro* cytotoxicity experiments employing MDCKII cells stably expressing human MDR1. Compared with their parental counterparts, MDR1 – expressing cells showed 14-fold resistance to BMS-275183. The observed resistance was clearly lower than the resistance factor for paclitaxel (RF: 39), in line with previous publications demonstrating that paclitaxel is a good Pgp substrate (Sparreboom 1997) and that treatment of a MDR1 expressing colon cancer cell line (HCT-116/MDR) with BMS-275183 resulted in less reduction of cytotoxic activity compared with paclitaxel. (Rose WC 2001) Moreover, co-incubation with a nontoxic concentration of the selective Pgp inhibitor zosuquidar resulted in complete reversal of resistance to BMS-275183 and to paclitaxel, suggesting that Pgp is involved in the observed resistance to the two taxane compounds. Results of transport studies with LLCPK and MDKCII cell monolayer revealed active transport of BMS-275183 mediated by MDR1. LLCPK cells were employed due to low endogenous Pgp expression in MDCKII cells. Transport of BMS-275183 from the basolateral to the apical side in MDR1-expressing monolayers was >30- fold higher than the translocation of the compound from the apical to the basolateral compartment. Further evidence of the active Pgp-mediated transport of BMS-275183 was obtained in transwell experiments performed by co-incubation with zosuquidar, which completely inhibited the transport of BMS-275183. Transport of BMS-275183 could also be inhibited

by co-incubation with pantoprazole, whereas no effect was observed of co-incubation with the H2 antagonist ranitidine. This finding gives pre-clinical support to our hypothesis of a potential drug-drug interaction mediated by Pgp between BMS-275183 and proton pump inhibitors. Of note, the inhibition of BMS-275183 transport exerted by pantoprazole appears to be concentration-dependent in our *in vitro* system, with complete blockade achieved at high pantoprazole concentrations (1.25 mM). Accumulation and efflux studies supported Pgp-mediated transport of BMS-275183 too: MDR1-expressing cells extruded BMS-275183 more efficiently than the parental cells.

Interestingly, according to available guidelines recently issued by regulatory authorities and according to the recommendations of the International Transporters Consortium, the magnitude of the transport observed in our model would require further clinical testing to assess the potential for Pgp mediated drug-drug interactions in case of further clinical development of BMS-275183. (CPMP/EWP/560/95/Rev.1; http://www.fda.gov; Giacomini 2010 and 2013).

In contrast, no increase in cytotoxicity neither transport of BMS-275183 was observed in our cell systems using BCRP-expressing cells (MDCKII-BCRP) suggesting that BMS-275183 is not a substrate for BCRP. Results obtained with MRP2-expressing cells appear to indicate a moderate affinity of BMS-275183 for MRP2. Indeed, no difference in cytotoxicity was observed between MDCKII-MRP2 and parental cells, whereas a moderate transport of BMS-275183 (AB/BA ratio: 1.6) could be documented in transwell experiments performed with the same cells in monolayers. The *in vivo* relevance of such transport is not clear at this time, as recently MRP2 has been reported to be able to significantly affect the pharmacokinetics of paclitaxel in mouse models with selective deletion of the Mrp2 gene, where MRP2 appears to dominate the hepatobiliary elimination of paclitaxel and Pgp the intestinal absorption and secretion of the drug, although *in vitro* experiments had suggested less affinity of paclitaxel for Pgp compared with MRP2. (Lagas JS 2006; Hendrikx JJ 2013) However, the magnitude of the observed MRP2 transport in our transwell experiments was significantly lower than what has been reported in previous studies with paclitaxel in the same *in vitro* models (AB/BA ratio: 6), suggesting limited relevance of MRP2 for BMS-275183 disposition *in vivo*.(Huisman MT 2005).

To explore whether the observed Pgp mediated transport of BMS-275183 observed *in vitro* could be relevant *in vivo*, we investigated the pharmacokinetics of the drug after p.o. and i.v. administration in wild-type and Mdr1a/1b$^{-/-}$ mice. In additional experiments wild-type and knockout mice were pre-treated with oral pantoprazole, in order to evaluate whether the pharmacokinetics of BMS-275183 would be altered, thus potentially explaining the drug-drug interaction observed in the clinic.

Results obtained after oral administration revealed a statistically significant increase (4-fold) in plasma exposure ($AUC_{0\text{-}6}$, C_{max}) to BMS-275183 in Mdr1a/1b$^{-/-}$ compared with wild-type mice ($p<0.001$). In addition the bioavailability of BMS-275183 increased from 63% to 97%. These results clearly indicate that Pgp limits the gastrointestinal absorption of BMS-275183. Pharmacokinetic data obtained after i.v. administration suggest an increase in systemic clearance of the drug, as plasma $AUC_{0\text{-}6}$ was significantly (2.5-fold) higher in Mdr1a/1b$^{-/-}$ compared with wild-type mice. Significant accumulation of BMS-275183 in the brain of Mdr1b/1b knockout mice (both absolute and corrected for plasma values) was observed too, whereas concentrations in wild type mice were too low to be detected by our HPLC analysis. Again, these findings strongly indicate that Pgp plays a key role

in the oral and systemic pharmacokinetics of BMS-275183. This is not unexpected as all other taxanes available to date (i.e., paclitaxel and docetaxel) have been reported to be good substrates of Pgp. In previous experiments treatment of Mdr1a/1b knockout mice with paclitaxel or docetaxel resulted in a significant increase in systemic exposure; oral bioavailability improved from 11% wild type to 35% in Mdr1a/1b$^{-/-}$ mice for paclitaxel and from 3.6% to 22.7% for docetaxel, respectively. (Sparreboom 1997, Waterschoot van RA 2009)

In contrast, in our *in vivo* wild-type mice no significant pharmacokinetic interaction between pantoprazole and BMS-275183 could be demonstrated. Pre-treatment of wild-type mice with 40 mg of pantoprazole did not significantly affect the pharmacokinetics of BMS-275183 after p.o. or i.v. administration. Plasma AUC, C_{max} as well as tissue concentrations were not significantly higher in mice pre-treated with pantoprazole compared with control mice. These findings strongly suggest that it is very unlikely that the clinically observed interaction between BMS-275183 and benzimidazole proton pump inhibitors is fully related to pantoprazole-mediated inhibition of Pgp. An apparent discrepancy is observed between our *in vitro* experiments performed with pantoprazole and the *in vivo* findings. However, in our transwell experiments Pgp-inhibition mediated by pantoprazole appears to be concentration-dependent, with complete inhibition of Pgp transport obtained at millimolar concentrations. It is very unlikely that such high concentrations are achieved with the dose we administered to mice in our experiments (40 mg/kg) and also at the doses that are usually prescribed to patients in the clinical setting.

There are possible explanations for the *in vitro-in vivo* and apparent mice-human discrepancy. Species differences could explain that our model is not representative for the human situation. In effect the oral bioavailability of BMS-275183 observed in our wild-type mice is higher than the bioavailability reported in humans (~24%). In recent studies paclitaxel appears to be a good substrate for human OATP1B3 transporter, which would be responsible for its uptake into hepatocytes. However there is no orthology between rodents and humans for OATP. (Smith NF 2005, Steeg van de 2011) Moreover, in recent experiments mouse liver microsomes appears to metabolize paclitaxel far less efficiently than human or CYP3A4-transgenic liver microsomes, suggesting species differences in metabolism of taxanes. (Hendrikx JJ 2013) A masking effect exerted by food can be excluded, as in our experiments mice were fasted for 4 hours before treatment with BMS-275183 or other compounds (e.g., pantoprazole, ritonavir, elacridar). Moreover, in clinical studies food intake did not significantly affect the exposure to BMS-275183.(Broker LE 2008) Another possible limitation of our experiments is that pharmacokinetics was studied only after a single dose of pantoprazole, whereas in clinical practice proton-pump inhibitors are given as a chronic therapy. Up- or down-regulation of other transporters and/or drug metabolizing enzymes involved in oral absorption and metabolism of BMS-275183 during chronic exposure to proton-pump inhibitors cannot be excluded. In effect BMS-275183 appears to be metabolized primarily through CYP3A4 and to lesser extent through CYP3A5 enzymes, which catalyze the hydrolysis of the side-chain and the oxidation of the t-butyl groups of the side chain. (Zhang D 2009) This is in contrast with paclitaxel, which is essentially metabolized through CYP2C8 enzymes and to a lesser extent by CYP3A4. Benzimidazole proton-pump inhibitors are primarily metabolized by CYP2C19 enzymes and to a lesser extent by CYP3A4. However, in patients who are poor metabolizer for CYP2C19 due to genetic polymorphism or drug-interactions metabolism

of benzimidazoles is essentially carried out by the CYP3A4 system. Therefore it cannot be excluded that the observed drug-drug interaction between BMS-275183 and proton-pump inhibitors took place at the CYP3A4 level.
Finally, the high BMS-275183 plasma concentrations and toxicity observed in the clinic could be related to other factors rather than the concomitant use of proton-pump inhibitors. In the clinical studies performed to date with BMS-275183 high interpatient variability has been observed which may seriously hamper the clinical development of the drug, in view of its narrow therapeutic window. As in our models Pgp appears to play a crucial role in the pharmacokinetics of BMS-275183, modulation of Pgp expression and/or activity due to genetic polymorphism, pathophysiological conditions, concomitant exposure to Pgp inhibitors/modulators as co-medications or within food or herbal supplements could result in enhanced oral bioavailability of BMS-275183, resulting in high plasma concentrations and associated toxicity. This could explain at least in part the high interpatient variability observed in patients. In effect, although contrasting results have been published in the literature, several studies have reported an association between several single nucleotide polymorphisms in the ABCB1 gene and toxicity of taxanes. (Jaber RS 2012, Levy P 2013) Furthermore, BMS-275183 is extensively metabolized by CYP3A4, as demonstrated by metabolism and excretion studies performed in rats and dogs, where mainly oxidative metabolites were observed and unchanged parent drug accounted for <3.5% of the administered dose in feces and urine. Fecal excretion was also the major route of BMS-275183 elimination in both species (85-86% and <9% of the dose was recovered in feces and urine, respectively). (Ly van T 2009) Therefore, genetic polymorphism or other factors altering the activity of the CYP3A4 system could also lead to altered pharmacokinetics of the drug and unwanted toxicity. Of course, a combination of both (i.e., modulation of Pgp and CYP3A4 activity) could impair more extensively BMS-275183 pharmacokinetics. Indeed, combined genetic deletion of CYP3A and Mdr1a/1b in mice resulted in dramatically increased oral bioavailability of docetaxel and toxicity. (Waterschoot van RA 2009)
In conclusion, our *in vitro* experiments show that BMS-275183 is a good substrate for Pgp, a moderate substrate for MRP2 and not transported by BCRP. *In vivo*, genetic deletion of Pgp in mice resulted in increased BMS-275183 oral bioavailability, systemic exposure and brain penetration indicating that Pgp plays a crucial role in the pharmacokinetics of the drug. However, co-administration of BMS-275183 with pantoprazole did not affect the pharmacokinetics of BMS-275183 after p.o. or i.v. administration in wild-type mice, suggesting that different mechanisms, other than modulation of Pgp, are involved in the reported interaction. Eventually, interaction studies in humans are considered necessary to further explore this potentially clinically relevant interaction.

Acknowledgments
We thank M.A.J. van Eijndhoven and M.J. Boljin (Department of Experimental Therapy, The Netherlands Cancer Institute) for the precious technical assistance.

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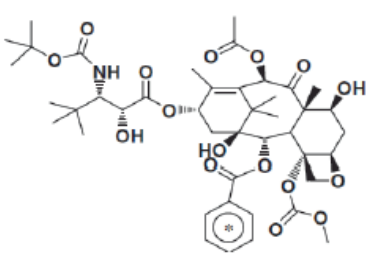

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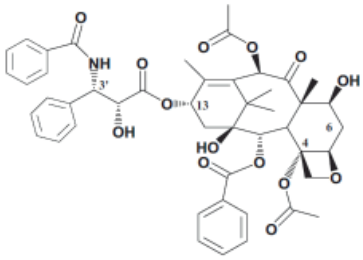
Molecular weight = 845.9



Paclitaxel
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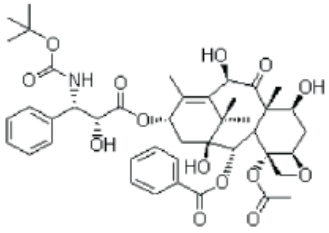
Molecular weight = 853.9



Docetaxel
Molecular formula = $C_{43}H_{53}NO_{14}$
Molecular weight = 807.8

Fig. 6 (Supplementary figure).
Chemical structure of BMS-275183, paclitaxel and docetaxel.

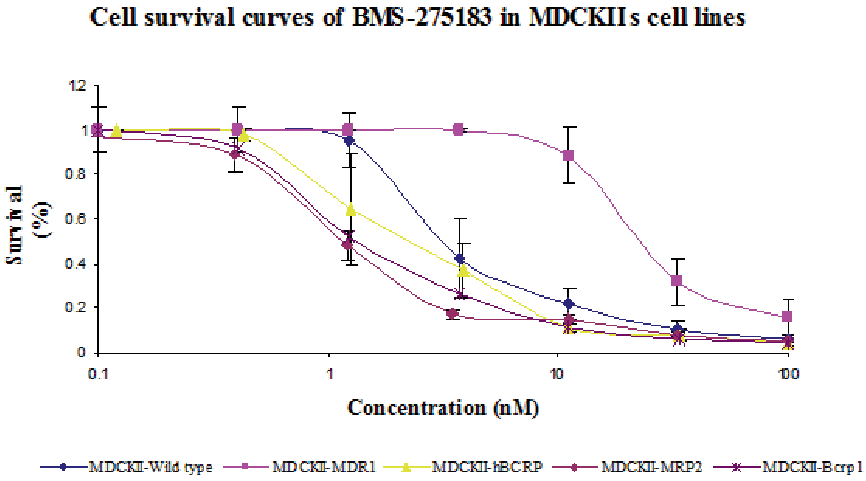


Fig. 7 (Supplementary figure)
Survival curves of BMS-275183 in MDCKII- Wild type, MDR1, MRP2, Human BCRP, mouse Bcrp1 cells after 72 hours of drug exposure.

10

The effect of hydroxyurea on P-glycoprotein/BCRP-mediated transport and CYP3A metabolism of imatinib mesylate

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Cancer Chemother Pharmacol (2007) 59:855–860

ABSTRACT

Purpose: It has been reported that the combination therapy of imatinib mesylate, a tyrosine kinase inhibitor, plus hydroxyurea, a ribonucleotide reductase inhibitor, is associated with remarkable antitumor activity in patients with recurrent glioblastoma multiforme. However, the mechanism of the added activity of hydroxyurea to imatinib is not known. The purpose of this study was to investigate *in vitro*, whether hydroxyurea could enhance the central nervous system penetration of imatinib, by inhibition of the ATP-dependent transporter proteins P-glycoprotein (ABCB1; MDR1; Pgp) and Breast Cancer Resistance Protein (ABCG2; BCRP), or by inhibition of cytochrome P450 3A (CYP3A) metabolism of imatinib.
Methods: The effect of hydroxyurea on the Pgp and BCRP mediated transport of imatinib was investigated by the sulforhodamine-B (SRB) drug cytotoxicity assay and transepithelial transport assay. *In vitro* biotransformation studies with supersomes expressing human CYP3A4 were performed to investigate whether hydroxyurea inhibited CYP3A4.
Results: In both *in vitro* cytotoxicity and transport assays, hydroxyurea did not affect Pgp and BCRP mediated transport of imatinib. In a biotransformation assay, hydroxyurea had no influence on the metabolic degradation of imatinib either.
Conclusion: The results indicate that hydroxyurea does not interact with imatinib by inhibition of Pgp and BCRP mediated transport or by CYP3A4 mediated metabolism of imatinib.

INTRODUCTION

Imatinib mesylate (STI-571, Gleevec®, imatinib), a potent and selective receptor tyrosine kinase inhibitor was shown to be clinically effective and well tolerated in Bcr/Abl-expressing chronic myeloid leukemia [6] and c-Kit-expressing gastro-intestinal stromal tumors (GIST) [4]. In addition, imatinib effectively inhibits platelet-derived growth factor (PDGF)-induced glioblastoma cell growth preclinically [11]. However, trials with imatinib in patients with recurrent glioblastoma multiforme showed limited penetration of imatinib into the central nervous system and modest antitumor activity [19, 24]. A plausible explanation for this low efficacy of imatinib is the efficient protection of the brain against drugs by the blood–brain barrier, containing various efflux transporters, including P-glycoprotein (ABCB1; MDR1; Pgp) and Breast Cancer Resistance Protein (ABCG2; BCRP). Pgp and BCRP are located in apical membranes of epithelia and vascular endothelial cells, which can actively extrude a variety of structurally diverse drugs and drug metabolites from the central nervous system and from tumor cells into the blood circulation [7, 22]. *In vitro* and *in vivo* studies have shown that Pgp and BCRP play an important role in the transport of imatinib and limit the distribution of imatinib to the brain [1, 3]. Furthermore, effective Pgp and/or BCRP inhibitors, such as elacridar (GF120918), zosuquidar (LY335979) and pantoprazole, significantly improved the brain accumulation of imatinib [1, 3]. This concept raises the possibility that co-administration of a transport inhibitor improves therapy with imatinib.
Two recent reports suggested that the combination of imatinib plus hydroxyurea, a ribonucleoside reductase inhibitor, is a safe and effective therapy for a subpopulation of

glioblastoma multiforme patients who have experienced disease progression after prior radiotherapy and at least temozolomide-based chemotherapy [5, 20]. This is the first report that a signal transduction inhibitor combined with a chemotherapeutic agent has activity in glioblastoma multiforme. However, the mechanism of action underlying the activity of this regimen is unknown. Based on the preclinical results of Dai et al. [3] and Breedveld et al. [1], we hypothesized that hydroxyurea interferes with the penetration of imatinib through the blood–brain barrier by inhibition of the efflux transporters Pgp and/or BCRP or by inhibition of cytochrome P450 3A (CYP3A) metabolism of imatinib.

MATERIALS AND METHODS

Chemicals

Imatinib, [^{14}C]imatinib (both as the mesylate salt) and its main metabolite N-desmethyl-STI (CGP74588) were kindly provided by Novartis Pharma AG (Basel, Switzerland). Pantoprazole (Pantozol®, Altana Pharma, Hoofddorp, The Netherlands) was obtained from the pharmacy of the Netherlands Cancer Institute. Zosuquidar trihydrochloride (LY335979) was kindly provided by Eli Lilly (IN, USA). Ritonavir was provided by Abbott (Chicago, IL, USA). Hydroxyurea was purchased from Sigma (St Louis, MO, USA).

Cell lines and culture conditions

The Madin–Darby Canine Kidney II (MDCKII) epithelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 units penicillin/streptomycin per ml [13]. Cells were grown at 37°C with 5% CO2 under humidifying conditions. Polarized MDCKII cells stably expressing human MDR1 (ABCB1) or murine Bcrp1 (ABCG2) cDNA have been described previously [8, 10].

Cytotoxicity assay

MDCKII-parental, -MDR1 and -Bcrp1 cells were cultured as described above and used in a sulforhodamine B (SRB) drug cytotoxicity assay for single and combination experiments as described by Ma et al. [15]. Briefly, 1,000 exponentially growing MDCKII cells/200 µl/well in 96-well plates were allowed to attach for 1 day followed by imatinib administration in the presence or absence of hydroxyurea for three more days.

Transport across MDCKII monolayers

MDCKII-parental, -MDR1 and -Bcrp1 cells were seeded on microporous polycarbonate membrane filters at a density of 1 x 10^6 cells/well in complete medium. Transepithelial transport assays were performed as described previously [2]. To exclude any contribution of Pgp in the MDCKII-Bcrp1 and MDCKII-parental cells, LY335979 was added. As the expression of BCRP in the MDCKII-parental and -MDR1 cells is negligible, co-administration of a BCRP inhibitor is redundant.

Biotransformation assay

The main metabolite of imatinib, CGP74588, was formed in in vitro incubations with supersomes that contained cDNA expressing human CYP3A4. Supersomal incubations (final volume = 50 µl) were performed at 37°C according to the BD Gentest procedure/

catalogue (BD Bioscience, Erembodegem, Belgium) and contained, per incubation: supersomes CYP3A4 (10 pmol), NADPH regenerating solutions A (2.5 µl) and B (0.5 µl) from BD Gentest/Bioscience, 0.1 M phosphate buffer, water and imatinib 20 µM in the presence or absence of hydroxyurea 300 µM or the known CYP3A4 inhibitor ritonavir 100 µM. Supersomal incubations were started by the addition of imatinib in water. Control incubations were performed on ice instead of 37°C. Incubations were performed for 1 h and stopped by the addition of 50 µl acetonitrile. Protein precipitations were obtained by the centrifugation of the incubates (8,000 rpm for 10 min). Supernatants were transferred and injected into the analytical column (method described below).

HPLC analysis of imatinib and CGP74588

Imatinib, CGP74588 and the internal standard 4-hydroxybenzophenone were separated using a narrow bore (2.1 x 150 mm) stainless steel packed column packed with 3.5 µm Symmetry C-18 material and detection was accomplished with a UV detector set at excitation and emission wavelengths of 265 nm and 460 nm, respectively. The mobile phase consisted of 28% (v/v) acetonitrile in 50 mM ammonium acetate buffer pH 6.8 containing 0.005 M 1-octane sulfonic acid and was delivered at 0.2 ml/min.

Statistical analysis

Statistical evaluation was performed using the two-sided unpaired Student's *t* test to assess the statistical significance of difference between two sets of data. Differences were considered to be statistically significant when $P < 0.05$.

RESULTS

The effect of hydroxyurea on the cytotoxicity of imatinib

In the first part of this study we investigated *in vitro* the effect of hydroxyurea on the cytotoxicity of imatinib in parental MDCKII and MDCKII cells stably transfected with human Pgp or BCRP, for which we used the mouse homolog Bcrp1 (MDCKII-MDR1 or MDCKII-Bcrp1, respectively). Hydroxyurea and imatinib alone were not less cytotoxic to the MDCKIIMDR1 and MDCKII-Bcrp1 cells compared to parental MDCKII cells ($P > 0.05$; Table 1). Furthermore, the cytotoxicity of imatinib was not significantly affected by co-incubation with a non-toxic dose of 50 or 100 µM hydroxyurea ($P > 0.05$; Table 1).

Table 1 Cytotoxicity of imatinib in MDCKII-parental, -Bcrp1 and -MDR1 cell lines in the absence or presence of hydroxyurea

	MDCKII-parental	MDCKII-Bcrp1	MDCKII-MDR1
	$IC_{50\ (\mu M)}$	$IC_{50\ (\mu M)}$	$IC_{50\ (\mu M)}$
Hydroxyurea	740 ± 66	679 ± 69	698 ± 60
Imatinib	8.0 ± 0.2	9.1 ± 1.3	8.5 ± 0.9
Imatinib + Hydroxyurea 50 μM	8.4 ± 0.6	9.3 ± 2.0	8.4 ± 0.8
Imatinib + Hydroxyurea 100 μM	8.2 ± 0.4	9.2 ± 1.7	8.9 ± 1.6

Inhibiting concentrations of 50% (IC50, in μM) of 3 day incubations are shown as mean ± SD from >3 independent experiments

The effect of hydroyurea on the active transport of imatinib by Pgp and Bcrp1

Secondly, we investigated in vitro, employing polarized MDCKII-parental, -MDR1 and -Bcrp1 monolayers, whether hydroxyurea is capable of inhibiting the active transport of imatinib by Pgp and Bcrp1. Imatinib alone resulted in an increased transport by Pgp and Bcrp1 from the basolateral to the apical side (BA) compared with the transport from the apical to the basolateral side (AB), i.e., active transport (BA/AB is 10.3 and 20.4, respectively) (Fig. 1a, b). These results are comparable to those shown previously by Dai et al. [3] and Breedveld et al. [1]. Furthermore, the effect of hydroxyurea and the Pgp and BCRP inhibitors, LY335979 and pantoprazole as positive controls, on the active transport of imatinib by Pgp and Bcrp1 were investigated. LY335979 and pantoprazole inhibited the MDR1 and Bcrp1-mediated transport of imatinib, respectively, as upon co-incubation the transport from BA was approximately equal to the transport from AB, i.e., no active transport. In contrast, hydroxyurea did not affect Pgp and Bcrp1-mediated transport of imatinib (BA/AB is 12.0 and 18.5, respectively) (Fig. 1a, b).

Imatinib biotransformation by human CYP3A supersomes in the absence and presence of hydroxyurea

We then tested whether hydroxyurea inhibited cytochrome P450 3A (CYP3A). Although hydroxyurea is not a known CYP substrate, recent studies of the 5-lipoxygenase inhibitor, zileuton, the structure of which includes a hydroxyurea moiety, indicate that it inhibits CYPs, including CYP3A, which isozyme is mainly responsible for the biotransformation of imatinib [14, 16]. We performed *in vitro* biotransformation studies with supersomes expressing human CYP3A4. The CYP3A4 supersomes metabolized 12.3 ± 1.2% of imatinib to its main metabolite CGP74588 over 1 hour of incubation. Subsequently, we incubated imatinib with hydroxyurea or ritonavir; the latter is a known CYP3A4 inhibitor. Ritonavir was able to inhibit imatinib biotransformation completely. In contrast, hydroxyurea had no inhibitory effect on the biotransformation of imatinib. The CYP3A4 supersomes metabolized 11.8 ± 0.9% of imatinib to CGP74588 in the presence of hydroxyurea, which is not significantly different from the rate of biotransformation of imatinib in the absence of hydroxyurea ($P > 0.05$).

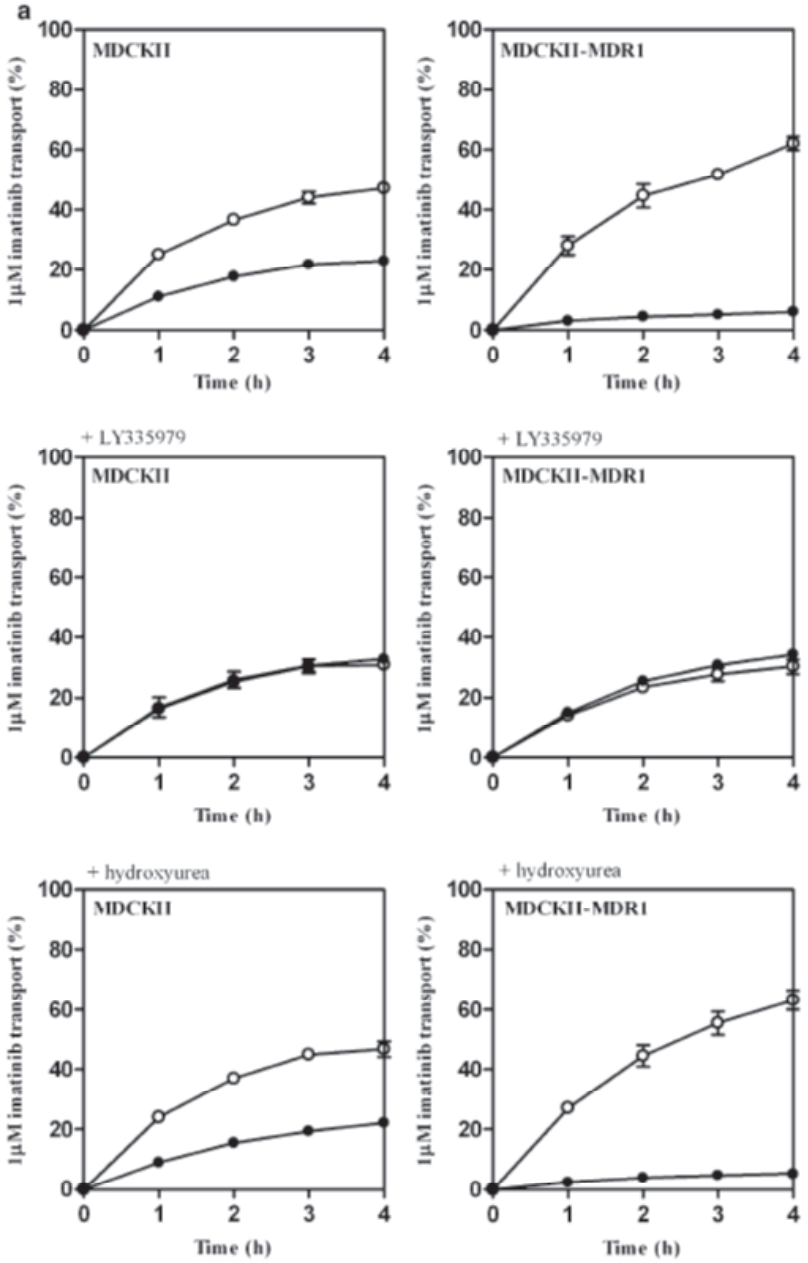
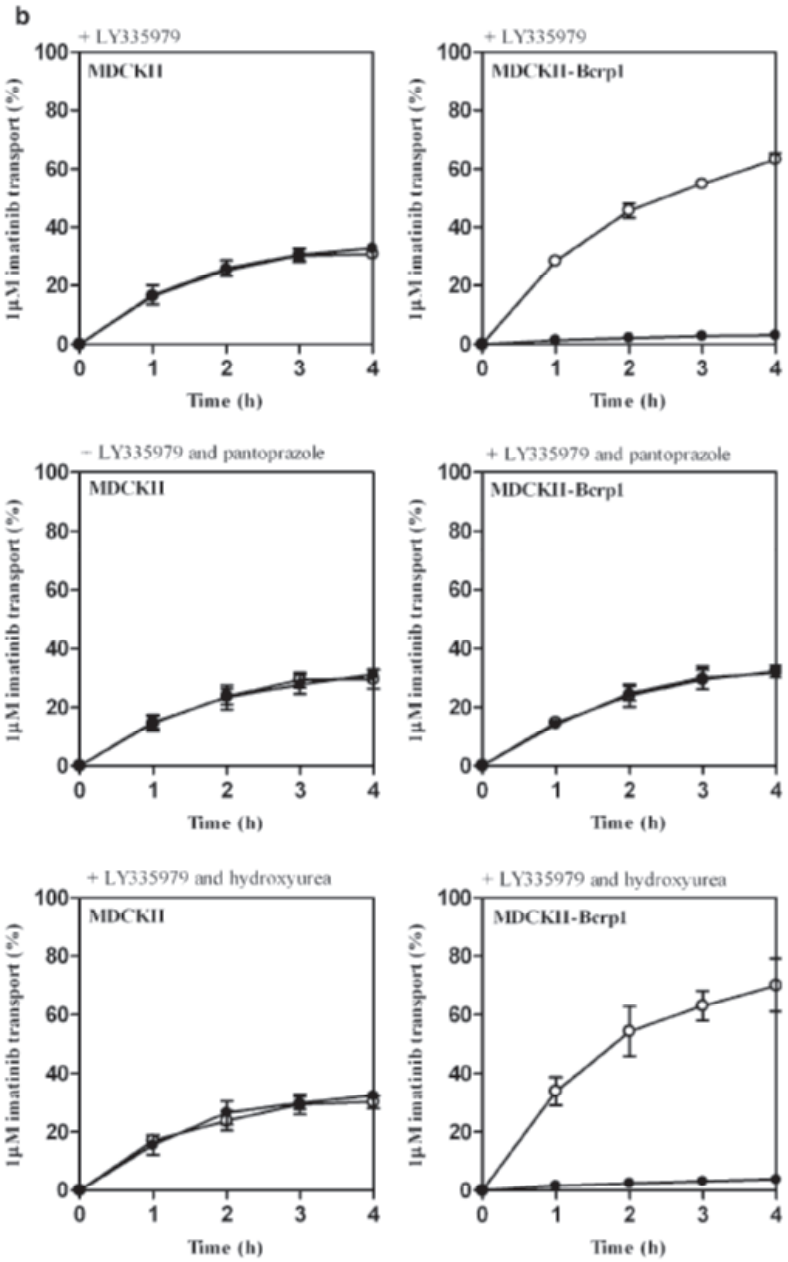


Fig.1 Transport of imatinib by MDR1 (Fig. 1a) and Bcrp1 (Fig. 1b) in the absence or presence of hydroxyurea, LY335979 and pantoprazole.
a MDCKII parental and MDCKII-MDR1 cells were pre-incubated for 2 h with and without (control) 5 µM LY335979 or 30 mM hydroxyurea. One µM of [^{14}C] imatinib and the indicated concentration of LY335979 or hydroxyurea were applied at t = 0 to the apical or basal side and the amount of [^{14}C] imatinib appearing in the opposite basal compartment (AB; closed symbols) or apical compartment (BA; open symbols) was determined. Samples were taken at t = 1, 2, 3 and 4 h. Points, means of each experiment in triplicate; bars, SD.



b MDCKII parental and MDCKII-Bcrp1 cells were pre-incubated for 2 h with 5 µM LY335979, and without (control) or with 500 µM pantoprazole or 30 mM hydroxyurea. One µM of [^{14}C]imatinib and the indicated concentration of LY335979 or hydroxyurea were applied at t = 0 to the apical or basal side and the amount of [^{14}C] imatinib appearing in the opposite basal compartment (AB; closed symbols) or apical compartment (BA; open symbols) was determined. Samples were taken at t = 1, 2, 3 and 4 h. Points, means of each experiment in triplicate; bars, SD.

DISCUSSION

The combination therapy of imatinib plus hydroxyurea is associated with remarkable antitumor activity in patients with recurrent glioblastoma. Thus far the mechanism of the added activity of hydroxyurea to imatinib is unknown. We hypothesized that the effect could be due to increased exposure of the tumor to imatinib. As imatinib is a high affinity substrate drug for Pgp and BCRP and is extensively metabolized by CYP3A, we investigated the effect of hydroxyurea on Pgp/BCRP mediated transport and CYP3A metabolism of imatinib. This study shows for the first time that hydroxyurea does not interact with imatinib by inhibition of Pgp and BCRP mediated transport or by CYP3A mediated metabolism of imatinib.

There are several other possible mechanisms of action that have to be investigated and could underlie the positive activity of this regimen. First, preclinical studies support that imatinib may enhance hydroxyurea mediated cytotoxicity by improving its delivery to the tumor microenvironment [9, 18]. Imatinib can diminish the tumor interstitial pressure. This could lead to increased capillary-to-interstitium transport and enhanced chemotherapy delivery, e.g., of hydroxyurea [17]. A clinical trial of imatinib with temozolomide, a cytotoxic agent with more established single-agent activity against glioblastoma multiforme than hydroxyurea, may be of interest in this respect.

Imatinib can also diminish tumor cell DNA repair after radiotherapy or chemotherapy by reducing Rad51 expression. Rad51 is an essential component of the DNA double-strand break pathway and has been implicated as a determinant of cellular radiosensitivity [21]. Imatinib-related decreased DNA repair may potentiate the cytotoxicity of hydroxyurea.

A final potential mechanism of action may be that PDGFR inhibitors exhibit significant antiangiogenic activity primarily by targeting perivascular cells, as shown in preclinical models [12, 23]. Furthermore, several chemotherapeutic agents suppress tumor angiogenesis and enhance the antitumor activity of vascular endothelial growth factor inhibitors. Therefore, PDGFR inhibition by imatinib combined with chemotherapy, e.g., hydroxyurea may provide complementary antiangiogenic activity, thereby limiting tumor growth, e.g., in glioblastoma multiforme.

In conclusion, hydroxyurea and imatinib do not interact at the level of Pgp, BCRP and CYP3A4 and further research is needed to clarify the beneficial activity against glioblastoma multiforme of the combination
of hydroxyurea and imatinib.

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11

Conclusions & perspectives

CONCLUSIONS

The review of the literature provided in **chapter two** and **three** clearly substantiate the crucial role of ATP Binding Cassette (ABC) and several SLCO transporters in drug disposition and in clinically relevant drug-drug, food-drug and herbal-drug interactions. Understanding of the pathophysiological functions of transporters and their role in drug disposition, efficacy and toxicity may help in the end to improve therapeutic efficacy of new drugs and to allow safer drug treatment. Moreover, it could open new treatment strategies for various diseases, by optimizing drug delivery to intracellular targets, selected organs and to sanctuary sites.

Chapter four to **nine** represent examples of evaluation of affinity of new drug under development for selected transporters. As recently described in the literature evaluation of affinity of new compounds for drug transporters can be explored from either a "bottom up" or a "top down" approach [1]. In the "bottom up" approach, affinity for transporters is explored early in drug development (in the pre-clinical or early clinical phase) in order to gain insight in pharmacokinetics and target-reaching properties and their potential clinical applications. The "top down" approach relies first on the generation of clinical data and subsequently on assessment of affinity for transporters in order to explain clinical findings and, for instance, observed clinical interactions. Essentially in chapters **four** up to **eight** examples of drugs where a bottom up approach is applied have been provided, as affinity of erlotinib, gimatecan and AZD1152 for ABC transporters has been explored early in the clinical development in order to evaluate potential clinical consequences and applications. In **chapter eight** and **nine** two examples where a top down approach has been employed can be seen, as affinity for ABC transporters has been checked in order to explain high interpatient variability in drug exposure (BMS-275183) and drug-drug interactions (between BMS-275183 and proton pump inhibitors, and between imatinib and hydroxyurea, respectively) observed in clinical practice.

Our experiments reported in **chapter four** show that erlotinib is a good substrate for BCRP and a moderate substrate for Pgp *in vitro*, whereas no transport by MRP2 could be documented. BCRP and Pgp appear to be involved in the intestinal uptake and brain penetration of erlotinib as demonstrated by the significant increase in oral bioavailability (1.5-fold) and plasma concentrations (1.5-fold) in Mdr1a/1b/Bcrp1 knockout compared with control mice. These findings provide justification for further evaluation of drug-drug interactions between erlotinib and drugs or herbal/food supplements able to modulate activity of Pgp and BCRP. Moreover, as Pgp and BCRP expression may inhibit brain penetration of erlotinib, these findings provide the theoretical basis for the development of a new strategy for the treatment of patients with brain metastases by concomitant administration of erlotinib and a Pgp/BCRP inhibitor. In effect, a study where erlotinib is combined with the Pgp and BCRP inhibitor elacridar in patients with brain metastases is planned to be performed in our institute.

In **chapter five**, the results of *in vitro* testing for affinity of gimatecan, a novel oral camptothecin derivative, for BCRP, MDR1 and MRP2 have been reported. The molecular structure of gimatecan would suggest less affinity for these ABC transporters when compared with other camptothecins, such as topotecan, irinotecan and its active metabolite SN-38. However, our results indicate that BCRP transports gimatecan efficiently. The magnitude of the transport and the effect of BCRP on the cytotoxicity of gimatecan in

human BCRP and mouse Bcrp1-overexpressing cell lines (MDCKII and T8) and monolayers (MDCKII) was significantly lower compared with topotecan, but it was still sufficient to affect the pharmacokinetics of the drug after oral administration when compared wild type and Bcrp1/Mdr1a/1b$^{-/-}$ knockout mice as further evaluated in **chapter six**.
Indeed in **chapter six**, deletion of BCRP and MDR1 in mice led to a significantly (1.4-fold) increased systemic exposure to gimatecan after oral administration compared with control mice. Elacridar significantly increased systemic exposure to oral gimatecan in wild type, but also in Bcrp1/Mdr1a/1b$^{-/-}$ mice, whereas the Pgp and BCRP inhibitor pantoprazole did not significantly affect the pharmacokinetics of gimatecan. Probably the inhibitory potency of pantoprazole is insufficient to cause a significant interaction.
In **chapter seven** the effect of ABCC4, as well as ABCC2 on the brain penetration of gimatecan and other campthotecins (i.e., topotecan, irinotecan, and SN-38) was evaluated. Additional genetic deletion of Abcc4 in Abcb1a/1b/Abcg2$^{-/-}$ (Mdr1a/1b/Bcrp1$^{-/-}$) mice significantly increased the brain concentrations of all camptothecin analogs by 1.2-fold (gimatecan) to 5.8 fold (SN-38), making gimatecan in principle a better candidate for clinical evaluation in the treatment of intracranial tumors.
As described in **chapter eight**, results of our *in vitro* models suggest that the aurora B kinase inhibitor AZD1152 (barasertib) and its active pro-drug AZD1152-hydroxy QPA are also good substrates for BCRP and Pgp. BCRP and Pgp transport appears to contribute to the low oral bioavailability of the drug, as well as to modulate brain penetration, kidney and liver distribution and myelotoxicity associated with treatment with AZD1152 as observed in *in vivo* experiments performed in Mdr1a/1b/Bcrp1$^{-/-}$ and wild type mice.
As illustrated in **chapter nine**, in our *in vitro* models BMS-275183 appeared to be a good substrate for Pgp and a moderate substrate for MRP2, whereas it was not transported by BCRP. *In vivo* experiments conducted in Mdr1a/1b$^{-/-}$ and wild type mice indicate that Pgp is able to affect the pharmacokinetics and brain penetration of BMS-275183 after i.v. and p.o. administration. Although *in vitro* pantoprazole was able to reverse the Pgp-mediated transport of BMS-275183 in Pgp-overexpressing cell monolayers in a concentration-dependent manner, no effect on the pharmacokinetics of BMS-275183 was observed by co-administration of pantoprazole, suggesting that, at least in our *in vivo* model, pantoprazole-mediated inhibition of BMS-275183 is not the most relevant contributor to the interaction reported in the clinic. By extrapolation of our results to the human situation the affinity of BMS-275183 for Pgp could explain, at least partly, the high interpatient variability in drug exposure observed in the clinic.
Finally, in **chapter ten**, our experiments appear to indicate that the interaction observed in the clinic between imatinib and hydroxyurea is not mediated by ABC transporters as in our cell survival and transwell experiments cytotoxicity and transport of imatinib mediated by BCRP and Pgp was not affected by co-incubation with different doses of hydroxyurea. Similarly, no effect of hydroxyurea on the CYP3A4 mediated metabolism of imatinib was observed. Other mechanisms appear to be involved in this interaction.

PERSPECTIVES

The newly gained knowledge obtained in the studies described in this thesis together with advancement of the field over the past few years could be exploited to achieve a number of important goals. These concern improvement of our understanding of the complex interplay between the different drug transporters and transporter families in handling exogenous as well as endogenous compounds. Exogenous compounds comprise food components, as well as drugs, party drugs, and complementary alternative medicines (CAM) frequently taken by chronically ill patients because of claims of healing capacity and by healthy subjects aiming to prevent disease. Better understanding of the molecular pharmacology of drug transporter processes might enable prediction of in vitro and in vivo handling of existing and novel compounds thereby potentially improving therapeutic application. This may help to prevent undertreatment by identifying unwanted food-drug and drug-drug interactions (DDIs) resulting in low oral absorption or high clearance or reduced drug concentration at the target organ as well as to prevent side-effects caused by overexposure due to unexpected low clearance, enhanced oral absorption and/or altered tissue distribution. That a more comprehensive understanding of DDIs is very much needed can be illustrated by numerous examples of clinically important DDIs, for instance between methotrexate and the proton pump inhibitor (PPI) omeprazole that has been reported already in 1993 [2]. However, it took more than a decade to unravel the pharmacological mechanism behind this serious and potentially lethal DDI [3]. Availability of easily accessible comprehensive databases enabling prediction of drug transporter mediated DDIs is thus a prerequisite for safe clinical application of existing and novel chemotherapeutics. The recently established UCSF-FDA database, together with other similar initiatives, is in this regard helpful and is worth further expanding [http://bts.ucsf.edu/fdatransportal; http://www.membranetransport.org; http://pharmacogenetics.ucsf.edu; http://www.druginteractioninfo.org] [4].

Understanding of the possible influence of genetic polymorphism in drug transporters on drug disposition and therapeutic efficacy also needs to be expanded in the near future and clinical consequences documented and made available to the community in an easily accessible way. To try to standardize studies about pharmacogenetics (of transporters) in drug development, recently a new guidance document has been issued by the European Medicines regulatory authority, where recommendations are given for evaluation of pharmacogenetic factors (genetic variability in drug transporters and metabolizing enzymes) affecting drug exposure and therapeutic efficacy in the same manner as for other intrinsic factors like organ function, age, weight, and gender [5]. Hereby, the concert played by the drug transporter families does not stand on its own as there is also a narrow interplay between drug transporter activity and drug biotransformation by the cytochrome P450 family (CYP), which in studies addressing in vivo pharmacology of substrate drugs for drug transporters cannot be disregarded [6, 7]. An integrated approach or "systems approach" to drug disposition is therefore advocated not only in clinical practice, but also in drug research.

The increasing importance of drug transporters in clinical practice and drug development is underlined by the recent foundation of the International Transporter Consortium, a group of academic, industrial and regulatory scientists focused on the role of transporters in drug disposition, particularly as it pertains to the development of drugs.

Two workshops have been performed to date (in 2008 and 2012, respectively) where recommendations were issued about the transporters to be taken under consideration during drug development [8, 9]. Indications were given about when an investigational new drug should be evaluated as a substrate for key human transporters and about the preferred in vitro and in vivo testing to explore drug transport. Decision trees were drawn to help in decision making whether a clinical study of transporter mediated drug-drug interactions should be conducted. These recommendations have been reflected in guidance documents issued recently by regulatory authorities (i.e., FDA and EMA), with the intent to explain regulatory necessities and methods needed for drug transporter studies supporting drug development [10, 11]. The need for well selected transporter studies early on is exemplified by the increasing number of drug-label changes due to drug-drug interactions mediated by drug transporters and of post-marketing transporters studies requested by regulatory authorities. (for information about impact of transporters on drug registration and labeling the reader is referred to the recent reviews of Tweedie D et al. 2013 and of Maeda K et al. 2013)[12,13]. Target of the guidelines is to standardize the inclusion of the evaluation of affinities of new molecular entities for drug transporters (under specific circumstances/when indicated) and the risk of related interactions, during drug development. Of course these guidelines need frequent updates to keep up with novel insights and scientific and public demands.

The goals defined above could at least partly be achieved by employing novel tools that have shown to be extremely valuable in identifying the role of individual drug transporters in the pharmacokinetics of affected substrate drugs, especially genetically modified knockout mice models, such as the mdr1a Pgp knockout mouse model [14]. Since the development and characterization of this model a range of knockout mice models of single and combined drug transporter negative models, and recently also with genetic deletion of the CYP3A system, has been developed and made available for pharmacological studies. Furthermore, transgenic models expressing human transporters, after knocking out mouse homologs, have further improved insight into absorption and disposition processes of affected substrate drugs [15,16]. This includes drug disposition into so-called sanctuary sites, such as the CNS compartment often well protected behind the blood-brain barrier (BBB), that are often difficult to approach experimentally in man. For this aim also less costly and easier to handle models have become available like the zebra-fish model of which the BBB largely resembles human BBB physiology [17]. This model could be exploited as a high-throughput screening system. Similarly, new methods employing suspended hepatocytes and sandwich-cultured hepatocytes have been developed to evaluate hepatic uptake and/or biliary excretion of endogenous compounds and xenobiotics (for information about test models and preclinical pharmacology the reader is referred to excellent reviews, for example Brouwer KLR et al. 2013) [1]. Recently, transporter imaging study, which involves noninvasive measurements of tissue distribution of drugs and transporters has been also recently evolved. Positron emission tomography (PET) and single-photon emission computed tomography (SPECT) using tracers represented by endogenous compounds or drug substrates or inhibitors of transporters is an evolving field, and is expected to provide useful tools for noninvasive, dynamic in vivo evaluation of tissue concentrations of specific transporters or of drug substrates for specific transporters, in order to assess how efficiently drugs reach their target organs and accumulate not only in clearance organs, such as the liver and the kidney, but also in other normal organs and

in tumors. Penetration in sanctuary sites and tumors, as well as the expected effect of transporter inhibitors, can also be tested in vivo, in animal and humans. For information about the use of imaging in studies of drug transporters the reader is referred to excellent reviews, for example Kusuhara H et al. 2013, Syvanen S et al. 2010 and Mairinger S et al. 2011) [18–20]. Recommendations for modeling and simulation have been also developed for in vitro - in vivo extrapolation of transporters interaction data in order to evaluate the reliability of preclinical in vitro and in vivo testing in predicting the clinical relevance of such findings. Several and specific modeling approaches for uptake and efflux transport estimations based on standardized kinetic parameters have been explored and established for the in vitro and in vivo models. Along with the standard static models for transporter in vitro-in vivo extrapolation, a dynamic physiologically based pharmacokinetic modeling approach has been developed. For more detailed information about modeling for in vitro-in vivo extrapolation of transporter data, the reader is referred to the excellent review of Zamek-Gliszczynski MJ et al. 2013 [9].

Besides achieving a better understanding of DDIs and food-drug interactions mediated by drug transporters and the interplay with drug metabolism on the basis of gained knowledge and availability of novel experimental tools for drug transporter studies also novel therapeutic approaches could be developed. This concerns the possibility to engineer/design/model new drugs with specific pharmacokinetic characteristics by avoiding or taking advantages from affinity for several transporters, or the possibility to reverse multidrug resistance of cancer cells in order to improve therapeutic efficacy of anticancer drugs. Additional applications concern the development of strategies to improve oral pharmacokinetics and/or disposition into sanctuary sites. Many oral drugs show low oral bioavailability. This coincides invariably with wide variation in oral pharmacokinetics as predicted on the basis of the known relationship between absolute bioavailability and interpatient variation in systemic exposure to a drug [21]. Low and variable oral pharmacokinetics is associated with a number of obvious disadvantages. Low oral bioavailability can often be explained by one or more of the following characteristics: poor water solubility, poor stability in aqueous solution, high affinity for drug transporters in the epithelial layer of the gastro-intestinal tract, high pre-systemic elimination ("first-pass"). If affinity for the indicated drug transporters, especially ABCB1 and/or ABCG2, is a dominant factor then one could develop a strategy to improve oral pharmacokinetics of indicated substrate drugs by combining the drug of interest with an effective inhibitor of ABCB1 and/or ABCG2. This boosting strategy could be applied to a number of anticancer drugs that are currently only available for intravenous (i.v.) administration, such as the taxanes docetaxel and paclitaxel. Boosting oral pharmacokinetics might enable oral therapy with docetaxel and paclitaxel, which could benefit patients in several ways. If successful, this strategy might serve as a template for other anticancer drugs as well as non-anticancer drugs. There is however a number of prerequisites for a successful i.v. to oral switch in anticancer therapy with these drugs that need to be fulfilled in future research. Another strategy is to boost the disposition of drugs into sanctuary sites such as the CNS compartment. The CNS is well protected by the BBB, in which a range of drug transporters is expressed effectively extruding drugs back into the blood compartment, thereby keeping drug concentrations in the brain low. In different therapeutic areas increased exposure of the brain to drugs may be of benefit, such as in the case of primary brain tumors, neurodegenerative diseases and infectious diseases primarily affecting the

brain. If potentially active drugs would however show low CNS accumulation on the basis of affinity for the indicated drug transporters the boosting concept might increase CNS exposure and potentially improve therapeutic outcome. Obtaining proof of principle of increasing CNS accumulation of the EGFR tyrosine kinase inhibitor erlotinib by combining erlotinib with the booster drug elacridar is currently being pursued in our department.

Areas of uncertainty

Strategies and methods mentioned above will hopefully increase our understanding of drug absorption and disposition processes affected by drug transporters and may be exploited for the outlined boosting concepts and other clinically relevant applications, as well as for a safer and more effective administration of drugs to patients. However, currently there are still a number of significant uncertainties that need to be addressed in future studies.

Since the first identification of Pgp more than three decades ago, more than 400 membrane transporters in two major superfamilies (ATP binding cassette [ABC] and solute carrier [SLC] have been identified) in the human genome and for several of these the pathophysiological function is still unknown. Therefore, other transporters not (fully) characterized yet may play a crucial role in drug disposition increasing the difficulties in predicting pharmacokinetics of drugs. For instance, MATE transporters have been discovered in 2005, but they appear to have a key role in renal disposition of a range of drugs explaining several clinically relevant drug-drug interactions [22].

Crystallographic structures of drug transporters are being elucidated in order to provide tools for obtaining direct evidence of transport mechanisms and molecular interactions between proteins and ligands. Computational models are also used in order to predict and identify drug-transporter interactions. However, crystal structures not always fully represent the configuration of the proteins, as crystallization procedures (e.g. surfactants and cofactors) may lead to non-physiological protein conformations and also because proteins are dynamic systems. Moreover, validation of computational models remains a point of concern [23].

Currently, our understanding of the genetic, epigenetic, and other factors that affect expression and activity of transporters involved in drug disposition and response is far from comprehensive.

Despite the beauty of the outlined models extrapolation of results obtained in preclinical models to the clinic can still only be done qualitatively and not quantitatively. Moreover, every available approach/methodology to study activity of transporters in vitro or in vivo has several limitations. Static and dynamic translational modeling is based on several assumptions which not always correspond to the complex biology of the human situation too. For instance, an important limitation is related to the incapacity of all methods currently available to measure unbound intracellular drug concentrations and the influence of transporters on such relevant parameters [1,9,24]. Moreover, transporter expression and/ or activity may vary with age, gender, race, pathophysiological conditions [25]. Furthermore, several transporters may be involved in the uptake or efflux of a compound, as an overlap in substrate specificity and tissue distribution between different transporters and/or metabolizing enzymes is reported. Very specific transporters inhibitors are also lacking. For instance cyclosporin A is able to block Pgp, MRP, OATP, PEPT transporters [26]. All these issues limit for example prediction of the absolute magnitude

of DDIs in patients. Confirmatory studies in man will thus still be needed in the case of pre-clinically documented potentially relevant DDIs.
Drug absorption and disposition in man may be different from mice and other animal models because of a number of reasons. Physiology in the gastro-intestinal tract in humans may be different and more variable, humans show obviously very much wider genetic variation than inbred animal strains, the affinity constants for human drug transporters are known to be different from non-human drug transporters and drug solubility may be different between humans and laboratory animals. Moreover, differences in tissue distribution and orthology between mice and humans have been observed. Transporter-knockout animal models may present potential compensatory mechanisms. Transgenic mice expressing human transporters and/or enzymes have recently been developed, but the utility of such humanized mouse models in predicting drug pharmacokinetics and DDI remains to be determined. This further cautions direct extrapolation of pre-clinically obtained results of drug absorption and disposition to the clinic.
Concerning the boosting concepts other uncertainties need to be resolved, involving the exact pharmacokinetics of the interaction between affected substrate drug and booster drug, the optimal dose of both compounds, the safety of the booster drug and the potential for new DDIs because of the presence of the booster drug. As pivotal studies are currently lacking there is no proof of a positive benefit/risk yet of orally boosted anticancer drugs. The boosting concept into sanctuary sites is as yet clinically unproven.
Finally, regarding regulatory aspects, it is unclear at this time whether the available guidance documents recently issued by regulatory authorities will be sufficiently compelling to cover the complexity of the transporter system and whether their application will translate into an improvement of the drug development process and/or in development of safer and more efficient therapies for patients.
Notwithstanding these areas of uncertainty studies directed at boosting oral bioavailability and drug disposition into sanctuary sites are expected to pay off in the end. In the upcoming years it is expected that understanding of all the transporters genes and their functions as well as their role in physiology and pathology will be achieved providing exciting and possibly unexpected opportunities for the understanding and treatment of human diseases, and as well as for improving drug discovery and development.

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Appendix

Chemical structures of studied lead molecules in this thesis

Summary

Nederlandse samenvatting (Dutch summary)

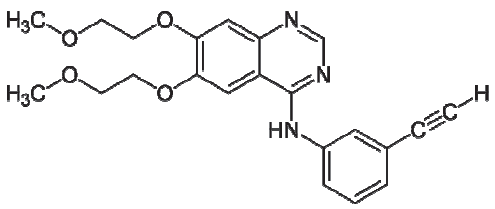
Dankwoord (aknowledgements)

Curriculum Vitae

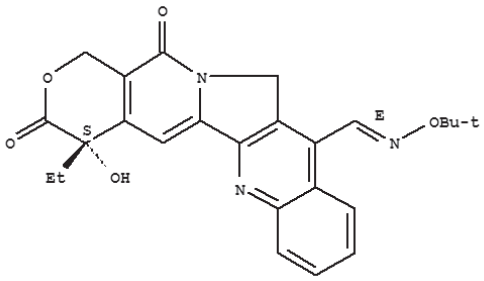
List of publications

APPENDIX 1

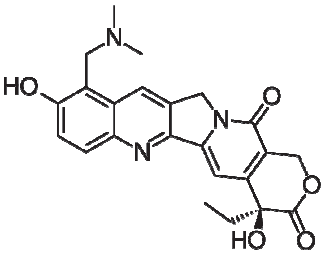
Chemical structures of studied lead molecules in this thesis



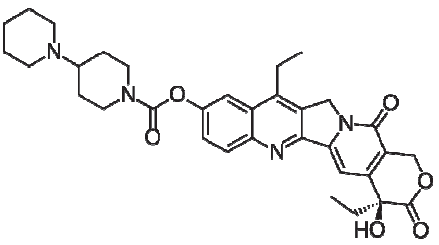
Erlotinib



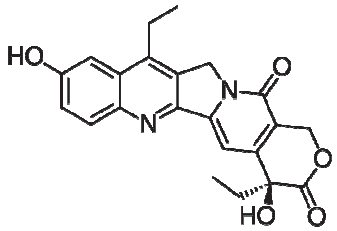
Gimatecan [Synonyms: (S)-Gimatecan;7-[(E)-t-Butyloxyiminomethyl]camptothecin]



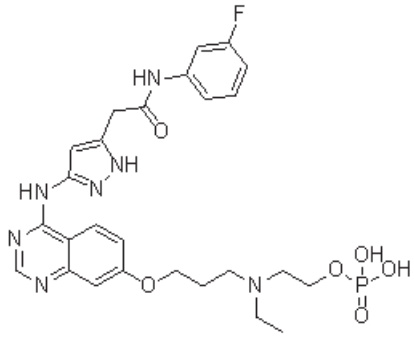
Topotecan



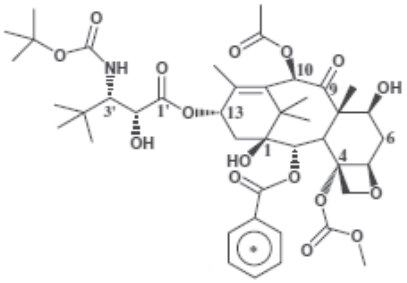
Irinotecan



SN-38, active metabolite of irinotecan



Barasertib (AZD1152)



BMS-275183

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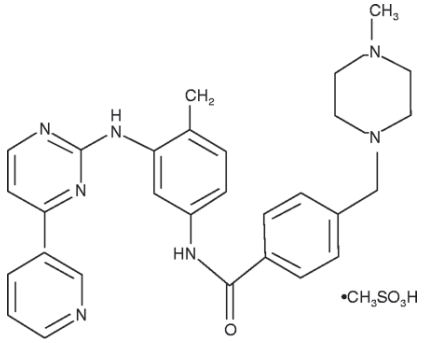
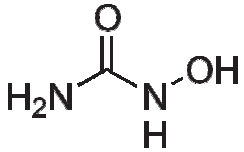


Imatinib mesylate



Hydroxyurea

SUMMARY

The ATP binding cassette (ABC) drug efflux transporters are proteins located in the plasma membrane of epithelial cells, where they mediate the ATP-dependent extrusion of substrates from cell. P-glycoprotein (P-gp, MDR1, ABCB1) was the first ABC transporter discovered by Juliano and Ling in 1976. (Over)-expression of P-gp in cancer cells was reported to confer resistance to substrate anticancer drugs, the so called multidrug resistance phenotype. Since then more than 70 membrane transporters have been discovered. The pathophysiological role of ABC transporters has also been increasingly studied. Together with the ability to confer the multidrug resistance phenotype to expressing cells, they appear to play a crucial role in protection of the body against toxic xenobiotics and metabolites by secreting these compounds into bile, urine and the intestinal lumen and by preventing their accumulation in several organs like brain, testicles, and fetus. Due to their strategic localization in organs that are implicated in absorption, distribution metabolism and excretion (ADME), ABC transporters are able to affect the pharmacokinetics of substrate drugs, toxins, endogenous and exogenous compounds. Moreover, interactions between substrates and inhibitors of such transporters can lead to clinically relevant drug-drug interactions.

On the other hand, under several circumstances the administration of a selective ABC transporter inhibitor may provide a useful strategy to improve treatment outcome. Inhibition of ABC transporters located at the apical (luminal) side of the intestine (like P-gp and BCRP) can lead to an increase in oral bioavailability of substrate drugs and to reduction of inter-patient variability on drug exposure. Similarly, inhibition of specific transporters located at the blood-brain barrier could increase the brain penetration of anticancer drugs substrates of ABC transporters, possibly leading to improvement of treatment of intracranial tumors. Evaluation of affinity of novel compounds for ABC transporters is also useful to predict the effect of such transporters on the pharmacokinetics of substrate drugs and to predict, and thereby avoid, clinically relevant interactions.

After a short introduction of this thesis in **chapter 1**, in **chapter 2 and 3** a review of the literature about the effect of selected ABC and SLC transporters on the pharmacokinetics and pharmacodynamics of substrate (anticancer) drugs and their contribution to clinically relevant drug-drug interactions is presented.

In **chapter 4** to **9** the affinity of several anticancer drugs, including the EGFR tyrosine kinase inhibitor erlotinib (Tarceva®), the campthotecin derivative gimatecan, the Aurora B kinase inhibitor barasertib (AZD1152) and its more active form barasertib hydroxy-QPA (AZD1152-hQPA), and the orally available C-4 methyl carbonate analogue of paclitaxel BMS-275183, for selected ABC transporters (i.e., P-gp [MDR1, ABCB1], BCRP [ABCG2], MRP2 [ABCC2]) was studied in several preclinical cell and mouse models. *In vitro*, cell survival, transport in transwell experiments, and competition experiments in membrane vesicles were performed. *In vivo*, the pharmacokinetics and tissue distribution of the tested compound were evaluated in Abcg2 and/or Mdr1a/b and/or Mrp4 knockout mice and compared with wild type mice.

In **chapter 4** affinity of erlotinib (Tarceva®) for P-gp, BCRP and MRP2 was evaluated in *in vitro* and *in vivo* models. Our results show that erlotinib is a substrate of P-gp and BCRP, whereas no transport by MRP2 was documented. The role of P-gp and BCRP in the ADME of erlotinib after oral administration was explored in mice knockout for P-gp and BCRP

(Abc1a/b$^{-/-}$/Bcrp1$^{-/-}$) genes and in control mice. The area under the curve (AUC) in plasma and oral bioavailability of erlotinib were 1.5-fold increased in Abc1a/b$^{-/-}$/Bcrp1$^{-/-}$ mice compared with control mice. These results provide justification for further evaluation of drug-drug interactions between erlotinib and drugs able to modulate P-gp and/or BCRP activity. On the other hand, concomitant administration of erlotinib and a P-gp/BCRP inhibitor could result in an improvement of the brain penetration of erlotinib, therefore allowing treatment of patients with intracranial tumors.

In **chapter 5** transport of the new campthotecin derivative gimatecan by P-gp, BCRP and MRP2 is explored in *in vitro* models. Although it was previously reported in the literature that gimatecan, together with other lipophilic campthotecin derivatives, would not be a good substrate for BCRP and P-gp, in this chapter we clearly demonstrate that gimatecan is efficiently transported by BCRP/Bcrp1. Overexpression of BCRP in Madin-Darby canine kidney II cells resulted in up to 10-fold increased resistance to gimatecan compared with parental (wild type) cells. In transwell experiments transport of gimatecan by BCRP/Bcrp1 was clearly documented and could be completely inhibited by the BCRP inhibitors elacridar and pantoprazole. In Sf9-BCRP membrane vesicles gimatecan inhibited the ATP-mediated transport of methotrexate by BCRP in a concentration-dependent manner. In contrast, gimatecan was not transported by P-gp or MRP2 in our *in vitro* models. To investigate whether the affinity of gimatecan for BCRP observed *in vitro* could be relevant *in vivo*, pharmacokinetics of gimatecan were evaluated after oral and intravenous administration in Mdr1a/1b$^{-/-}$/Bcrp1$^{-/-}$ and wild type mice. The results of these experiments are reported in **chapter 6**. Essentially, genetic deficiency of Bcrp1 in mice significantly affected the pharmacokinetics of gimatecan after oral administration. Of note, concomitant administration of pantoprazole (a proton pump inhibitor able to block P-gp and BCRP) did not significantly affect the pharmacokinetics of gimatecan in mice. In contrast, concomitant administration of the BCRP and P-gp inhibitor elacridar resulted in a significant 1.6- to 1.8-fold increase in systemic exposure to gimatecan in presence and absence of Bcrp1/Mdr1a/b. This suggests that the *in vivo* interaction between elacridar is partly mediated by other drug transporters than BCRP/Bcrp1. In **chapter 7** we show that Abcc4 (Mrp4), together with Abcg2 and Abcb1a/b, is able to affect the brain penetration of gimatecan and other camptothecin analogues (in particular topotecan, irinotecan, and SN-38). This could be demonstrated by the development of a new mouse model with combined deletion of Abcc4, Abcg2 and Abcb1a/b genes. Abcb1a/b$^{-/-}$/Abcg2$^{-/-}$/Abcc4-/- mice displayed a significant 1.2-fold higher brain penetration of gimatecan compared with Abcb1a/b$^{-/-}$/Abcg2$^{-/-}$ mice. Importantly, the brain distribution of gimatecan in wildtype mice was more than 40-fold and 220-fold higher than that of topotecan and SN-38, respectively, likely due to the more lipophilic nature of gimatecan. This finding suggests that gimatecan might be a better candidate than irinotecan in the treatment of intracranial tumors.

In **chapter 8** we explored the affinity of barasertib (AZD1152) and its more active form barasertib-hydroxy-QPA (AZD1152-hQPA) for P-gp, BCRP and MRP2. Barasertib is a selective Aurora B kinase inhibitor currently under clinical testing for the treatment of haematological malignancies. Active transport of barasertib by P-gp and BCRP was documented in transwell experiments employing MDCKII and LLCPK cell monolayers overexpressing P-gp and BCRP. In Sf9-BCRP membrane vesicles barasertib inhibited the ATP-mediated transport of methotrexate by BCRP in a concentration-dependent manner.

Overexpression of P-gp and BCRP in MDCKII cells resulted in increased resistance to barasertib compared with parental cells. The influence of P-gp and BCRP on the ADME of barasertib after intravenous and intraperitoneal administration was explored *in vivo* using wild type and Bcrp1$^{-/-}$/Mdr1a/b$^{-/-}$ mice. The systemic exposure as well as bioavailability, tissue distribution (brain, liver, kidney) and myelotoxicity of barasertib-hQPA were statistically significantly increased in Bcrp1$^{-/-}$/Mdr1a/b$^{-/-}$ compared with wild type mice. Our *in vivo* experiments indicate a potential for drug-drug interactions between barasertib and other clinically used BCRP/P-gp substrates or inhibitors.

In **chapter 9** the affinity of BMS-275183, a novel taxane administered orally, for P-gp, BCRP and MRP2 was evaluated in *in vitro* and *in vivo models*, in order also to explore the mechanism underlying a clinically relevant interaction observed in the clinic between the taxane analogue BMS-275183 and the proton pump inhibitor pantoprazole, a well-known BCRP and P-gp inhibitor. In our experiments BMS-275183 was efficiently transported by P-gp and to a lesser extent by MRP2 *in vitro*. The magnitude of the P-gp-mediated transport of BMS-275183 appears to be of lower magnitude compared with paclitaxel. Active P-gp transport of barasertib was completely inhibited by pantoprazole in a concentration dependent manner. *In vivo*, oral bioavailability, plasma $AUC_{0\text{-}6h}$ and brain concentrations of barasertib were significantly 1.5-, 4-, and 2-fold increased, respectively, in Mdr1a/b$^{-/-}$ compared with wild type mice. However, oral co-administration of pantoprazole did not alter the pharmacokinetics of BMS-275183 in wild type mice. Our results suggest that BMS-275183 is efficiently transported by P-gp and to a lesser extent by MRP2. However, additional studies are considered necessary in order to clarify the mechanisms responsible for the reported drug-drug interaction between BMS-275183 and pantoprazole.

In **chapter 10** we explored the mechanisms underlying the pharmacokinetic interaction between imatinib and hydroxyurea, a ribonucleotide reductase inhibitor. Imatinib mesylate (Gleevec®) is a tyrosine kinase inhibitor registered in 2001 by FDA and EMA for the treatment of patients with chronic myeloid leukemia and gastrointestinal stromal tumors (GIST). Preliminary results suggest a good antitumor activity of the combination imatinib-hydroxyurea in patients with recurrent glioblastoma multiforme. In this chapter we show that P-gp, BCRP and CYP3A4 are not responsible for the observed interaction between hydroxyurea and imatinib. Further research is needed to clarify the beneficial activity of the combination imatinib-hydroxyurea in patients with glioblastoma multiforme.

In conclusion, this thesis describes the crucial role of selected ABC-transporters on the pharmacokinetics and pharmacodynamics of new and already known anticancer drugs. A better understanding of the pathophysiological role of ABC transporters might lead to algorithms for prediction of clinically relevant drug-drug interactions, to improvement of treatment outcome due to the strategical administration of transporter inhibitors, and to a better understanding of the mechanisms of tumor resistance against anticancer drugs.

NEDERLANDSE SAMENVATTING (DUTCH SUMMARY)

De geneesmiddeltransporters van de ABC familie zijn eiwitten die zich bevinden in de plasmamembraan van epitheelcellen, waar ze de ATP-afhankelijke uitscheiding van substraten, waaronder bepaalde geneesmiddelen, bewerkstelligen. P-glycoproteine (P-gp, MDR1, ABCB1) is de eerste ABC geneesmiddeltransporter die ontdekt werd door Juliano en Ling in 1976. Toen werd vastgesteld dat expressie van P-gp in kankercellen kan leiden tot resistentie tegen anti-kankermiddelen, een fenomeen dat (multi)drug resistentie (MDR) wordt genoemd. Sindsdien zijn er meer dan 70 membraantransporters geïdentificeerd. Inmiddels zijn er vele en belangrijke inzichten verkregen in de farmacologische en de fysiologische betekenis van de ABC-geneesmiddeltransporters. Naast de rol in multidrugresistentie spelen deze transporters een belangrijke pathofysiologische rol in de bescherming van het lichaam, doordat zij de penetratie van toxines in een aantal belangrijke organen, zoals hersenen, testis en placenta beperken. Onderzoek heeft aangetoond dat deze transporters ook aanwezig zijn in organen die belangrijk zijn voor absorptie, distributie, metabolisme en eliminatie (ADME) van stoffen. Door deze strategisch gunstige aanwezigheid kunnen deze transporters de farmacokinetiek van een scala aan geneesmiddelen, toxines, maar ook lichaamseigen stoffen beïnvloeden. Interacties tussen substraten en remmers van transporters kunnen tot klinisch relevante geneesmiddel-geneesmiddel interacties leiden met risico's van toxiciteit of onderdosering. Toch kan de toediening van remmers van geselecteerde transporters leiden tot juist een beoogde verbetering van de behandeling van patiënten. Remming van geneesmiddeltransporters die zich bevinden in de plasmamembraan van cellen van de dunne darm aan de apicale zijde (bijvoorbeeld P-gp en BCRP) kan leiden tot een verbetering van orale beschikbaarheid en daarmee tot bijvoorbeeld een afname van de inter-patiëntvariabiliteit van geneesmiddelconcentraties. Geneesmiddeltransporters in de bloed-hersenbarrière kunnen de distributie van veel klassieke chemotherapeutica in de hersenen en de overige weefsels van het centrale zenuwstelsel beperken. Remming van die transporters zou de penetratie van antikankermiddelen in het centraal zenuwstelsel (CZS) kunnen verbeteren, waardoor de behandeling van intracraniale tumoren mogelijk beter kan geschieden.

Het vooraf screenen van antikankermiddelen op affiniteit voor ABC transporters is belangrijk om de invloed van deze transporters op de ADME van orale medicijnen en geneesmiddeleninteracties in de kliniek beter te kunnen voorspellen.

Na een korte introductie van het proefschrift in **Hoofdstuk 1** volgt in de **Hoofdstukken 2** en **3** een literatuuroverzicht over de rol van de specifieke ATP-afhankelijke Binding Cassette (ABC) en Solute Carrier (SLC) geneesmiddeltransporters in de farmacologie van geneesmiddelen met een discussie over de gevolgen van klinisch relevante geneesmiddel-geneesmiddelinteracties.

In de **Hoofdstukken 4** tot en met **9** werdt de affiniteit van een aantal antikankergeneesmiddelen voor specifieke ABC transporters, te weten P-gp (MDR1, ABCB1), BCRP (ABCG2) en MRP2 (ABCC2), onderzocht. Het betreft hier de EGFR tyrosine kinase remmer erlotinib (Tarceva®), het campthotecine-analoog gimatecan, de Aurora B kinase remmer barasertib (AZD1152) en het oraal taxaan-analoog BMS-275183. Verschillende *in vitro* geneesmiddel transporter modellen met cellen die een (over) expressie vertonen van specifieke ABC transporters werden gebruikt, onder andere het

transwell model en het membraanvesikel model. Invloed van specifieke ABC transporters op de farmacokinetiek van de bovengenoemde anti-kanker geneesmiddelen werd onderzocht *in vivo* in zogenaamde knock out muismodellen voor één (Bcrp1$^{-/-}$) of meer transporters (Mdr1a/b$^{-/-}$; Mdr1a/1$^{-/-}$/Bcrp1$^{-/-}$; Mdr1a/1b/Bcrp1/Mrp4$^{-/-}$) en vergeleken met (wildtype) controlemuizen.

In **Hoofdstuk 4**, werdt de affiniteit van erlotinib (Tarceva®) voor P-gp, BCRP en MRP2 onderzocht in *in vitro* en *in vivo* modellen. Enerzijds laten de resultaten zien dat erlotinib een substraat is voor BCRP en P-gp. Anderzijds kan geen affiniteit voor MRP2 worden aangetoond in de gebruikte modellen. De rol van P-gp en BCRP in de ADME bij oraal toegediend erlotinib werd ook onderzocht door middel van het gebruik van muizen met deletie van de P-gp en BCRP (Abcb1a/b$^{-/-}$/Bcrp1$^{-/-}$) genen en (wildtype) controlemuizen. De oppervlakte onder de plasmaconcentratie-tijd curve van nul tot oneindig ($AUC_{(0\text{-inf})}$) en orale biologische beschikbaarheid van erlotinib in Abcb1a/b$^{-/-}$/Bcrp1$^{-/-}$ muizen waren significant (1.5-voudig) verhoogd in vergelijking met controle muizen. Deze bevindingen suggereren dat het combineren van erlotinib met een van de veel gebruikte P-gp/BCRP remmers een effect kan hebben op de ADME van erlotinib met mogelijk invloed op de doeltreffendheid en de veiligheid van dit middel. Interactie van erlotinib met andere P-gp/BCRP remmers kan hieruit worden afgeleid. Aan de andere kant, de strategische remming van P-gp en BCRP bij de bloed-hersenbarriere zou kunnen leiden tot een verbetering van de hersenpenetratie van erlotinib en daardoor de behandeling van intracraniale tumoren toelaten.

Vervolgens werdt in **Hoofdstuk 5** het transport van de nieuwe campthotecine-analoog gimatecan door P-gp, BCRP en MRP2 onderzocht in *in vitro* modellen. Vroeger is gepubliceerd dat gimatecan, samen met andere in hoge mate lipofiele camptothecine derivaten, weinig tot geen affiniteit zouden vertonen voor BCRP (ABCG2) en P-gp (ABCB1). Echter, in dit hoofdstuk wordt getoond dat gimatecan wel degelijk getransporteerd wordt door BCRP/Bcrp1. Overexpressie van ABCG2 in Madin-Darby canine kidney II cellen blijkt geassocieerd met acht- tot tienvoudige resistentie tegen gimatecan in vergelijking met controle (wildtype) cellen. In transwell experimenten werd transport van gimatecan door BCRP aangetoond. Competitie tussen gimatecan en methotrexaat voor BCRP-gemedieerd transport in Sf9-BCRP membraanvesikels werd ook aangetoond. Echter, gimatecan toonde geen affiniteit voor P-gp en MRP2 in onze *in vitro* modellen. Om de *in vivo* gevolgen van de vertoonde affiniteit van gimatecan voor BCRP verder te onderzoeken, werd de farmacokinetiek van gimatecan na orale en intraveneuze toediening onderzocht in muizen die geen BCRP en P-gp eiwit aanmaken (Bcrp1$^{-/-}$/Mdr1a/b$^{-/-}$ of triple knockout) en bij zogenaamde controlemuizen die wél beide eiwitten aanmaken. De resultaten gerapporteerd in **Hoofdstuk 6** laten zien dat BCRP een significant effect heeft op de farmacokinetiek van oraal ingenomen gimatecan in muizen. Opmerkelijk is dat bij gelijktijdige toediening van gimatecan en pantoprazol (een protonpompremmer die P-gp en BCRP remt) er geen significant effect optreedt op de farmacokinetiek van gimatecan in muizen. Hiertegenover staat dat gelijktijdige toediening van elacridar (een andere BCRP en P-gp remmer) en gimatecan leidde tot een significante 1.6- tot 1.8-voudige toename in systemische blootstelling aan gimatecan in BCRP/P-gp proficiënte muizen in vergelijking met BCRP/P-gp deficiënte muizen. Dit suggereert dat elacridar naast BCRP en P-gp ook andere eliminatieroutes remt.

In **Hoofdstuk 7** werdt aangetoond dat Abcc4, samen met Abcg2 en Abcb1a/b, de hersenpenetratie van gimatecan en andere camptothecine derivaten (te weten topotecan, irinotecan, en metaboliet SN-38) beïnvloedt. Dit kon worden aangetoond middels een nieuw-ontwikkeld muismodel, dat naast Abcb1 en Abcg2, ook deficiënt is voor Abcc4 (Abcb1a/b$^{-/-}$/Abcg2$^{-/-}$/Abcc4$^{-/-}$). Abcb1a/b$^{-/-}$/Abcg2$^{-/-}$/Abcc4$^{-/-}$ muizen toonde een significante 1.2-voudige hogere hersenpenetratie van gimatecan in vergelijking met Abcb1a/b$^{-/-}$/Abcg2$^{-/-}$ muizen. Opmerkelijk is dat gimatecan een meer dan 40- en 220-voudige hogere spiegel in de hersenen van wildtype muizen bereikte in vergelijking met respectievelijk topotecan en SN-38. Dit wordt waarschijnlijk verklaard doordat gimatecan een veel lipofielere verbinding is dan de andere camptothecines. Deze resultaten suggereren dat gimatecan een beter geneesmiddel dan irinotecan zou kunnen zijn voor de behandeling van, met name, intracraniale tumoren.
In **Hoofdstuk 8** is onderzoek naar de affiniteit van barasertib (AZD1152) en zijn actievere vorm barasertib-hydroxy-QPA voor P-gp, BCRP en MRP2 beschreven. Barasertib is een selectieve Aurora B kinaseremmer die in klinisch onderzoek is voor de behandeling van acute leukemia. Middels het gebruik van MDCKII en LLCPK cellen, zowel wildtype als met (over)expressie van P-gp, BCRP en MRP2, werd actief transport van barasertib door P-gp en BCRP gedocumenteerd *in vitro* in het transwell model. Competitie tussen barasertib en methotrexaat voor BCRP-gemedieerd transport in Sf9-BCRP membraanvesikels werd ook duidelijk aangetoond. In vergelijking met wildtype lijken MDCKII-BCRP en MDCKII-MDR1 cellen meer resistent tegen barasertib *in vitro*. De invloed van BCRP en MDR1 op de ADME van barasertib na intraveneuze en intraperitoneale toediening werd onderzocht middels het gebruik van Mdr1a/b$^{-/-}$/Bcrp1$^{-/-}$ en wildtype (controle)muizen. De systemische blootstelling, orale biologische beschikbaarheid, weefseldistributie in hersenen, lever en nier, en de beenmergtoxiciteit die kan worden toegeschreven aan een behandeling met barasertib waren significant hoger in Mdr1a$^{-/-}$/Bcrp1$^{-/-}$ muizen wanneer die parameters werden vergeleken met die in controlemuizen. Gezien het effect van Mdr1a/b en Bcrp1 gendeletie op de farmacokinetiek van barasertib in muizen, kunnen in patiënten geneesmiddeleninteracties tussen barasertib en veelvuldig gebruikte P-gp en BCRP-remmers worden verwacht.
In **Hoofdstuk 9** is de affiniteit van de nieuwe orale taxaananaloog BMS-275183 voor P-gp, BCRP en MRP2 onderzocht aan de hand van *in vitro* en *in vivo* modellen. Dit onderzoek is ook uitgevoerd om het mechanisme van de mogelijke famacokinetische interactie tussen BMS-275183 en de protonpompremmer pantoprazol (een bekend BCRP en P-gp remmer), eerder beschreven bij een patient, te kunnen verklaren. Transport van BMS-275183 door P-gp in verschillende *in vitro* modellen met LLCPK en MDCKII cellen is duidelijk aangetoond. Een bescheiden affiniteit van BMS-275183 voor MRP2 werd ook aangetoond *in vitro*. Opmerkelijk is dat het transport van BMS-275183 door P-gp in het gebruikte transwell model volledig werd geblokkeerd door pantoprazol, op een wijze die afhankelijk was van de concentratie van het middel. *In vivo*, orale biologische beschikbaarheid, AUC en hersenpenetratie van BMS-275183 waren respectievelijk 1.5, 4, en 2-voudig hoger in de Mdr1a/b$^{-/-}$ muizen in vergelijking met controlemuizen. Echter, gelijktijdige toediening van pantoprazol en BMS-275183 had nauwelijks tot geen effect op de ADME van BMS-275183. Meer onderzoek is nodig om het mechanisme van de geneesmiddelinteractie zoals waargenomen in de kliniek te kunnen verklaren.

In **Hoofdstuk 10** is het mechanisme van de mogelijke farmacokinetische interactie tussen imatinib en hydroxyurea onderzocht. Imatinib mesylaat (Glivec®) is een tyrosine-kinaseremmer die in 2001 door de FDA en EMA is geregistreerd voor de behandeling van patiënten met chronische myeloïde leukemie en gastrointestinale stromale tumoren (GIST). Preliminaire resultaten laten opmerkelijke antitumoractiviteit zien van imatinib in combinatie met hydroxyurea, een ribonucleotide reductaseremmer, in patiënten met terugkerende glioblastoma multiforme. Wij tonen in dit hoofdstuk aan dat P-gp, BCRP en CYP3A4 géén effect hebben op de interactie tussen hydroxyurea en imatinib. Ook hier is meer onderzoek noodzakelijk om het mechanisme van de hoge effectiviteit van dit regime vast te stellen.

Samengevat beschrijft dit proefschrift de rol van ABC geneesmiddeltransporters in de farmacologie van nieuwe en al bekende relevante anti-kankermiddelen. Kennis van de farmacologische rol van ABC geneesmiddeltransporters zal leiden tot een beter begrip van geneesmiddelinteracties. Een verbetering van de behandeling van (kanker)patiënten met geneesmiddelen door strategische toediening van remmers van transporters kan mogelijk ook worden bereikt.

DANKWOORD (AKNOWLEDGEMENTS)

Working on the PhD and on the writing of a thesis is an exciting and educational experience that requires the collaboration of several people. I'm very grateful to everybody who contributed to the researches described in this thesis.

First of all I would like to express my gratitude to my promoters prof. dr. Jan H.M. Schellens and prof. dr. Roberto Mazzanti. Dear Jan, I have no words to thank you for giving me the great opportunity to work in your department first as a student and afterwards as a medical oncologist. It is a genuine pleasure to work under your supervision. You have taught me so many things that it is not possible to summarize them in this short paragraph! Your immense knowledge, your critical thinking, your brilliance, your enthusiasm, your enormous motivation and the passion you have for your work together with your innate attitude towards education and the patience make you an extraordinary source of inspiration and motivation for everybody as well as a magnificent promoter.

Dear Roberto, thank you very much for supporting and guiding me during my internship in Italy to become a medical oncologist first and to achieve my PhD later. You set a great example for me as a motivated researcher, a good clinician, a handy lab expert and a critical thinker. I'm very grateful for all I've learn under your supervision, as well as the several advices, the critical comments, and … I'll never thank you enough for giving me the opportunity to come to the Netherlands during my Italian PhD.

Dear Dick, without your precious assistance and collaboration in the lab this thesis would not have been possible. Thank you!! You are not only an excellent technician but also a funny, kind and very helpful person. I really enjoyed working with you in the lab. Monique and Marja, thank you for teaching me all I know regarding cell culture and related *in vitro* experiments. I'll never forget your kindness and patience too!

I'm also very grateful to all the other co-authors of our papers published or to be published, in particular to prof. Olaf van Tellingen and prof. Jos Beijnen. Many thanks for the critical reading of my manuscripts and the valuable advices! Olaf, thank you also for the precious assistance and help during the *in vivo* experiments.

Dear Jolanda, I have no words to thank you for your continuous support. Your friendly and sympathetic attitude is a gift. You are much more than a trusty assistant: since I met you I've got the pleasant and warm feeling that somebody is ready to take care of me when necessary. Thank you!

Dear Suzanne, you are one of the most talented people I know. Your creativity and versatility is impressive! And…you have a good heart! Thanks for your help and … for the realization of the nice cover of this thesis.

George, thank you for your support, help and motivation during the writing of this thesis. I have so much respect for your perseverance!

Georg and Heleen, thank you very much for your continuous and pleasant support during these years. Heleen, you are a spectacular example of kindness, perseverance and intelligence!

Last but not least, I want to express my special gratitude to my parents and to the rest of my family. Dear babbo and mamma, you are the most important source of motivation in my life! Together with Michela, Leonardo and Matteo you are constantly in my heart! And … dear Andrè, I'll describe you with few attributes: patience, reliability, tenderness, integrity, and flexibility. Many thanks for your support in this last difficult period!

Finally, I want to thank everybody who contributed to the researches described in this thesis and I did not mention due to forgetfulness. Thank you very much! Without your contribution, the accomplishment of this thesis would have never been possible!

CURRICULUM VITAE (DUTCH)

Serena Marchetti werd op 9 juni 1974 geboren in Pescia, Italie. Zij behaalde haar middelbare school eindexamen cum laude in 1993 op het Liceo Gymnasium Niccolò Forteguerri in Pistoia, Italie. Vervolgens begon zij de studie Geneeskunde aan de Universiteit Florence (Italie). Zij studeerde cum laude af in 1999 als basisarts met een afstudeerscriptie getiteld "Cancer in the elderly: experience of the Department of Radiotherapy of the Florence University in the treatment of breast cancer patients ≥70 years old".
Aan het einde van dat jaar begon zij aan de Universiteit Florence met haar opleiding inwendige geneeskunde & medische oncologie. In 2003 studeerde zij af aan de Universiteit Florence met een afstudeerscriptie getiteld "18-FDG-PET in gynaecological cancers: first experience in ovarian cancer".
Aan het einde van 2003 begon zij een PhD promotieonderzoek in klinische en experimentele oncologie aan de Universiteit Florence onder leiding van prof. dr. R. Mazzanti. Gedurende deze periode volgde zij een wetenschappelijke stage van ongeveer een jaar in het laboratorium van prof. dr. J.H.M. Schellens op de afdeling Experimentele Therapie van het Nederlands Kanker Instituut. In 2007 verkreeg zij in Italië de PhD graad met een proefschrift getiteld "Affinity of novel anticancer drugs for ABC-transporters (P-gp, BCP, MRP2): potential clinical implications". Hierna startte zij als internist bij de spoedeisende hulpafdeling van het Del Ceppo ziekenhuis in Pistoia (Italie) en vervolgens als medisch oncoloog in het Campo di Marte ziekenhuis in Lucca (Italie).
Sinds mei 2008 werkt Serena als medisch oncoloog en staflid van de afdeling Klinische Farmacologie van het Nederlands Kanker Instituut in Amsterdam. Tevens is zij werkzaam voor het Nederlands College ter beoordeling van Geneesmiddelen (CBG) als klinisch beoordelaar voor de registratie van nieuwe anti-kankermiddelen. Sinds 2012 is zij lid van de Oncology Working Party (ONCWP) van het European Medicines Agency (EMA).
Het onderzoek beschreven in dit proefschrift werd uitgevoerd op de afdelingen Experimentele Therapie en Klinische Farmacologie van het Nederlands Kanker Instituut/ Antoni van Leeuwenhoek ziekenhuis (NKI/AvL) onder leiding van prof. dr. J.H.M. Schellens en prof. dr. R. Mazzanti.

CURRICULUM VITAE (ENGLISH)

Serena Marchetti was born on June 09, 1974 in Pescia (Italy). In 1993 she completed her final secondary school exam cum laude at the Liceo Gymnasium Niccolò Forteguerri in Pistoia (Italy). Afterwards, she started the study of Medicine at the University of Florence (Italy). She graduated cum laude in 1999 as MD with a thesis entitled "Cancer in the elderly: experience of the Department of Radiotherapy of the Florence University in the treatment of breast cancer patients ≥70 years old".

At the end of the same year she started her fellowship internal medicine & medical oncology at the University of Florence. In 2003 she graduated at the University of Florence as medical oncologist with a thesis entitled "18-FDG-PET in gynaecological cancers: first experience in ovarian cancer".

At the end of 2003 she began a PhD project in Clinical and Experimental Oncology at the Florence University under the supervision of prof. dr. R. Mazzanti. During this period she spent about one year at the Department of Experimental Therapy of the Netherlands Cancer Institute, in Amsterdam, in the laboratory of prof. dr. J.H.M. Schellens. In 2007 she achieved her PhD degree with a thesis entitled "Affinity of novel anticancer drugs for ABC-transporters (P-gp, BCP, MRP2): potential clinical implications". Afterwards she worked as MD at the emergency department of the Del Ceppo hospital in Pistoia (Italy) and subsequently as medical oncologist at the department of medical oncology of the Campo di Marte hospital in Lucca (Italy).

Since May 2008 Serena is employed as medical oncologist and staff member of the Department of Clinical Pharmacology of the Netherlands Cancer Institute in Amsterdam. She also works for the Dutch Medicines Evaluation Board as clinical assessor for the registration of anticancer drugs. Since 2012 she is member of the European Medicines Agency (EMA) Oncology Working Party (ONCWP).

The work described in this thesis was performed at the Department of Experimental Therapy and Clinical Pharmacology of the Netherlands Cancer Institute, under supervision of prof. dr. J.H.M Schellens and prof. dr. R. Mazzanti.

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1: Mazzanti R, Arena U, Pantaleo P, Antonuzzo L, Cipriani G, Neri B, Giordano C, Lanini F, **Marchetti S**, Gentilini P. Survival and prognostic factors in patients with hepatocellular carcinoma treated by percutaneous ethanol injection: a 10-year experience. Can J Gastroenterol. 2004 Oct;18(10):611-8.

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3: Oostendorp RL, **Marchetti S**, Beijnen JH, Mazzanti R, Schellens JHM. The effect of hydroxyurea on P-glycoprotein/BCRP-mediated transport and CYP3A metabolism of imatinib mesylate. Cancer Chemother Pharmacol. 2007; 59: 855-60.

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