ABC-transporters in the pig

ABC-transporters in het varken

(met een samenvatting in het Nederlands)

Proefschrift

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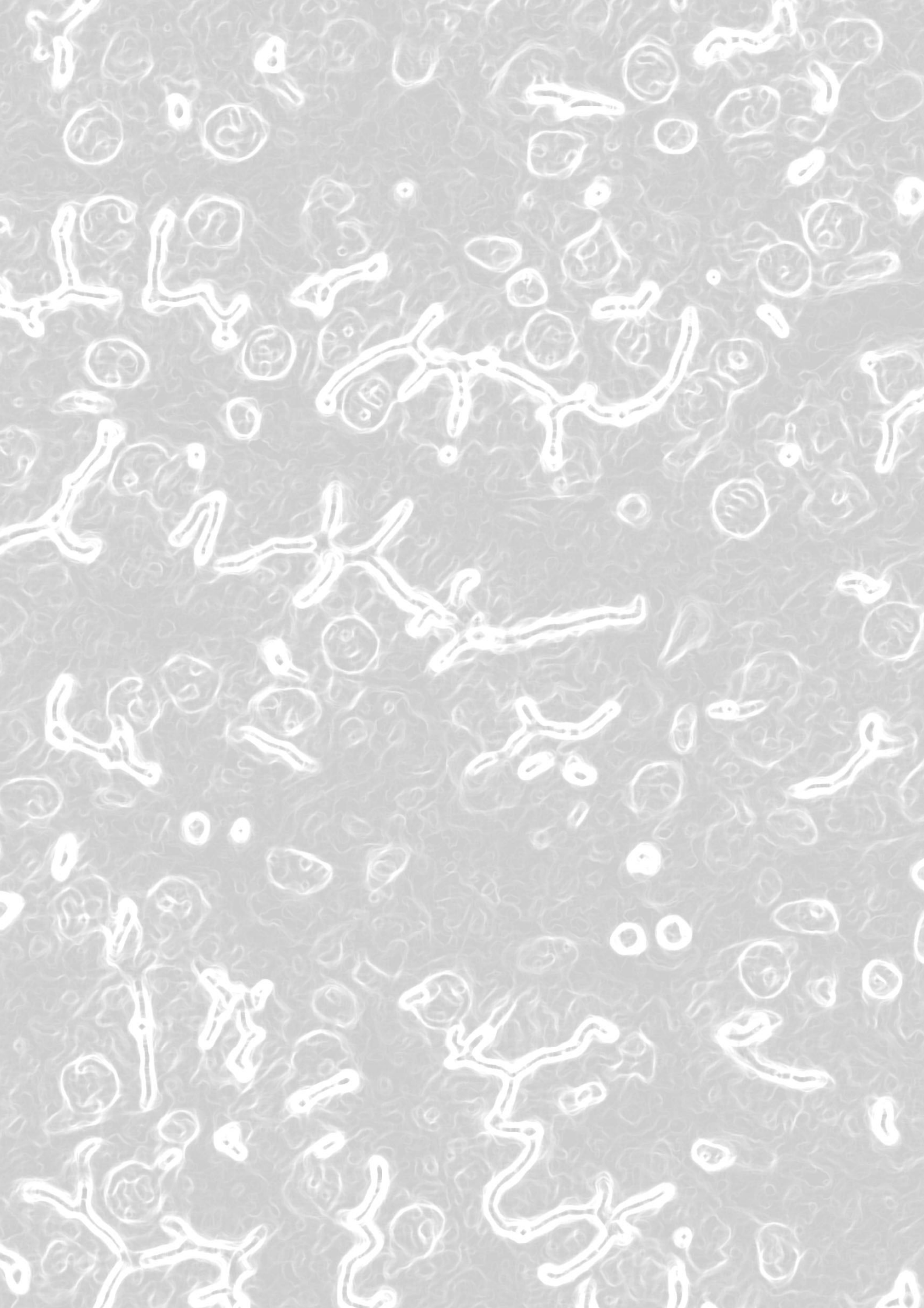

CHAPTER 1

General introduction

1. Introduction

The efficacy of any drug depends on its intrinsic activity in the modulation of the target structure that can be a receptor, ion channel or enzyme (pharmacodynamics) and its tissue distribution and residence time in the body (pharmacokinetics). This implies that a substance can be highly effective in an in vitro system, but without a practical value in a clinical setting, only because it can not reach its target in the body. The pharmacodynamic and pharmacokinetic properties of a drug are therefore equally important and need to be assessed during the development of new molecules, and in the assessment and comparison of existing medicinal products

The pharmacokinetic properties of a drug are largely influenced by its ability to cross biological membranes to reach the target organ or structure, and its residence time in the organisms that is determined by its resistance to rapid metabolic inactivation and excretion. Physicochemical characteristics of a drug, such as molecular size, lipophilicity and pKa (degree of ionisation under physiological conditions) drive the passive membrane permeation. These parameters also largely determine the affinity of a given compound to membrane-spanning transport proteins, whereas the absolute structural configuration determines its biotransformation pathways.

During the last decades, inter-individual differences and genetic polymorphisms in drug metabolising enzymes have been considered as a major cause of unexpected adverse drug reactions (Guengerich, 2003). These drug biotransformation processes are generally grouped into Phase I and Phase II reactions. Phase I reactions involve molecular changes such as oxidation, reduction and hydrolysis and are to a large extend facilitated by the cytochrome P450 enzyme system. Phase II reactions enzymatically conjugate the parent drug or its metabolite(s) to glucuronic acid, glutathione, sulphate or amino acids, increasing the hydrophilicity and in turn the excretability. In some cases glutathione conjugates are further processed in the kidneys via the β-lyase pathway, and this phase had been denoted Phase III metabolism. However, more recently the term Phase III is being drawn on excretory mechanisms at the cellular level. The fact that conjugated metabolites have a higher water solubility introduced the question how for example hepatocytes excrete these metabolites into the biliary ducts. Detailed investigations revealed that specific transmembrane proteins, belonging to the class of ATP-Binding Cassette (ABC)-transporters, facilitate this excretory permeability. In the hepatocyte, common transcription factors such as PXR, CAR and LXR (co-) regulate both, the expression of many CYP450 isozymes as well as the expression of these efflux transporters indicating the linkage between both systems.

The ATP-Binding Cassette (ABC) comprise transporting ATP-ases, which are integrated in the cellular membrane but also in the membranes of intracellular compartments. Depending on their subcellular location, these proteins are importing substrates into the cell, or efflux substrates out of the cell into the extracellular space, independent of a concentration gradient.

To date, 49 transporters have been identified in humans, phylogenetically allocated to 7 subfamilies, denoted ABCA-ABCG (http://nutrigene.4t.com/humanabc.htm). These ABC transporters play an important role in diverse physiological processes (for review see Borst and Elferink, 2002) and various inherited or acquired human diseases, such as the Dubin-Johnson syndrome, cystic fibrosis, Tangier disease, immune deficiency, Pseudo-Xanthoma Elasticum and several forms of cholestasis (http://nutrigene.4t.com/humanabc.htm). Some of the ABC transporters are highly substrate specific, recognizing a limited number of physiological substrates, whereas others accept divers compounds as substrates, including drugs, toxins and plant metabolites that are present in the daily diet.

Three ABC-transporters, P-gp, BCRP and MRP2 are localized in the luminal membrane of secretory and barrier cells, and recognize a wide range of drugs and metabolites as substrates, transporting these towards the extra-cellular compartment. This subcellular localization and their specific expression-pattern in tissues explain their significant impact in drug disposition.

2. Specific efflux transporters involved in drug transport

2.1. P-gp

P-gp, encoded by the ABCB1 gene, was the first mammalian member of ABC proteins discovered by Juliano and Ling (1976). Initially, a decreased drug permeability was noticed in Chinese hamster ovary cells, displaying resistance to colchicines and a wide range of ampiphilic, but otherwise unrelated chemotherapeutic agents. The decreased sensitivity was associated with the expression of a 170 kDa protein, and hence named Permeability glycoprotein (P-gp). P-gp was subsequently referred to as Multi-Drug Resistance protein 1 (MDR1) in the field of cancer therapy, as it confers resistance of cancer cells to various structurally unrelated cytostatic agents. Studies on the expression of P-gp in normal tissues indicated a substantial expression in the adrenal gland, intestines, liver, pancreas and kidney of rodents as well as humans (Fojo *et al.*, 1987; Thiebaut *et al.*, 1987; Cordon-Cardo *et al.*, 1990). P-gp was found at high levels in the apical surface of epithelial cells, and on the (biliary) canalicular surface of hepatocytes. However, despite this subcellular localization and the suggestion that P-gp plays a role in drug elimination (Thiebaut, 1987), its importance for pharmacokinetics of compounds other than cytostatics remained unrecognised for many years. It was only in 1994 that its role as an important factor in drug disposition became evident, when a colony of *mdr1a* (-/-) knock-out mice needed to be treated for a mite infection with the endectocidal drug ivermectin. Most of the knock-out mice died, while the wild-type animals remained unaffected by the given (standard) therapy. The observed ivermectin toxicity (ivermectin affects the function of GABA-receptors in the central nervous system) resulted from an increased permeability of the blood-brain barrier, and this incident clearly demonstrated that not only cytostatic agents, but also other therapeutics are substrates for P-gp. In the follow-up of this incident it was shown that the mice had an overall increased

accumulation of ivermectin in tissues including the central nervous system (Schinkel *et al.*, 1994).
The discovery of the functions of P-gp was a milestone in the understanding of ABC transporters in drug pharmacokinetics. Its physiological role, however, is yet not fully understood. Physiological substrates for P-gp are steroid hormones including cortisol and aldosterone (Ueda *et al.*, 1992), and various studies have suggested a role of P-gp in the transport of lipids (van Helvoort *et al.*, 1996; for review see Pohl *et al.*, 2005), cytokines (Drach *et al.*, 1996). Moreover, P-gp seems to contribute to NK-mediated cytolysis (Klimecki *et al.*, 1995), migration of antigen-presenting dendritic cells (Randolph *et al.*, 1998), proliferation and differentiation of stem cells, inhibition of T-cell activation, and inhibition of apoptosis (reviewed by Johnstone *et al.*, 2000).

Forthcoming studies with the aim to explain multi-drug resistance in cancer cells and the advances in genomic analyses lead to the discovery of other efflux transporters, different from P-gp. Closely related transporters were cloned from hamster cell lines, termed mdr2 or abcb1b (Gros *et al.*, 1986; Van der Bliek *et al.*, 1986; Jongsma *et al.*, 1987), and from the human liver, initially designated MDR3 or ABCB4 (Van der Bliek *et al.*, 1987). Later MRP1 or Multi-drug Resistance associated Protein 1 (Cole *et al.*, 1992) and related transporters, including MRP2, were described.

2.2. MRP2

MRP2 was formerly called canalicular Multi-specific Organic Anion Transporter (cMOAT), but is now classified as a product of the ABCC2 gene (Mayer *et al.*, 1995; Buchler *et al.*, 1996; Paulusma *et al.*, 1996). cMOAT was originally identified in mutant rat strains deficient for the excretion of bilirubin-glucuronides and other organic anions (Oude Elferink *et al.*, 1990). An inherited disease, the human Dubin-Johnson syndrome, characterized by persistent hyperbilirubinemia, was found to be associated with the absence of cMOAT/MRP2 (for review see Konig *et al.*, 1999).
Next to bilirubin-glucuronides (and other conjugates of glucuronic acid including conjugated estradiol-17β-glucuronosyl estradiol), the oxidized form of glutathione, GSSG, is a substrate for MRP2. The efflux transport mediated by MRP2 thus reduces the level of the electrophilic agent GSSG in hepatocytes and other cells (Leier 1996). On the contrary, many substrates require a co-transport with GSH to be excretable by MRP2. In summary, while its role in multi-drug resistant cancer cells is only modest, MRP2 contributes significantly to the excretion of endogenous conjugated metabolites, and of xenobiotics and their conjugates localized in the hepatocyte canalicular membrane and at the luminal membrane of renal proximal tubule cells. Prominent examples for endogenous substrates of MRP2 are the leukotriene C_4 (LTC_4), as well as bilirubin and estrogen glucuronides as mentioned above. Exogenous substrates are glucuronide conjugates of divers drugs and toxins, as well as some unconjugated drugs such as vinblastine (GSH dependent), cisplatin, and a number of fluoroquinolones.

2.3. BCRP

BCRP, the ABCG2 gene product, is one of the latest discovered members of the ABC-transporter family (Allikmets *et al.*, 1998; Doyle *et al.*, 1998; Miyake *et al.*, 1999) and again its expression was first associated with resistance to chemotherapeutics used in breast cancer patients, resulting in the name breast cancer resistance protein (Allen *et al.*, 1999). At the same time the gene was characterized in the human placenta (Allikmets, 1998) and it appeared that BCRP plays an important role in the protection of the fetus preventing the trans-placental passage of drugs and toxins. BCRP is a so-called half transporter with six membrane-spanning domains and requires homodimerization for its function in contrast to P-gp that consists of twelve membrane-spanning domains. Its expression has been demonstrated in nearly all organs (Doyle, 1998), where it acts similar to P-gp as part of tissue-barriers, limiting the penetration into the brains and the foetus, and the absorption of toxins from the intestinal lumen. The physiological role of BCRP is yet not fully elucidated and only a few endogenous substrates such as heme and other porphyrins were recognized as substrates (Krishnamurthy *et al.*, 2004) and it has been suggested that BCRP plays a role in cell survival under hypoxic conditions by reducing intracellular concentrations of toxic heme metabolites (Krishnamurthy, 2004). Other endogenous substrates of BCRP are folic acid (Chen *et al.*, 2003) and sulphated estrogens (Imai *et al.*, 2003). With the aim to study the function of BCRP *in vivo*, a *bcrp* knock out mouse was developed by The National Cancer Institute (NKI) in the Netherlands, and again accidentally, an unintended modification of the diet with an increase in dietary chlorophyll leading to a skin phototoxicity in the knock-out mice, apparently associated with an increased absorption and cellular accumulation of a chlorophyll degradation product, pheophorbide a (Jonker *et al.*, 2002). However, as yet, little data is available about the clinical relevance of BCRP in the disposition of individual classes of drugs, but the list of BCRP substrates is currently increasing rapidly.

3. ABC-transporters in drug absorption and disposition

The relevance of the individual transporters in drug disposition dependents on its tissue distribution and substrate recognition. The organ-specific and subcellular distribution of ABC transporters has been evaluated by immunohistochemistry mainly in human tissues (Thiebaut, 1987; Cordon-Cardo, 1990; Konig, 1999/ review ; Maliepaard *et al.*, 2001; Scheffer *et al.*, 2002; Campbell *et al.*, 2003; Fetsch *et al.*, 2006). However, pharmacokinetic studies were mostly done in laboratory animals, comprising also experiments in knock-out mice. Substrate identification studies used *in vitro* systems with defined cell cultures, such as Caco-2 cell monolayers as described below. These studies indicated that P-gp and BCRP have a wide and overlapping tissue distribution affecting drug absorption, distribution and elimination, whereas MRP2 was found to be the most important transporter for the excretion of conjugated drugs into bile and urine.

3.1 Modulation of drug absorption by ABC-transporters

The ABC-transporters P-gp, BCRP and MRP2 are situated in the apical membrane of intestinal epithelial cells mediating the efflux of their substrates that counteract the intestinal absorption. Although the intestinal absorption of drugs and xenobiotics dependents on a wide range of factors, an impaired absorption was often observed for P-gp substrates (Sparreboom *et al.*, 1997; Kim *et al.*, 1998; Greiner *et al.*, 1999; Kruijtzer *et al.*, 2002; Yamaguchi *et al.*, 2002; Adachi *et al.*, 2003) as well as in some cases for BCRP substrates (Jonker *et al.*, 2000; Jonker, 2002; van Herwaarden *et al.*, 2003; Sesink *et al.*, 2005; Zaher *et al.*, 2006). Moreover, the unidirectional transport to the luminal surface (exsorption or desorption) has been recognized as an additional route for drug-elimination, competing with the excretion into the bile and urine, and depending on the expression and activity of ABC-efflux transporters (Mayer *et al.*, 1996).

The barrier function of the intestinal wall, i.e. the prevention of the absorption of (bacterial) toxins and xenobiotics (including drugs) thus seems to be associated with the expression of P-gp. In addition to the expression of P-gp, enterocytes are generally rich in CYP3A enzyme activity (Kolars *et al.*, 1994), although the activity is still lower than that of hepatic CYP3A4. Cellular uptake of toxins and drugs and subsequent efflux by P-gp results in repeated exposure of substrates to CYP 3A4 in enterocytes and hence in many cases an increased rate of bio-inactivation (pre-systemic elimination) (Wacher *et al.*, 1995; Kirn *et al.*, 1999). P-gp efflux enhances in this way the intestinal biotransformation capacity of CYP 3A4, whilst accumulating evidence suggest that P-gp mediated efflux decreases metabolism by CYP3A4 in the liver (Benet *et al.*, 2004).

3.2. Excretion

3.2.1. Intestinal elimination

The role for the intestines with a high expression of P-gp as an excretory organ has previously been demonstrated in *mdr 1a/1b* knock-out mice. A pronounced decrease in intestinal secretion of various P-gp substrates, including among others digoxin, was observed in these animals as compared to the corresponding wild-type strain (Schinkel *et al.*, 1997). In humans, intestinal perfusion experiments demonstrated that the enteral elimination of digoxin, administered IV, was decreased in the presence of quinidine (Drescher *et al.*, 2003). The role of P-gp in intestinal elimination of ivermectin (an important veterinary drug) was demonstrated in an experiment with rats. The intestinal elimination of systemically applied ivermectin appeared to be five fold higher than the elimination via the bile, and co-administration of verapamil, an known inhibitor of P-gp, markedly decreased the intestinal secretion (Laffont *et al.*, 2002). It is highly suggestive that also MRP2 and BCRP contribute to intestinal elimination, but specific data are limited (Dietrich *et al.*, 2001; van Herwaarden, 2003).

3.2.2. Renal excretion

The kidneys are well equipped for the excretion of compounds into the urine (for review see Chandra and Brouwer, 2004). Various transporters belonging to the SLC-superfamily are present in the basolateral and apical membrane of the tubule cells along the nephron and contribute to cellular uptake and efflux (van Montfoort *et al.*, 2003). With regard to ABC-transporters, is appears that MRP2 is highly expressed in the proximal tubule cells of various animal species. Its functional characterization at this specific location, however, is rather limited (van de Water *et al.*, 2005). Renal transporters have to take over the excretory capacity of liver cells in cases of an impaired function of these transporters, for example in cases of cholestasis, as demonstrated for MRP2 in rats (Tanaka *et al.*, 2002). The role of P-gp and BCRP in renal excretion seems to be limited: some reports suggested a role for P-gp (Kazuyo Nishihara, 1999; Shimizu *et al.*, 2004), while others deny this (Hedman *et al.*, 1991; Smit *et al.*, 1998; Kageyama *et al.*, 2006). BCRP is highly expressed in the kidneys of rodents and seems functionally involved in the urinary excretion of its substrates (Herwaarden *et al.*, 2006), and particular in the excretion of sulphated conjugates in mice (Mizuno *et al.*, 2004). In contrast, the expression of BCRP in the human kidney is apparently very low, as it could be detected only by RT-PCR analysis and not by immunological methods, moreover no data are available about its functional role in mechanisms of renal excretion.

3.2.3. Biliary elimination

The liver is still considered to be the major detoxifying organ, despite the increasing recognition of the pre-systemic elimination of drugs and toxins in the intestines, outlined above. Parenchymal cells (hepatocytes) are specialized in the uptake, metabolism and excretion of endo- and xenobiotics. Phase I and phase II metabolizing enzymes are highly expressed in the liver and form sequential elements in the detoxification process prior to active excretion of the drug and metabolites. The vectorial transport from the blood into the bile dependents on transporters located in the sinusoidal (basolateral) membrane and in the apical (canalicular) membrane. Drug-transporters in the basolateral membrane are mainly members of the SLC family (SLC, solute carrier such as OATP, OAT and OCT), while members of the ABC-transporter family, including ABCB1, ABCB4, ABCC2 and ABCG2, facilitate the canalicular drug transport. Several members of the ABCC-family in the basolateral membrane transport also substrates out of the hepatocytes back into the sinusoidal blood stream.

P-gp is a major canalicular transporter for neutral and cationic substances (Hiroyuki Kusuhara, 1998; Smit, 1998), with a limited role in the excretion of conjugates. The overlap in substrate affinity of both, human CYP3A4 and P-gp, seems to ensure that the compounds that were not metabolized by CYP3A4 are excreted into the bile as well, while inhibition of hepatic P-gp increases the intracellular residence time of these compounds and results in an increased metabolism. Human MDR3 (ABCB4) is related to P-gp (MDR1) and shows a certain degree of overlap in substrate affinity. Its overall transport affinity, however, is much lower that that of P-gp (Smith 2000). MRP2 transports some organic anions into the bile (such as for example as

for ampicillin and ceftriazone), and is a major transporter of phase II conjugates (Oude Elferink *et al.*, 1995; Hiroyuki Kusuhara, 1998; Konig, 1999; Dietrich, 2001). The role of BCRP in hepatic xenobiotic excretion is still unclear. Although various substrates of BCRP are excreted into the bile (van Herwaarden, 2003; Hirano *et al.*, 2005; Merino *et al.*, 2005), this transport could as yet been demonstrated only in mice. As mice show a relatively high expression of BCRP in the liver comparable to that in the jejunum and ileum, these findings may not be representative for other animal species.

Phase II metabolites, such as glucuronides, sulfates and glutathione conjugates are generally too hydrophilic to leave the hepatocyte by passive diffusion. Therefore carrier-mediated processes are required to transport these conjugates across either the basolateral or the apical membrane. Although members of the SLC-family in the basolateral membrane in principle could facilitate a bi-directional transport, their dependence on ion exchange (here GSH and α-ketoglutarate) explains that the mainly act as uptake transporter in hepatocytes. In contrast, members of the ABCC-subfamily allow the active secretion of phase II conjugates into the sinusoïdal blood stream that is limited under physiological conditions, while under pathological conditions, such as cholestasis, their expression is increased and thus contribute to the secretion of conjugated metabolites into the sinusoidal and systemic circulation. The major transporter involved in the biliary excretion of conjugates is MRP2, as mentioned before. But various conjugates are also substrates for BCRP (Suzuki *et al.*, 2003; Adachi *et al.*, 2005; Zamek-Gliszczynski *et al.*, 2005), and hence BCRP transport may potentially act as an alternative route for their secretion.

3.3. Blood-tissue barriers

An additional, but prominent role of transporters in the disposition of drugs is their role as essential part of biological barriers, such as the blood-brain barrier. In contrast to endothelial cells in other organs, the capillary endothelial cells in the central nervous system have tight junctions and a continuous basal membrane, thus preventing paracellular passage of drugs or toxins. The prominent role of P-gp in the function of the blood-brain barrier was first recognized with the penetration of ivermectin (and later digoxin) into the central nervous system in *mdr1* (-/-) mice (Schinkel, 1994; Schinkel *et al.*, 1995), and genetic defects in the MDR1 gene have been found to explain the increases sensitivity of certain dog breeds, towards ivermectin. While for many years, P-gp was reviewed only as protective transporter, preventing the entrance of potentially neurotoxic compounds into the central nervous system, recent interest focus on the use of P-gp inhibitors as adjunct drugs to facilitate the transport of pharmacological agents that require for their efficacy high intracerebral concentrations, including anti-epileptics, anti-psychotic drugs and agents used for the treatment of brain cancers (for review see Loscher and Potschka, 2005). Correspondingly, other vulnerable tissues such as the testis are depending on functioning efflux transporters (for review see Fromm, 2004). The foetus is protected via the placental barrier, at which P-gp and BCRP, that are expressed in the cellular membrane of the human placental syncytiotrophoblast cells, prevent

the passage of potentially harmful substrates (for review see Ceckova-Novotna *et al.*). In contrast, in the mammary gland an increased expression of BCRP was found during lactation where it transports substrates into the milk (Jonker *et al.*, 2005; Merino, 2005; Herwaarden, 2006; Merino *et al.*, 2006). Excretion of xenobiotics is associated with a potential risk for the neonate, and in veterinary medicine this mechanism is of specific interest as it is involved in the excretion of drug and contaminants into dairy milk.

Despite the fact that P-gp and BCRP are expressed in many other organs of the body where they fulfil distinct physiological roles, such as for example in the excretion of (steroid) hormones out of the cells, their effect on the disposition of drugs or toxins in these organs has not been investigated in much detail. In this scope, some authors have recently reviewed the current knowledge about ABC-transporters in the lung (van der Deen *et al.*, 2005) and in the heart (Couture *et al.*, 2006; Solbach *et al.*, 2006).

4. Transcriptional co-regulation of biotransformation enzymes and ABC-transporters

Many transporters and enzymes are subject to direct post-transcriptional regulation, enabling very rapid changes in their activity (Sekine *et al.*, 2006). Intermediate and long term changes are achieved by complex transcriptional regulation mechanisms by specific members of the nuclear receptor family of transcription factors.

The xenosensor Pregnane X Receptor (PXR) and the Constitutive activated Androstane Receptor (CAR), are the master regulators in the expression of ABC transporters (and of the major drug metabolizing enzymes). PXR is highly expressed in the liver and moderately in the intestines (Blumberg Sabbagh 1998, Kliewer 1998) and is an activator of P-gp expression (Geick *et al.*, 2001). At the same time it regulates the expression of CY3A4 (Goodwin *et al.*, 1999), CYP2C9 (Chen *et al.*, 2004), CYP2B6 (Goodwin *et al.*, 2001).

Although the PXR orthologues in various species show a sequence identity of more than 95% in the DNA binding domains, the sequence identity for the ligand binding domains is as low as 75%-80% among mammalians, while in the chicken the PXR homologue, CXR, has a sequence identity with human PXR for the ligand binding domain of only 50% and with human CAR of 56%, a level that is comparable to that between mammalian PXR and CAR sequences (Handschin *et al.*, 2000; Moore *et al.*, 2002). The ligands for PXR are chemically and structurally diverse, and include endogenous steroids, xenobiotics such as rifampicin, phenobarbital, phenytoin, clotrimazole, paclitaxel, hyperforin, natural (phytoestrogens), synthetic steroids such as pregnelenone, androstanol, dexamethasone, and bile acids.

CAR is abundantly expressed in the liver, and to a lesser extend in the kidneys and the intestines (Lamba *et al.*, 2004). It is involved in the regulation of P-gp, and controls the

expression CYP2B enzymes (Honkakoski *et al.*, 1998). CAR is constitutively expressed and activates target genes ligand-independently or in a ligand-dependent manner, as for example the 'phenobarbital-type' of CYP inducers. The constitutive expression is negatively regulated by androstane metabolites that dissociate CAR from its co-activator (Forman *et al.*, 1998).

CAR, PXR and also FXR (Farnesoid X receptor), VDR (Vitamine D Receptor) and LXR (liver X Receptor) bind as heterodimers with RXR to overlapping sequences in the promoter regions of ABC-transporters (Kast *et al.*, 2002) and various enzymes involved in biotransformation processes, such as SULT2A1 (Song *et al.*, 2001), UDP-glucuronosyl transferase 1A (UGT1A) (Junko Sugatani, 2001), gluthatione S-transferase A2 (GSTA2) (Falkner *et al.*, 2001). Ligands and their potency for PXR and CAR highly vary among species, such is the case for rifampicin that is a potent inducer of human and rabbit CYP3A by PXR, but does not activate rat and mouse PXR (Jones *et al.*, 2000; Moore *et al.*, 2000). Another example is the activation by PCB's of mouse PXR, but not human PXR (Tabb *et al.*, 2004)

The bile acid sensor FXR, is a regulator of bile acid and lipid metabolism inducing ABCB11 (BSEP), ABCC2 (MRP2) and ABCB4 (MDR3) transcription (Ananthanarayanan *et al.*, 2001; Kast, 2002; Huang *et al.*, 2003), exemplified by a reduced intestinal MRP2 expression during cholestasis (Dietrich *et al.*, 2004). In addition, ligand-activated FXR induces the expression of the inhibitory nuclear receptor small heterodimer partner (SHP). SHP is predominantly expressed in the liver and represses the transcriptional activity of several nuclear receptors including HNF4α (hepatocyte nuclear factor), CAR, LXRα, ER (estrogen receptor), thyroid hormone receptor (TR) and GR (glucocorticoid receptor), by competing with their transcriptional coactivators (Seol *et al.*, 1996; Johansson *et al.*, 1999; Brendel *et al.*, 2002; Bae *et al.*, 2004).

Hepatocyte nuclear factors (HNF1, 3, 4 and 6) regulate the expression of genes for metabolism and transport in the liver either directly or as coregulators. Ligands for HNF's are largely unknown, but fatty acids seems to regulate the function of HNF4α (Wisely *et al.*, 2002; Duda *et al.*, 2004). HNF4α is predominantly expressed in hepatocytes, but was also found in intestinal epithelial cells, pancreatic β-cells and kidney proximal tubule cells. HNF4α directly induces the expression of the UGT1A9 that is expressed in the liver and activates the induction by PXR and CAR (Akihide Kamiya, 2003; Tirona *et al.*, 2003; Barbier *et al.*, 2005).

Other receptors that are likely involved in the regulation of drug metabolism and transport are VDR, GR, the Aryl hydrocarbon Receptor (AhR) and Nuclear factor-erythroid 2 p45- related factor (Nrf2). VDR induces the expression of CYP3A (Thummel *et al.*, 2001) and SULT2A1 (sulfonation of endogenous steroids and sterols) (Chatterjee *et al.*, 2005). GR induces UGT1 (Sugatani *et al.*, 2005), and AhR regulates the induction of the CYP1 family and UGT1A1 (Yueh *et al.*, 2005). Nrf2 is activated by electrophils and recognizes a promoter sequence similar to the antioxidant responsive element (ARE) and induces the expression of phase II detoxifying enzymes including glutathione S-transferases (Ikeda *et al.*, 2002).

As yet, there are no conclusive data about the transcriptional regulation of BCRP. Only some indications that suggest the involvement of HIF-1, AhR, the androgen Receptor and the estrogen receptor (Ebert *et al.*, 2005; Anapolsky *et al.*, 2006; Krishnamurthy and Schuetz, 2006; Szatmari *et al.*, 2006).

5. Drug-drug interactions and inter-individual variation in drug disposition attributable to differential expression of ABC-transporters

Drug-drug interactions leading to inhibition of efflux transporters can lead to an increased rate of exposure and possibly to drug toxicity. An example is the interaction of digoxin with other cardiac drugs, such as verapamil and quinidine, or cyclosporine, resulting in marked increases in digoxin plasma concentrations and the appearance of clinical signs of intoxication (Fromm *et al.*, 1999; Verschraagen *et al.*, 1999; Sachiyo Funakoshi, 2003).
Changes in function or expression of ABC transporters, originating either from genetic variation, including species differences or from physiological, pathological conditions, or from exogenous factors, determine the individual variability in drug disposition and kinetics and subsequently the individual pharmacological response.

As mentioned above, the expression of transporters (and enzymes) is directly or indirectly regulated by endogenous and exogenous factors via transcription factors and co-regulators. Endogenous factors are steroids (including sex hormones), thyroid hormones and cytokines (Miyoshi *et al.*, 2005). Exogenous factors include Herbs, such as St John's Wort (Hennessy *et al.*, 2002), food constituents or supplements that may inhibit or induce transporters resulting in an increased and decreased exposure of drugs, respectively. This accounts in particular for flavonoids as substrates and modulators of multiple ABC-transporters (for reviews see Lin, 2003; Zhou *et al.*, 2004; Morris and Zhang, 2006). Additionally, genetic heterogeneity in drug transporters may affect the disposition and metabolism of drugs (Kerb, 2006; Lamba *et al.*, 2006).

6. ABC-transporters in stem cells and progenitor cells

One of the phenotypic markers for hematopoietic stem cells and progenitor cells is the fact that these cells efflux of the fluorescent dye Hoechst33342. This capacity is used in the isolation of the stem and progenitor cell fraction from tissues by means of FACS sorting. Hoechst33342 is a substrate for P-gp, and the rapid efflux was therefore attributed to a high expression of P-gp. However, the progenitor cells of P-gp knock out mice displayed an unchanged dye efflux that was subsequently attributed to the activity of BCRP in these cells (Zhou *et al.*, 2001). At present, BCRP is considered to be one of the typical phenotypic makers of progenitor cells of many organs (Scharenberg *et al.*, 2002; Islam *et al.*, 2005; Sainz *et al.*, 2006; Umemoto *et al.*, 2006). The physiological relevance of the high expression of this transporter in progenitor cells

is still unclear, but a protection of these cells against toxic heam metabolites, abundantly appearing under hypoxic conditions, has been suggested (Krishnamurthy, 2004).

7. Species differences in the expression of ABC-transporters with emphasis on veterinary target animal species

Although the incident with ivermectin sensitive knock-out mice was suggestive, it was only in 2001 that a defect in the P-gp gene was recognized that explained the ivermectin sensitivity in Collie-dogs (Mealey *et al.*, 2001) and other herding breeds (Neff 2004). Thereafter, an increased brain penetration in the mutant breeds was observed for various chemotherapeutic agents (Mealey *et al.*, 2003), loperamide (Sartor *et al.*, 2004) and doramectin (Geyer 2005).

The expression of P-gp, MRP1 and MRP2 in canine tissues was documented by Conrad *et al.* (2001), and Ma *et al*. (2002), and later MRP2 was functionally characterized (Ninomiya *et al.*, 2004). Functional studies indicated an important difference between species: in contrast to human MRP1, in dogs, mice, rats and cattle, MRP1 failed to confer the resistance to doxorubicin, despite an otherwise high functional similarity (Stride *et al.*, 1997; Ma, 2002; Taguchi *et al.*, 2002; Nunoya *et al.*, 2003). The clinical relevance of this finding relates to the multidrug-resistance phenotypes of canine tumours, and to the penetration of doxorubicin into the central nervous system. A significant species difference in expression was demonstrated also for MRP2, showing a high level of expression in the rat livers, but a relatively low expression in human and canine livers (Ninomiya *et al.*, 2005).

The activity of canine transporters can be estimated from *in vitro* studies using the canine derived kidney epithelial cell line Madin-Darby canine kidney cells (MDCK). MDCK cells transfected with individual ABC transporters are widely used for functional studies in comparison wild type (wt) cells and the use of selective inhibitors. For example it could be shown that the secretory transport of the P-gp substrates paclitaxel, vinblastine and digoxine in the wt-MDCK cells was sensitive for the P-gp inhibitors cyclosporine A, ketoconazole, loperamide, verapamil, nicardipine and quinidine (Taub *et al.*, 2005), suggesting similar characteristics as human P-gp. The feline homologue of P-gp was cloned in 2000 and shown to display a high homology of 90.7% (Okai *et al.*, 2000). This feline P-gp, however, was never functionally characterized. The rabbit homologue to MRP2 has been cloned and to a limited extend characterized (van Kuijck *et al.*, 1997). The P-gp of the chicken was cloned and its expression and function have been partially characterized (Edelmann *et al.*, 1999; Barnes, 2001).

Data about the transporters in other species became available when certain cell types served as a model for the human blood-brain barrier, such as endothelial cells isolated from the capillaries of bovines (Bachmeier and Trickler 2006) or porcine species (Eisenblatter and Galla 2002, Bauer *et al.*, 2003). The expression of BCRP was observed in the human, murine and bovine lactating mammary glands, emphasizing the potential role of these transporters in the

transmission of toxins and drugs into milk, which might comprise a risk for the neonate, but also to the consumer of dairy milk (Jonker, 2005).

8. Common veterinary drugs that are substrates for ABC-transporters

During the last decade is was recognized that ABC-transporters play a crucial role in the effectiveness of various cytostatic drugs, but also in the induction of undesirable site effects of these drugs, when the function of these transporters was modified by co-medication (for review see Leonard *et al.*, 2003). Co-medication involving substrates and/or specific inhibitors of ABC efflux transporters is currently evaluated in human medicine as an aid to overcome transporter-dependent drug resistance, and will offer new strategies in veterinary pharmaco-therapy as well. This applies also to the increasing use of cytostatic agents in dogs, an increased use of chemotherapeutic agents in veterinary patients can be expected the coming years, due to the increasing willingness of pet owners to apply advanced therapies.
Chemotherapeutics that are regularly used in veterinary therapy include anthracyclines (doxorubicin), vinca-alkaloids (vincristine and vinblastine), oxazaphosphorines (cyclophosphamide) and antifolates (methotrexate), all of them being identified as substrates of one or more ABC-transporters in other animal species and in humans.
Other prominent examples of P-gp substrates used as therapeutic agents in veterinary medicine are: ivermectin and the related avermectins moxidectin and selamectin; verapamil, diltiazem, nicardipine, digoxin, dexamethasone, hydrocortisone, prednisolone, domperidone, morphine, fentanyl and loperamide, lidocaine, quinidine, erythromycin, trimethoprim, phenytoin, omeprazol, raninitidine, phenytoin, phenobarbital. Examples of BCRP substrates are enrofloxacin and its metabolite ciprofloxacin, cimetidine, sulfasalazine, and benzimidazoles as well as conjugates and sulphated conjugates of many drugs, including acetaminophen sulphate. Recognized MRP2 substrates are, among others, enalaprilat (enalapril) and acetaminophen glucuronide.

Scope of the thesis:

The presented literature overview suggests an increasing recognition of the role for ABC-transporters also in veterinary therapy. The work presented here, focussed initially on the description of the involvement of ABC transporters in the kinetics of antibiotics and toxins, which are of relevance in pig husbandry. Based on these results, it seemed necessary to gain more insight into the species-specific expression and functionality of porcine ABC transporters.

The first part of the thesis aimed at the characterization of 3 different substances with respect to their role as substrates for one or more ABC transporters. Like in many other studies, for the identification of ABC transporters involved in the transport of these compounds, a standardized Caco-2 cell model was used.

In **Chapter 2**, Oxytetracycline was investigated because this antibiotic plays a prominant role in pig husbandry, as the most widely used therapeutic agents. The fact that many tetracyclines including oxytetracycline are poorly absorbed following oral application (the common application form in pigs), initiated the question to what extent the studies in Caco2 cells would identify means to increase the oral availability by the simultaneous application of competitors or inhibitors of intestinal efflux proteins.

Chapter 3 is devoted to danofloxacin-mesylate, a fluoroquinolone antibiotic licensed exclusively for the use in veterinary medicine. Kinetic studies, in particular those in calves and lactating cows indicated an unusual distribution pattern after parenteral injection, with unexpected high tissue levels in lungs and the intestinal lumen. It was hypothesized that this distribution pattern might be related to an active secretion of danofloxacin-mesylate into the luminal space of the lungs and into the intestines. Hence it was the aim of this study, to identify the transporters involved in this excretory process, as this knowledge could support the use (and licensing) of danofloxacin-mesylate in other animal species, including the pig.

Chapter 4 aimed to identify whether or not the isocoumarin ochratoxine A (OTA) is a substrate for ABC-transporters as well. The mycotoxin OTA is a common undesirable contaminant of animal feeds, particularly of feeds for pigs and poultry. Moreover, toxicological studies suggested that the pig is the most sensitive animal species in terms of toxin-induced renal damage, which was attributed to its accumulation in proximal tubule cells. Hence, the factors involved in renal excretion and re-absorption are of interest, to understand the bioaccumulation.

When the results obtained in Chapters 2, 3 and 4 where discussed in respect of their consequences for pigs, it became evident that the lack of knowledge regarding the expression of ABC-transporters in pigs was at that time too limited to draw any conclusions about species-specific features of the selected compounds in pigs. Hence the second part of the thesis was

devoted to a structural and functional characterization of ABC-transporters in pigs. The expression of mRNA (Real-time PCR analyses) in various organs of pigs, are presented in **Chapter 5** and **Chapter 6**. In addition, immuno-histochemical studies on the subcellular location of these transporters are presented. The results presented in these chapters demonstrate that the ABC-transporters ABCB1 (p-gp), ABCC2 (MRP2) and ABCG2 (BCRP) are expressed in pigs with an organ distribution that resembles many characteristics of the expression in human tissues. Moreover, sequence analyses were conducted, suggesting a high homology between the selected porcine ABC-transporters and their human counterparts, which suggest that the pig is also an interesting model species for studies devoted to the implication of ABC-transporters in (liver) diseases.

Finally, a convenient screening method to assess the functionality of transporters, particularly P-gp, in porcine peripheral blood lymphocytes was established to allow rapid *ex vivo* testing of a number of specific substrates and inhibitors (**Chapter 8**). This method might be helpful in the assessment of new and old pharmaceuticals, and the characterization of toxins emerging in pig husbandry.

These original chapters are preceded by a **General introduction** and results are put into a broader context in the **General discussion**.

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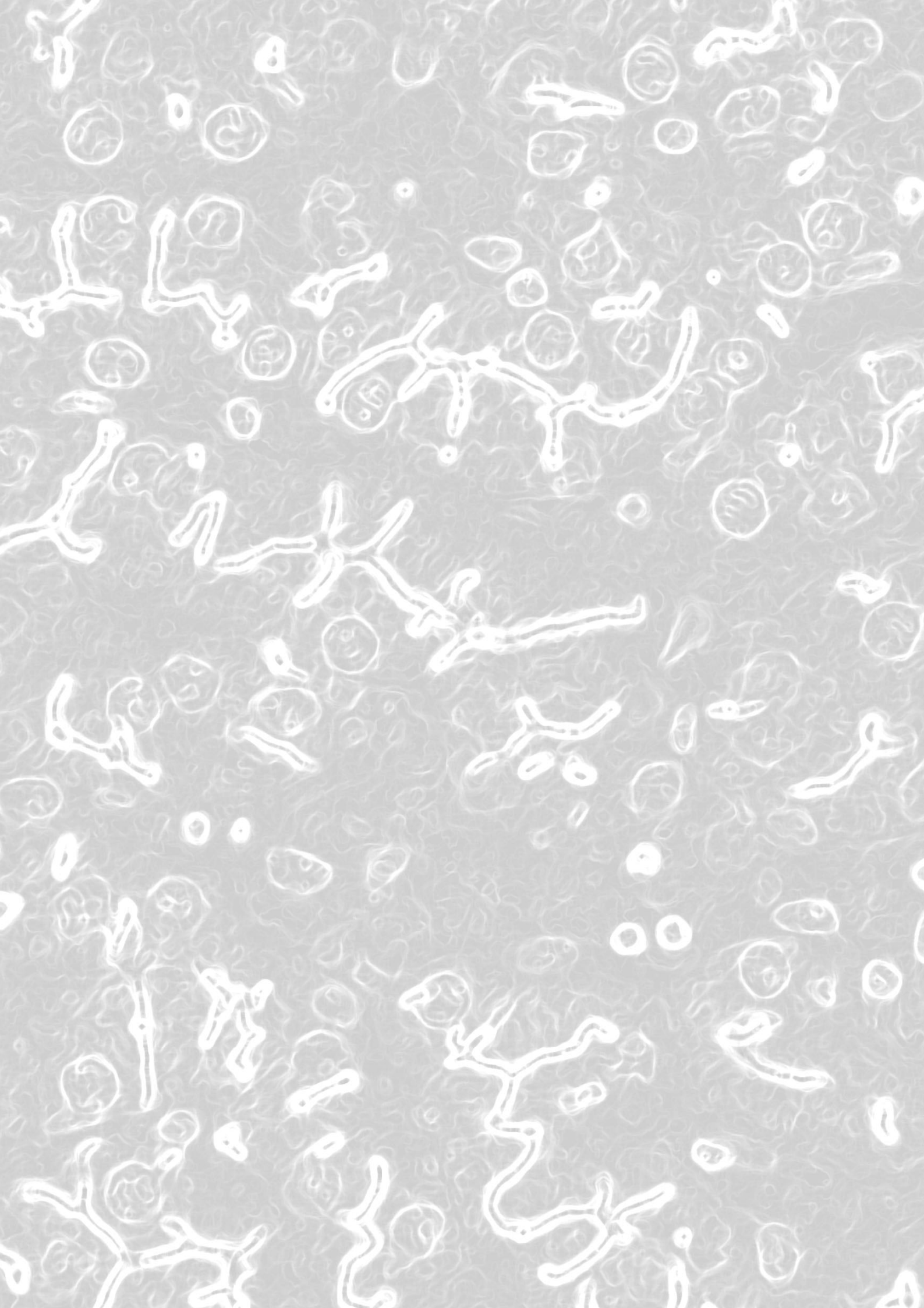

CHAPTER 2

P-glycoprotein mediated transport of oxytetracycline in the Caco-2 cell model.

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Abstract

ATP-dependent drug transporters such as P-gp (P-glycoprotein), MRP-2 (multi-drug resistance associated protein) and BCRP (breast cancer resistant protein) are expressed at the brush border membrane of enterocytes. These efflux transporters excrete their substrates, among them various classes of antibiotics, into the lumen thus reducing net absorption as indicated by a low bioavailability after oral administration. Oxytetracycline (OTC) has been used for decennia in veterinary medicine due to its extensive spectrum of antimicrobial activity. A major limitation has been, and still remains, its low bioavailability following oral administration. The present study aimed to investigate to what extent this low bioavailability is attributable to the fact that OTC is a substrate for one or more efflux transporters. As an experimental model to study the transmembrane transport of OTC, differentiated Caco-2 cells grown as monolayers on permeable supports were used. With this model it was shown that the secretion of OTC is slightly higher than its absorption. PSC833, a potent inhibitor of P-gp, decreased the secretion of OTC without affecting its absorption, while the MRP-inhibitor MK571 did not exert any effect. These data indicate that OTC is a substrate for P-gp and possibly for BCRP. The affinity of OTC to these transporters seems to be rather low, as suggested by the low efflux ratio of 1:1.3. In competition experiments, OTC decreased the effluxes of other P-gp substrates such as Rhodamine 123 and ivermectin. These findings are of clinical relevance, as they clearly indicate potential drug-drug interactions at the level of P-gp mediated drug transport.

Introduction

Efflux transporters are a family of ATP-dependent transport proteins (ABC transporters) that are located in the body at typical sites of substrate elimination, as for example, in the canalicular membrane of hepatocytes, in renal tubular cells, as well as at biological barriers, such as the blood-brain barriers and also the intestinal barrier. Major representatives of the ATP-dependent efflux transporters are P-gp (P-glycoprotein, the MDR1 gene product also classified as ABCB1), MRP2 (multi-drug resistance associated protein, denoted ABCC2) and BCRP (breast cancer resistance protein, denoted ABCG2). The popular abbreviations refer to the initial findings, when multi-drug resistance to cytostatic agents was associated with the upregulation of these efflux transporters (Goldstein *et al.*, 1992; Leonard *et al.*, 2003). More recently, the superfamily of ABC transporters has been reclassified into distinct gene families (Kaminski et al., 2006) and an increasing number of commonly used drugs have been found to be a substrate for one or more members of the ATP–dependent transporters (Schinkel and Jonker, 2003; Mealey, 2004).

P-gp plays a specific role in drug absorption, disposition and elimination as indicated by its tissue distribution and cellular localization. For example, high concentrations of P-gp can be found in the brush borders of epithelial cells in the intestinal tract, limiting absorption after oral administration and facilitating secretion into the intestinal lumen. In the liver, P-gp is found in the canalicular membrane of hepatocytes, facilitating drug excretion into the bile, and in the kidneys the expression of P-pg at the apical surfaces of epithelial cells may supports renal elimination of drugs. At physiological barriers such as the blood-brain barrier or blood-placenta barrier, P-gp prevents the passage of drugs into these vulnerable tissues (Leslie *et al.*, 2005)

Among the drugs that are substrates for P-gp are various classes of antibiotics, including fluoroquinolones as well as tetracyclines. Oxytetracycline (OTC), a congener of tetracycline, is a commonly used broad-spectrum antimicrobial agent with bacteriostatic activity. In veterinary medicine, typical indications are the treatment and prophylaxis of intestinal and respiratory tract infections in a variety of animal species, including poultry and pigs. Absorption of OTC from the gastro-intestinal tract is incomplete, with major differences between species. In turkeys the oral bioavailability is as low as 9.4% in fed animals, increasing to 47.6% in fasted animals (Dyer, 1989). In pigs the oral bioavailability of oxytetracycline varies between 3% and 9% (Mevius et al., 1986; Pijpers et al., 1991; Nielsen and Gyrd-Hansen, 1996), with no significant differences between fasted and fed animals. As a result, high oral dosages are needed to obtain effective plasma concentrations for the treatment of systemic infections, and a considerable fraction of the administered dose is excreted unchanged in the faeces. Subsequently, tetracyclines have been detected in manure and in the soil, affecting the natural soil microbial community and essential decaying processes (Halling-Sorensen et al., 2002; Hamscher et al., 2002; Thiele-Bruhn, 2003). Moreover, resistance genes for tetracyclines have been found in soil microorganisms and may contribute to the overall spread of these resistance

genes (Esiobu et al., 2002; Thiele-Bruhn, 2003). These findings initiated a critical reappraisal of the veterinary use of tetracyclines and stimulated investigations to understand and possibly improve the bioavailability of commonly used tetracyclines after oral administration.

Previous data suggests that the parent compound tetracycline is a substrate for P-gp (Grandi and Giuliani, 1988; Kavallaris M. et al., 1993; George et al., 1996). The low oral bioavailability of OTC observed in clinical studies in pigs and poultry poses the question to what extent P-gp expression in the intestines accounts for its limited absorption, as this would offer the possibility to improve the oral availability by competitive inhibitors, many of which are present in natural feed materials (Bergmann et al., 2005; Nishimura et al., 2005; Ofer et al., 2005). An established model to investigate the mechanisms involved in the bioavailability after oral administration is the Caco-2 cell line, derived from a human colon carcinoma. Grown as confluent monolayers, Caco-2 cells form tight junctions, and thus resemble the intestinal barrier (Calcagno et al., 2006). Depending on the chemical characteristics of the compound under consideration, passive diffusion and active transport, contributing to absorption and secretion through a Caco-2 cell monolayer, can be measured (Yamashita et al., 1997; Artursson et al., 2001).
The aim of the present study was to evaluate the potential role of P-gp in the absorption of oxytetracycline. In addition, potential interactions between OTC and other substrates of P-gp were studied.

Materials and methods

Materials

Oxytetracycline dihydrate, Rhodamine123, cyclosporine A and ivermectin, were purchased from Sigma (St. Louis, MO, USA). MK-571 sodium salt was from Alexis Biochemicals (Grünberg, Germany). [^{3}H]Oxytetracycline (5 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). PSC833 was a generous gift of Novartis Pharma AG (Basel, Switzerland). [^{3}H]Ivermectin was kindly provided by Merial (NJ, USA).
Dulbecco's Modified Eagle Medium (DMEM), foetal bovine serum, non-essential amino acids and trypsin-EDTA were purchased from Gibco (Grand Island, NY, USA). L-Glutamine and penicillin-streptomycin were from BioWhittaker (Maine, USA).

The monoclonal antibody (Mab) C219 directed against P-gp was obtained from Signet Laboratories (Dedham, USA) and Mab M_2III-6 directed against MRP2 was from Monosan (Uden, the Netherlands). Biotinylated goat anti-mouse IgG and streptavidin-AP conjugate were obtained from Biorad (Veenendaal, the Netherlands) and Boehringer (Mannheim, Germany), respectively. The molecular weight marker, Precision Plus Protein standard, was obtained from Biorad (Veenendaal, the Netherlands).

Western blotting

Protein samples from Caco-2 cell lysates were separated by electrophoresis on an 8% sodium dodecyl sulphate-polyacrylamide gel and transferred to a nitrocellulose membrane. P-gp was then identified by probing with the Mab C219 (1:200 dilution) and MRP2 by probing with M_2III-6 (1:20 dilution). Biotinylated goat anti-mouse IgG and streptavidin-AP conjugate was used for the detection of the primary antibody.

Caco-2 cell culture

Caco-2 cells (American Type Culture Collection, ATCC HTB-37), passage 95-105, were cultured in DMEM with 4.5g/l glucose, supplemented with 10% Foetal Bovine Serum (FBS), 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained in humidified air (5% CO_2) at 37^{o}C. For transport experiments, Caco-2 cells were seeded on cell culture inserts (pore size 0.4μm, high pore density, polyethylene terephthalate (PET) microporous membranes, surface area 0.3 cm^2 (BD Falcon, NJ USA) at a density of 30,000 cells per insert and maintained for 19-21 days, with medium replacements every 2-3 days. Cell-monolayers on inserts were only used when the transepithelial electrical resistance (Millicel-ERS ohmmeter, Millipore Corporation, Bedfort, MA) exceeded 225 $\Omega.cm^2$.

Transport studies

All transport experiments were conducted in transport medium consisting of DMEM without FBS and phenol red, supplemented with 1% (v/v) non-essential amino acids and 2 mM L-glutamine. Solutions containing the test compounds were added either to the basolateral compartment at a volume of 700 μl, or to the apical compartment at a volume of 300 μl, for measuring secretion and absorption, respectively. After 1 h of incubation at 37^{o}C in humidified air with 5% CO_2, samples of 200 μl were taken from the receiver compartment. Radioactivity of oxytetracycline and ivermectin was measured using a liquid scintillation counter (Tri-Carb 1900CA, Packard) after dispersion of the sample in 4 ml scintillation fluid (Ultima Gold, Perkin Elmer, Groningen). At least 10,000 counts were recorded, except for the background samples.

Fluorescence of Rhodamine123 was measured using filters for excitation at 485 nm and emission at 530 nm read by a multiplate reader (Fluostar Optima, BMG Labtechnologies, Offenburg, Germany).

Inhibition experiments, to assess the involvement of transporters were conducted with the inhibitors MK571, cyclosporine A and PSC833. Inhibitors were added to both apical and basolateral compartment. In each inhibition experiment, transport was also assessed in the absence of inhibitors as a control.

For the competition experiments, Caco-2 cell monolayers were incubated with 5 μM Rhodamine123, in the absence or presence of increasing concentrations of OTC added to the same compartment as Rhodamine123. For the competition experiment with ivermectin, 5 μM oxytetracycline or PSC833 was added to the same compartment as ivermectin (1 μM).

In all experiments the final concentration of the solvents in the transport medium was 0.2% for dimethylsulfoxide (DMSO) and 0.1% for ethanol.

Data analysis

All statistical analyses were calculated using Graph Pad Prism software (version 2.01, Graph Pad software Inc., San Diego, Calif.). Group comparisons were made by analysis of variance ANOVA, followed by Bonferroni post test correction. The level of significance was set at $p = 0.05$.

The apparent permeability (Papp) was calculated according to the following equation:

$$Papp = (V \times dC/dt) / (A \times C_0) \quad [cm\ s^{-1}]$$

V: Volume [cm^3] of receiver compartment
dC/dt: Rate [mol dm^{-3} s^{-1}] of time dependent increase in the concentration in the receiver compartment
A: surface area [cm^2] of microporous membrane of the inserts
C_0: Initial concentration [mol dm^{-3}] of substrate in the donor compartment

Results

P-gp expression by Western blot analysis.

Caco-2 cells grown on inserts were harvested for western blot analysis. The monoclonal antibodies C219 and M_2III-6 were used to identify protein expression of P-pg and MRP2, respectively (Fig. 1). A specific band was detected at ~150kDa for P-gp and at ~190kDa for MRP2.

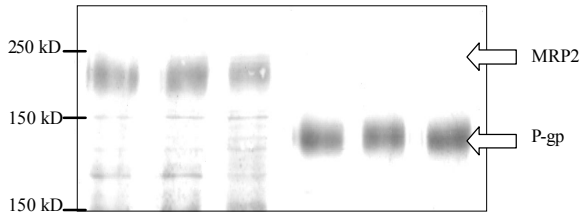

Figure 1. Western blot analysis of Caco-2 cells. The left three lanes are cell-lysate proteins from Caco-2 cells, grown on permeable inserts that were incubated with the monoclonal antibody M2III-6. The right three lanes show the same samples after incubated with the monoclonal antibody C219.

Polarized transport of Rhodamine123 and effect of inhibitors

The trans-epithelial passage of the proto-typical P-gp substrate Rhodamine123 across Caco-2 cell monolayers, in the basolateral-to-apical direction (secretion) was five times higher than the passage in the apical-to-basolateral direction (absorption). Secretion of 5 μM Rhodamine123 was blocked completely with PSC833, while MK571 did not affect secretion at equimolar

concentrations when added to both compartments. PSC833 and MK571 had no effect on absorption of Rhodamine123 (Fig. 2).

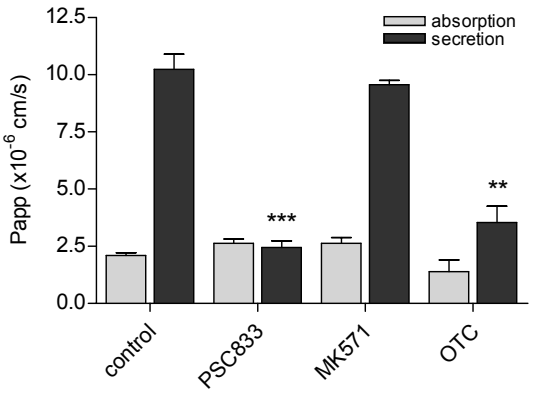

Figure 2. Absorptive and secretory permeability of Rhodamine123 across Caco-2 cell monolayers.
*5 μM Rh123 in the absence or presence of 5 μM PSC833, MK571 and OTC added to the apical and basolateral compartment. Data represent means ± SD of three replicates. Significant differences are marked *** and ** indicating P<0.001 and P<0.01.*

Transport of OTC.

Secretion of ^{3}H-labeled OTC at a concentration of 0.2 μM was higher than absorption, as shown in Fig. 3a . The calculated efflux ratio ($Papp_{secretion}$/$Papp_{absorption}$) of 1.3 indicated that the difference between absorption and secretion of OTC is less pronounced than that for Rhodamine123. When unlabeled OTC was added to the basolateral compartment at increasing concentrations, a decrease in secretory permeability was observed, as shown in Fig. 3b. This indicates that at higher concentrations, passive membrane passage of oxytetracycline prevails. When cellular passage is expressed as Papp, the passive component remains constant, and hence the carrier-mediated transport apparently decreases.

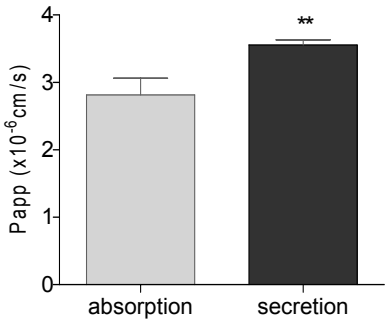

*Figure 3a. Absorptive and secretory permeability of 0.2 μM Oxytetracycline across Caco-2 cell monolayers. Data represent means ± SD of three replicates. ** indicates significant difference (P<0.01).*

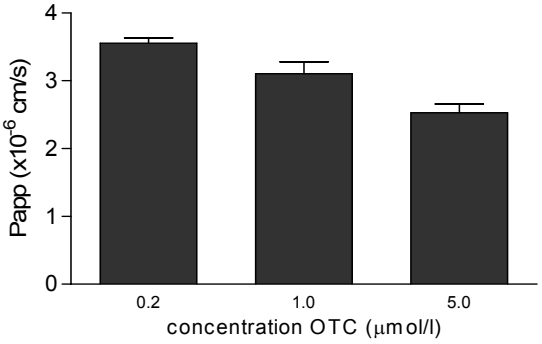

Figure 3b. Concentration dependent secretory permeability of oxytetracycline across Caco-2 cell monolayers. Data are expressed as mean P_{app} values ± SD of three replicates.

Concentration dependent inhibition of Rhodamine123 and OTC

PSC833, added to both compartments, decreased the secretion of Rhodamine123 (Fig. 4) and OTC (Fig. 5) in a concentration-dependent manner, confirming that OTC is transported by P-gp. Absorption of OTC was not affected by PSC833.

To evaluate the role of MRP2 in the polarized secretion of oxytetracycline, MK571 (5 μM) was added to both compartments. No effect was observed in the secretion or absorption of OTC (data not shown).

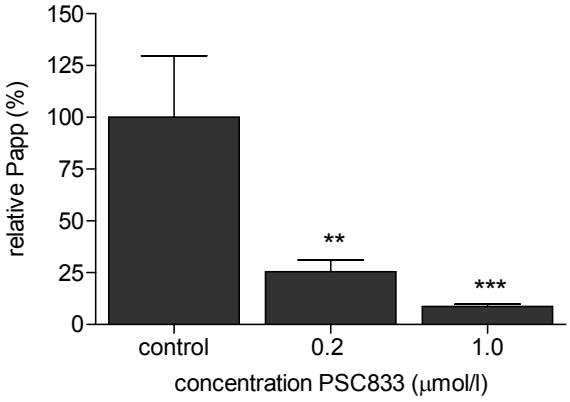

*Figure 4. Inhibition of Rhodamine 123 secretory permeability across Caco-2 cell monolayers by PSC833. Caco-2 cell monolayers were incubated with 5 μM Rh123 in the absence or presence of 0.2 or 1.0 μM PSC833 added to the apical and basolateral compartment. Data represent means ± SD of three replicates. Significant differences are marked *** and ** indicating P<0.001 and P<0.01.*

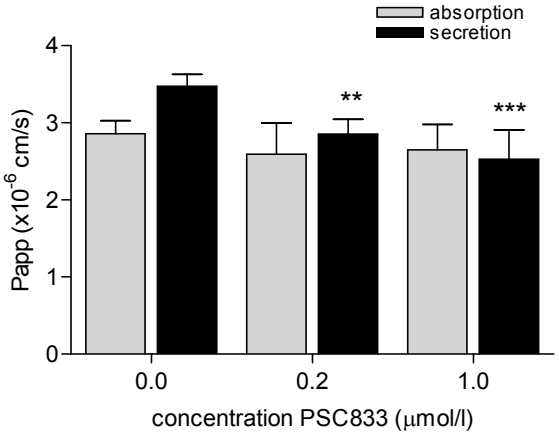

*Figure. 5. Inhibition of oxytetracycline secretory permeability by PSC833. Caco-2 cell monolayers were incubated with 0.2 μM OTC in the absence or presence of 0.2 or 1.0 μM PSC833 added to the apical and basolateral compartment. Data represent means ± SD of six replicates. Significant differences are marked *** and ** indicating P<0.001 and P<0.01*

Interaction between OTC and the P-gp substrates Rhodamine123 and ivermectin.

When Rhodamine123 and OTC were added together to the basolateral compartment, secretion of Rhodamine123 decreased in the presence of increasing concentrations of OTC. (Fig 6). When ivermectin (1 μM) was added together with OTC (5 μM) to the basolateral compartment secretion of ivermectin decreased as well, as shown in Fig. 7. Likewise, PSC833 reduced ivermectin secretion.

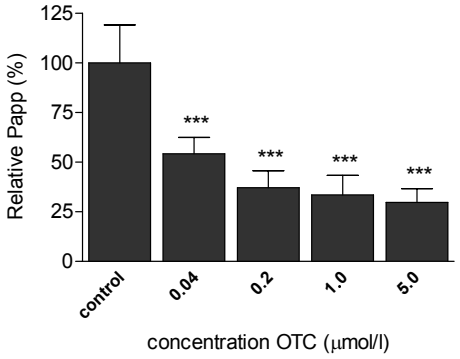

*Figure 6. Inhibition of Rhodamine123 secretory permeability across Caco-2 cell monolayers by oxytetracycline. Caco-2 cell monolayers were incubated with 5 μM Rh123 in the absence or presence of increasing concentrations OTC added to the same compartment as Rhodamine123. Data represent means ± SD of ≥ three replicates. Significant differences are marked *** indicating P<0.001*

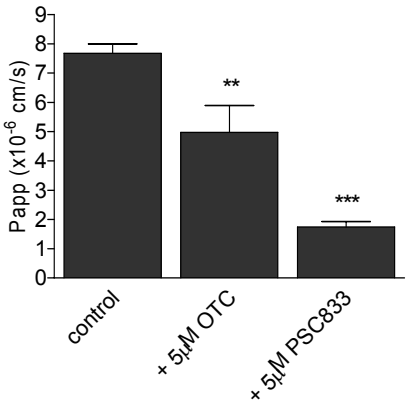

*Figure 7. Inhibition of ivermectin secretory permeability across Caco-2 cell monolayers by oxytetracycline. Caco-2 cell monolayers were incubated with 1 μM ivermectin in the absence or presence of 5.0 μM PSC833 or 5 μM OTC, added to the same compartment as ivermectin. Data represent means ± standard deviation of three replicates of one representative experiment. Significant differences are marked *** and ** indicating P<0.001 and P<0.01 respectively.*

Discussion

Clinical experience and pharmacokinetic studies have shown that the bioavailability of OTC after oral administration is very limited. Previous studies suggested that some tetracyclines are substrates for P-gp and hence we hypothesized that the observed limited bioavailability of OTC might be attributable to the secretory activity of P-gp that is located at the apical membrane of intestinal epithelial cells. P-gp dependent transport of drugs can be estimated by *in vitro* experiments with Caco-2 cell monolayers, resembling the intestinal barrier (Calcagno *et al*., 2006). Prior to using this model for the assessment of individual compounds, validation studies have to be conducted to confirm the functionality of the model under the selected experimental

conditions. To this end, we determined by Western blot analysis the level of expression of P-gp protein, using the murine monoclonal antibody C219 as primary antibody (Kartner *et al.*, 1985). In addition, the presence of the MRP2 protein was demonstrated by Western blot analysis using the specific monoclonal antibody M_2III-6 raised against human MRP2 (Scheffer *et al.*, 2000). Functional validation studies confirmed that under the selected experimental conditions Rhodamine123, a typical P-gp substrate, crosses the Caco-2 cell layer in the basolateral-to-apical direction. PSC833, a known inhibitor of P-gp inhibited the secretion of Rhodamine 123 in a concentration-dependent manner, whereas MK571, an MRP inhibitor, had no significant effect on Rhodamine 123 transport. Taken together, these data confirmed the suitability of the selected experimental conditions for the transport studies undertaken.

The relevance of this model to measure the trans-membrane transport as a surrogate in the estimation of the oral availability of veterinary drugs is based on the evidence for functional P-gp homologies across species. In our laboratory we have analysed almost the entire coding sequence of porcine cDNA prepared from various porcine tissues (NCBI accession no. AY825267) and found a nucleotide homology of 89.5% with human P-gp (ABCB1, NCBI accession no. NM_000927). Functional expression of porcine P-gp, assessed by Rhodamine uptake has been measured in different cell lines and in peripheral porcine lymphocytes (Laffont, 2002; Schrickx and Fink-Gremmels, 2006/ chapter 7).

Transport of OTC across these Caco-2 cell monolayers was investigated in both directions at different concentrations. Results show that transport from the basolateral-to-apical direction predominated. This secretory transport was concentration-dependent and saturable. The P-gp inhibitor PSC833 decreased the secretion of OTC in a concentration-dependent manner, whereas the MRP-inhibitor MK571 had no effect. These results suggest that OTC is primarily a P-gp substrate, and that MRP2 is not involved in OTC transport. However, the experiments with the PSC833 also demonstrated that inhibition of P-gp does not result in an increase of the absorption (apical-to-basolateral transport) of OTC in the selected concentration range. Calculation of the efflux ratio (secretory permeability / absorptive permeability) showed a ratio of 1:1.3 at a concentration of 0.2 μM OTC, indicating a low substrate affinity towards P-gp. A comparison of the present in vitro findings with data from animal experiments indicates that, under in vivo conditions, P-gp would readily be saturated by the administered OTC doses. For example, given with drinking water, OTC is administered generally in a concentration of 400 g /1000 L. This dose approximately equals a concentration of 800 μmol/L. In the present experiments, a tendency for saturation of the secretory transport was observed even at a concentration 0.2 μmol/L. This implies that in vivo other factors such as binding to feed components (Welling and Tse, 1982; Dyer, 1989) and dissolution of the drug in the gastro-intestinal tract (Amidon et al., 1995; Tongaree et al., 1999) seem to have a larger impact on the bioavailability of OTC after oral administration than the expression of P-gp in epithelial cells lining the intestines.

The present data indicate also that the secretion of Rhodamine123 was impaired in the presence of OTC, suggesting substrate competition. These initial findings provided the rationale for the experiments devoted to the interaction of OTC and ivermectin, another well known P-gp substrate (Schinkel *et al.*, 1995; Pouliot *et al.*, 1997; Griffin *et al.*, 2005) commonly used in veterinary practice. As expected, OTC also decreased the secretion of ivermectin by substrate competition for P-gp transport. In the present *in vitro* experiments with Caco-2 cells, the concentrations of OTC used correspond to those found *in vivo* in the blood serum following oral administration of OTC according to common dose regimens, as discussed above. Hence, the experiments suggest that OTC may be able to alter the pharmacokinetic parameters of ivermectin (and probably other P-gp substrates) also *in vivo* following co-medication.

In general, P-gp mediated transport is saturated either by high individual substrate concentrations, or following co-exposure to different substrates (drugs). In turn, drug-drug interactions at the level of P-gp will result in altered drug absorption and an increase in total body exposure (Lau *et al.*, 2004; Wu and Benet, 2005), impair hepatic clearance and biliary excretion and even may decrease the barrier function of blood-tissue barriers (Balayssac *et al.*, 2005). Undesirable side-effects resulting from these drug-drug interactions have been described in human patients (Ayrton and Morgan, 2001; Lin and Yamazaki, 2003). These findings suggest that the rapid saturation of intestinal P-gp by OTC carries the risk of changes in the kinetics of other drugs that might be used in the same animal population in the course of therapy.

In conclusion, the present data show that OTC is a substrate for the efflux transporter P-pg. At clinically relevant doses given orally, OTC is able to saturate P-gp. Subsequently, the net absorption of other drugs increases, which might be favourable in many cases but bears the risk of undesirable side-effects if the second drug has a small margin of safety. The present experiments also confirm that Caco-2 cells grown on permeably inserts are not only a model to estimate oral bioavailability, but may serve also to study potential drug-drug interactions of commonly used veterinary drugs at the level of P-gp mediated membrane transport.

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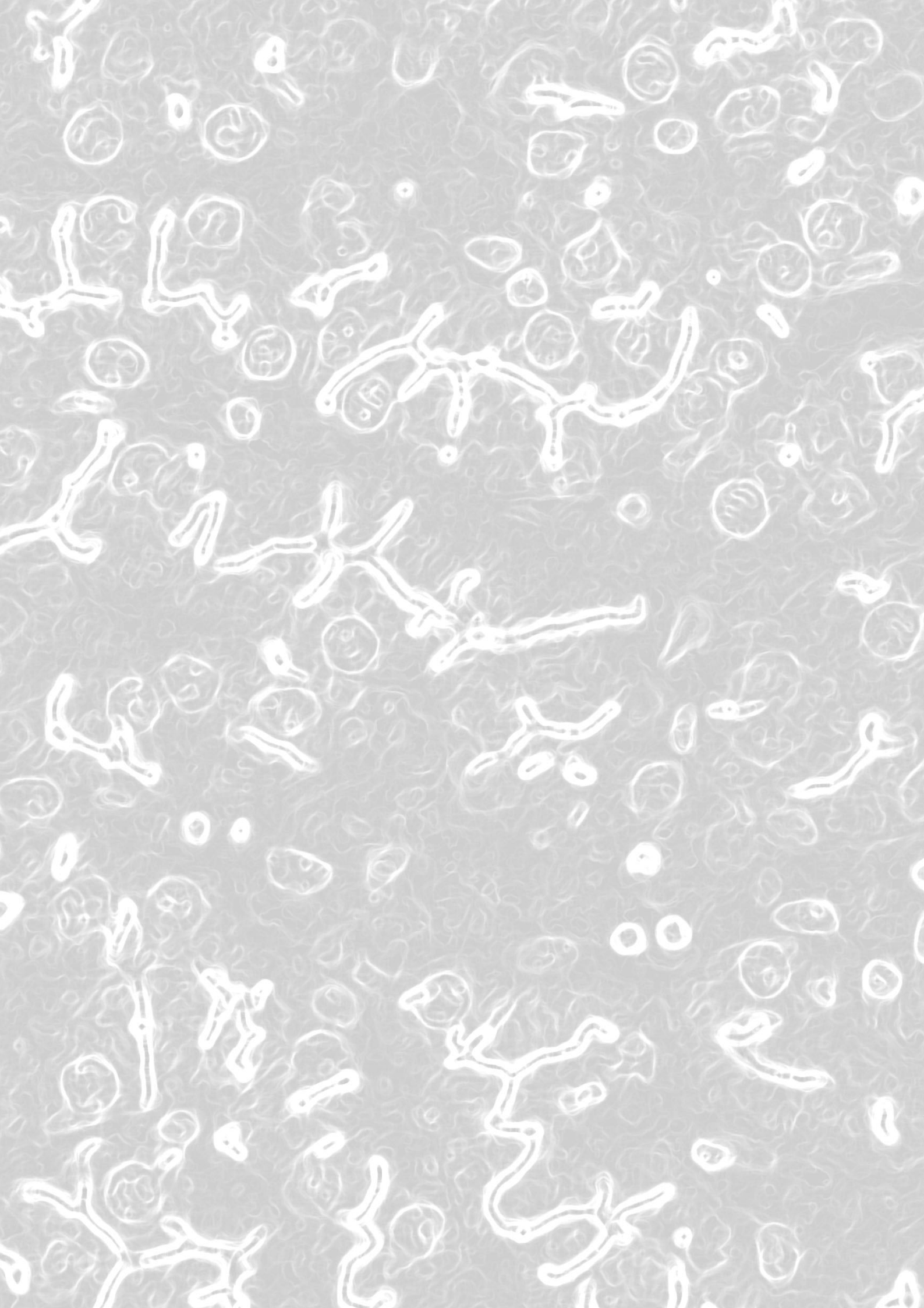

CHAPTER 3

Danofloxacin-mesylate is a substrate for ATP-dependent efflux transporters

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Abstract

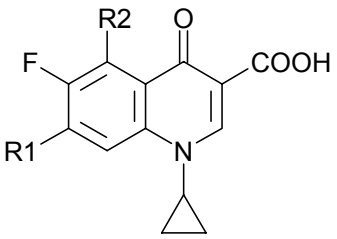
Background and purpose: Next to its broad antimicrobial spectrum, the therapeutic advantages of the fluoroquinolone antimicrobial drug Danofloxacin-Mesylate (DM) are attributed to its rapid distribution to the major target tissues such as lungs, intestines and the mammary gland in animal species. Previous analyses revealed that effective drug concentrations are achieved also in luminal compartments of these organs, suggesting that active transport proteins facilitate excretion into the luminal space. Members of the ATP-Binding Cassette (ABC) superfamily, including P-gp, BCRP and MRP2 are known to be expressed in many tissue barriers and in cell-membranes facing luminal compartments. Hence we hypothesized that DM is a substrate for one of these efflux-transporters. Experimental approach: As experimental model, tight monolayers of Caco-2 cells, grown on microporous membranes in bicameral tissue culture devices were used. Key results: Results show that DM transport across Caco-2 cells is asymmetric, with a rate of secretion exceeding that of absorption. The P-gp inhibitors PSC833 and GF120918 and the MRP-inhibitor MK571 partially decreased the secretion of DM and increased its absorption rate. The BCRP inhibitor, Ko143, decreased secretion only at a concentration of 1 μM. When DM was applied together with ciprofloxacin, secretion as well as absorption of DM decreased. Conclusions and Implications: The presented results suggest that DM is a substrate for the efflux transporters P-gp and MRP2, whereas the specific role of BCRP in DM transport needs further evaluation. These findings provide the mechanistic basis to understand the kinetic properties of DM in healthy and diseased individuals.

Introduction

Fluoroquinolone antimicrobials are widely used in the therapy of infectious diseases in consideration of their broad antimicrobial activity as well as their appreciated kinetics characterized by a large volume of distribution. Fluoroquinolones can be applied orally or by parenteral injection, and initial data indicated that the major route of elimination is renal excretion as only a small fraction was found to be eliminated with biliary fluid. Despite this low rate of biliary elimination, the drug concentrations are high of for example ciprofloxacin, the first widely marketed, fluoroquinolone in the gut lumen (Sorgel et al., 1989). These findings suggest a trans-intestinal elimination, and a few years later, Rabbaa et al. (1995) hypothesized that P-glycoprotein (P-gp) might be involved in the transepithelial secretion of ciprofloxacin as well as ofloxacin. P-gp is a member of the superfamily of ABC transporters, these transporters use ATP to pump compounds out off the cellular cytoplasma, hence contributing to the function of biological barriers, like the blood brain barrier and the intestinal barrier. Initially, inhibition of P-gp by specific substrates has been suggested as a strategy to improve efficacy for example of cytostatic drugs against tumour cells and intracellular pathogens. In turn, it was considered that secretion of antimicrobials from the basolateral site to the luminal surfaces of the alveolar space or the luminal space of the large intestines provides a therapeutic advantage against bacteria that colonize on these luminal surfaces.

Danofloxacin-mesylate (DM) is a fluoroquinolone antibacterial drug for veterinary use. It is indicated in cases of gram-negative infections of the respiratory tract, intestinal tract and the mammary gland in cattle. The recommended dose is 6 mg/kg b.w. with subcutaneous injection at a single occasion, based on the concept of concentration-dependent killing that is applied to this group of fluoroquinolones. Danofloxacin itself was initially selected by QSAR analysis, but never entered clinical studies due to its poor pharmacokinetic properties (Braish and Fox, 1990). These pharmacokinetic properties were significantly improved when the mesylated form was introduced. In contrast to the parent compound DM highly distributes into the target tissues, lung and intestines, and concentrations exceeding those in plasma were detected in the luminal compartments of these organs as well as in the milk (Friis and Nielsen, 1997; McKellar et al., 1998; Shem-Tov et al., 1998; Lindecrona et al., 2000). These luminal compartments are the major sites of Gram-negative infections and hence luminal drug concentrations accounts to a large extend for the therapeutic efficacy of DM. As the previously published kinetic data did not provide an explanation for the high drug concentrations that were measured in the luminal space of the intestines after parenteral injection, we hypothesized that DM, like enrofloxacin and grepafloxacin, is a substrate for efflux transporters. Among these transporters, P-gp (p-glycoprotein), BCRP (breast cancer resistant protein) and MRP2 (multi drug resistance protein) are known to be directed towards the luminal compartments in various organs, including the above-mentioned target organs for the clinical use of DM (de Lange et al., 2000; Nakajima et al., 2000; Naruhashi et al., 2001; Naruhashi et al., 2002; Yamaguchi et al., 2002; Sasabe et al., 2004; Merino et al., 2006). Hence we investigated the effects of the lumen-directed transporters on DM absorption and secretion in Caco-2 cell monolayers, known to express P-gp, MRP2 and

BCRP at the apical cell membranes (Taipalensuu et al., 2001; Prime-Chapman et al., 2004; Xia et al., 2005).
Our results indicate that DM secretion is P-gp and MRP2 dependent and are suggestive for an additional role of BCRP. Extrapolation of these data to the body disposition suggests that the widespread transporter P-gp will mediate the secretion of this fluoroquinolone antimicrobial drug into luminal compartments of organs, while MRP2 will have a similar role in the main drug-eliminating organs.



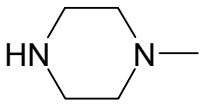
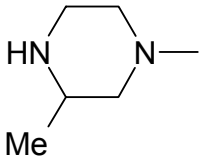
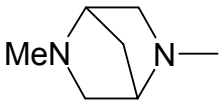
fluoroquinolone	R1	R2	Substrate for
Ciprofloxacin		H	BCRP
Grepafloxacin		CH_3	P-gp MRP1 MRP2
Danofloxacin (mesylate $C_{19}H_{20}FN_3O_3$ - CH_4O_3S)		H	

Figure 1: Chemical structure of Danofloxacin, Ciprofloxacin and grepafloxacin, fluoroquinolones that had been previously found to be a substrate for ATP-dependent drug transporters.

Materials and methods

Chemicals

Danofloxacin-mesylate was kindly supplied by Pfizer (Sandwich, UK). Ciprofloxacin was obtained from Fluka (Germany). MK-571 sodium salt was obtained from Alexis Biochemicals (Grünberg, Germany). PSC833 was a generous gift of Novartis Pharma AG (Basel, Switzerland), GF120918 was donated by GlaxoSmithKline (Stevenage, Herts, UK), and Ko143 was a generous gift of Prof. G.J. Koomen (University of Amsterdam, the Netherlands).

Caco-2 cell cultures

Caco-2 cells (American Type Culture Collection, ATCC HTB-37), passage 95-105, were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) with 4.5 g/l glucose, supplemented with 10% (v/v) foetal bovine serum (FBS), 0.1 mM nonessential amino acids, 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in 75-cm^2 tissue culture T-flasks at 37°C in a humidified atmosphere of 5% CO_2 in air for subsequent plating onto the cell culture inserts (0.4 μm pore size, high pore density, polyethylene terephthalate (PET) micro porous membranes, surface area 0.3 cm^2, BD Falcon NJ USA). Caco-2 cells were seeded at a density of 30,000 cells per insert. Transport experiments were conducted after 20-21 days, using only cell-monolayers on inserts with a transepithelial electrical resistance (TEER) value above 300 $\Omega.cm^2$, as measured by an epithelial volt-ohm meter (Millicell-ERS, Millipore Corporation, Bedford, MA).

Transport studies

Bidirectional transport studies were performed in DMEM without FBS and phenol red, supplemented with 1% (v/v) non-essential amino acids and 2mM L-glutamine. The experiments were initiated by adding medium containing DM, to either the apical compartment at a volume of 300 μl, or to the basolateral compartment at a volume of 700 μl, for measuring absorption and secretion respectively. In the subsequent experiments, stocks of the inhibitors PSC833, GF120918, MK571 and Ko143 were dissolved in DMSO, and were added to the medium in the given concentrations. The final concentration of the solvent DMSO was set to 0.1% in all experiments. The inhibitors, as well as ciprofloxacin, were added to both compartments of the bicameral system. After 1 hour of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air, samples from both compartments were collected separately for HPLC analysis.

HPLC analysis

The HPLC analyses were conducted according to the method previously described by Garcia et al. (2000), with minor modification. Briefly, samples of 20 μl were directly injected into the HPLC system, consisting of a high-pressure pump, an autoinjector (Gyna 50) and a fluorescence detector (Detector Jasco, Model FP 920). A spherisorb-ODS2 column was used for the separation of the samples, using a mobile phase of acetonitrile in an aqueous phase

(16:84, v/v) set to pH 3.0 and a flow rate of 1.0 ml/min. The aqueous phase consisted of tetrabutylammoniumhydrogenphosphate (TBAP), 0.02 M and potassium dihydrogenophosphate, 0.02 M dissolved in demi-water. Excitation and emission wavelengths of 280 and 440 nm, respectively, were used for the detection of DM. In order to allow a parallel measurement of DM and ciprofloxacin, in the corresponding experiments the percentage acetonitrile in the mobile phase was decreased to 10% (v/v). The area under curve was integrated by Chromeleon software (Separations, H.I. Ambacht, The Netherlands) and compared with standard curves prepared with cell culture medium for the quantification of DM concentrations.

Data analysis

The apparent permeability (Papp) was calculated according to the following equation:

(1) $$Papp = (Vx\ dC/dt) / (AxC_0)\ \ [cm\ s^{-1}]$$

V	Volume of receiver compartment [cm^3]
dC/dt	Rate of time-dependent increase in the concentration in the receiver compartment [mol dm^{-3} s^{-1}]
A	surface area of microporous membrane of the inserts [cm^2]
C_0	Initial concentration of DM in the donor compartment [mol dm^{-3}]

One-way ANOVA, followed by Dunnett's multiple comparison test (Graph Pad Prism software, version 2.01; Graph Pad software Inc., San Diego, California) was used to assess the statistical significance of observed differences. Differences were considered to be statistically significant when $p < 0.05$.

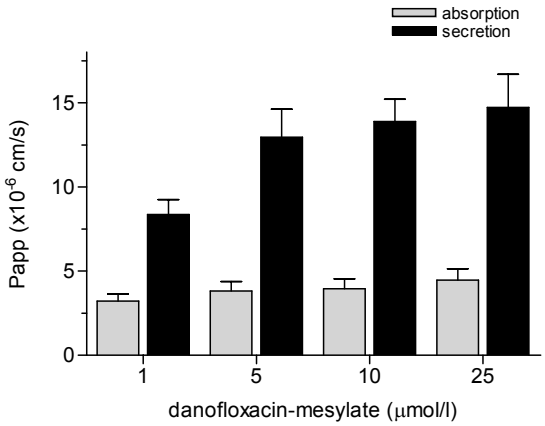


Figure 2. Concentration dependent transport of danofloxacin-mesylate: Caco-2 cell monolayers were incubated with increasing concentrations of danofloxacin-mesylate added to the basolateral or apical compartment. Data represent means ± SD of at least six replicates.

Results

Experiments with a concentration range between 1 and 25 µmol DM indicated an asymmetric transport of DM at all concentrations tested. Secretion, expressed as apparent permeability (P_{app}), increased with increasing concentrations, while absorption remained constant (fig. 2). The rate of secretion exceeded that of absorption 2.5 to 3.5 times. These findings suggest that the efflux of DM to the apical compartment is indeed a carrier-mediated process in Caco-2 cells. For the following experiment a series of inhibitors for ATP-dependent transporters was selected to assess the contribution of individual transporters to DM transport. In these experiments the inhibitors were added at different concentrations to both compartments, whereas a constant concentration (10 µM) of DM was used. As potent inhibitor for P-gp, PSC833 (Aouali *et al.*, 2005) was selected. PSC833 decreased the secretion of DM, and subsequently increased its absorption (fig. 3 left panel.), demonstrating that P-gp is involved in the secretion of DM.

However, as secretion was not completely blocked by PSC833, it was concluded that P-gp is not the only carrier responsible for the secretion of DM in Caco-2 cell model. Therefore we measured the effect of GF120918, a dual inhibitor for P-gp and BCRP (de Bruin *et al.*, 1999). Secretion was again decreased (fig. 3 right panel), while absorption remained apparently unaffected. With the aim to further analyse the role of BCRP, transport of DM was measured in the presence of Ko143 (fig. 4 left panel.). Ko143, considered to a potent inhibitor of BCRP-mediated transports (Allen *et al.*, 2002), decreased the secretion of DM, without a significantly effecting its absorption. Finally, an inhibitor for MRP's, MK571 (Gekeler *et al.*, 1995), was found to decrease the secretion and increase absorption of DM (fig. 4, right panel.), suggesting that MRP2, thus far the only MRP-transporter recognized at the apical membrane of Caco-2 cells, is also involved in DM secretion.

In a third experimental setting we evaluated the potential for common pathways in the transport of DM and ciprofloxacin, a related fluoroquinolone (figure 5). Co-incubation of DM with ciprofloxacin resulted in a decreased secretion of DM as well as a decreased absorption of DM, indicating drug-drug interactions at the level of membrane transport.

Discussion

The involvement of ATP-dependent efflux transporters in the disposition and excretion of DM had not been investigated before, despite suggestive findings in *in vivo* experiments, in which high drug concentrations could be measured at the luminal site of the intestines, the lung and the mammary gland following subcutaneous injection of DM. The aim of the present study was confirm that DM is like grepafloxacin, a substrate for ATP-dependent efflux transporters and to evaluate the potential role of individual transporters in danofloxacin secretion into luminal compartments. The applied Caco-2 model has been validated before and has been applied to

elucidate the transmembrane transport of various drugs and toxins. In the presented experiments, Caco-2 cells were grown in bicameral inserts and DM was added to the apical or basolateral compartment in the absence or presence of known inhibitors of ATP-dependent efflux transporters. With this model it could be demonstrated that DM is a substrate for more

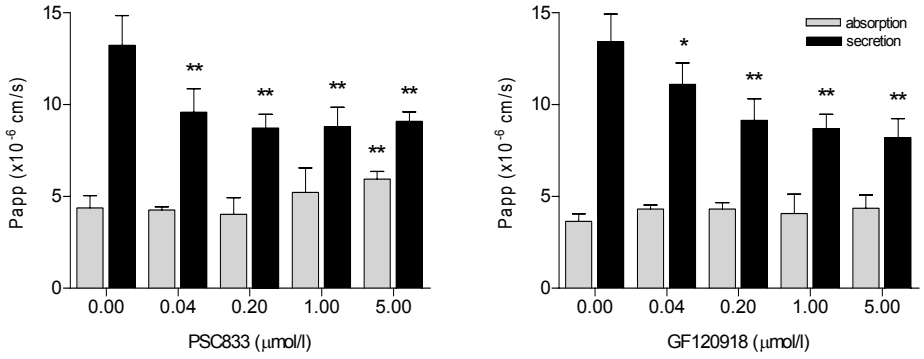


*Figure 3. The effect of PSC833 and GF120918 on absorptive and secretory transport of danofloxacin. Caco-2 cell monolayers were incubated with 10 μM danofloxacin in the absence or presence of increasing concentrations of PSC833or GF120918 added to the apical and basolateral compartment. Data represent means ± SD of six replicates. Significant differences are marked, * indicating P<0.05, ** indicating P<0.01.*

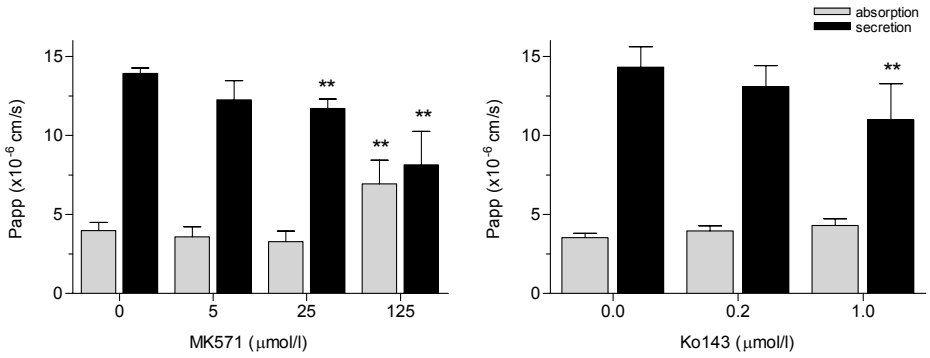


*Figure 4. The effect of MK571and Ko143 on absorptive and secretory transport of danofloxacin. Caco-2 cell monolayers were incubated with 10 μM danofloxacin in the absence or presence of increasing concentrations of MK571 or Ko143 added to the apical and basolateral compartment. Data represent means ± SD of six replicates. Significant differences are marked, ** indicating P<0.01.*

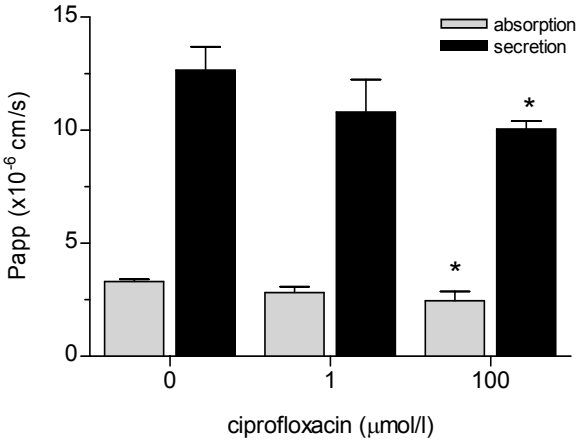


*Figure 5. The effect of ciprofloxacin on absorptive and secretory transport of danofloxacin. Caco-2 cell monolayers were incubated with 10 μM danofloxacin in the absence or presence of ciprofloxacin added to the apical and basolateral compartment at increasing concentrations. Data represent means ± SD of three replicates. Significant differences are marked, * indicating P<0.05.*

than one transporter. The inhibitory effects of PSC833 and GF120918 clearly show that secretion is P-gp dependent, while the other inhibitors, MK571 and Ko143, predict a role for MRP2 and BCRP in the organ-specific secretion. The limited effect of Ko143, used as inhibitor for BCRP, on DM secretion might be due to a compensatory increase in the transport by P-gp or to non-specified effects on other membrane transporters. Ko143 has been described to be a potent inhibitor of BCRP, but a minimal effect on P-gp and MRP1 function has been observed at a concentration of 0.50 μmol/L (which falls into the concentrations range tested here, see figure 4 upper panel), whereas it lacks any effect on MRP2 function in this concentration range (Allen, 2002). In the light of these previous findings it can be argued that the inhibitory effect of Ko143 on DM secretion that was only seen at the highest concentration used (1μM), results from an aspecific inhibition of P-gp. However, when PSC833 and Ko143 were used together (data not shown), DM secretion decreased further.

Ciprofloxacin, a substrate for BCRP, but not for P-gp or MRP2 (Lowes and Simmons, 2002; Merino, 2006), decreased the secretion and absorption of danofloxacin. A common pathway in the transport of danofloxacin and ciprofloxacin seems likely, however, with a limited role in danofloxacin transport. A basolateral carrier common to fluoroquinolones affecting the transepithelial transport of ciprofloxacin was previously suggested (Griffiths *et al.*, 1994). Basolateral carriers may belong to the MRP-family of active membrane carriers or to the family of solute (facilitated) carriers (SLC) (Griffiths, 1994; Zakelj *et al.*, 2006), the latter are mainly responsible for cellular uptake of substrates, but depending on the co-substrates and their concentrations they may serve as efflux carriers as well. Thus, whether inhibition of DM transport by ciprofloxacin has resulted from competitive inhibition for a basolateral and/or apical transporter (BCRP) needs further evaluation. However, a basolateral active efflux transporter involved in the absorption of DM is suggested by the increased secretion of DM

that was found when applying increasing concentrations of DM, leading to the saturation of this basolateral efflux transporter that counteracted partly the secretion . This would also explain the observed tendency for a decreased absorption at lower concentrations of MK571 (other MRP's than MRP2 are likely situated in the basolateral membrane of intestinal cells).

Our finding that DM is a substrate for multiple transporters is not surprising, since various drugs are substrates for more than one transporter, including the fluoroquinolone grepafloxacin (Lowes and Simmons, 2002; Naruhashi, 2002; Sasabe, 2004) and likely sparfloxacin (Cormet-Boyaka *et al.*, 1998). P-gp is expressed in alveolar epithelial type I epithelium within the human and rat lung tissue (Campbell *et al.*, 2003), while BCRP is expressed in the bronchial and bronchiolar epithelium, similar to P-gp, and in the endothelial cells (Scheffer *et al.*, 2002), but luminal staining of alveolus-cells has not been demonstrated yet. High concentrations of Grepafloxacin, a P-gp substrate (Lowes and Simmons, 2002; Zhao *et al.*, 2002), were previously measured in the epithelial lining fluid (ELF) of the rat lung when compared to plasma concentrations, while this was not observed for ciprofloxacin (Deguchi *et al.*, 2003). Similarly, concentrations of levofloxacin and sparfloxacin as substrates for P-gp (Ito *et al.*, 1997; Cormet-Boyaka, 1998; de Lange, 2000) in ELF exceeded those in plasma in humans (Wise and Honeybourne, 1996; Andrews *et al.*, 1997). It thus seems that P-gp determines the vectorial transport into the bronchiolar and alveolar space. Previous investigations had indicated that DM rapidly penetrates respiratory tract tissues and secretions of calves (Friis, 1993; McKellar *et al.*, 1999), suggesting an active secretion of danofloxacin into these secretions, that may be mediated by P-gp (Friis, 1993). Similar results were obtained in pigs (Friis and Nielsen, 1997). Other mechanisms involved in tissue distribution or accumulation are tissue uptake and tissue binding and high tissue concentrations may thus result from binding to cellular components as exemplified by binding to phophatidylserine in the case of Grepafloxacin (Suzuki *et al.*, 2002).

Expression of BCRP is highly upregulated in the lactating mammary gland of cows, human and mice (Jonker *et al.*, 2005). High concentrations of danofloxacin in the milk of cows exceeding those of serum were previously reported (Shem-Tov, 1998), and this phenomenon was explained by ion trapping. However, it is highly likely that BCRP plays an important role in the secretion of danofloxacin into the milk, as was shown for ciprofloxacin in rats (Merino, 2006) and enrofloxacin in ewes (Pulido *et al.*, 2006), and hence the mechanism should be further elucidated.

Expression of all three transporters is high in the small intestines. While the expression of MRP2 decreases along the human intestinal tract, P-gp and BCRP remain highly expressed in the large intestines (Maliepaard *et al.*, 2001; Langmann *et al.*, 2003). Active intestinal secretion, presumably mediated by one or more of these transporters, has been reported for grepafloxacin (Naruhashi, 2001; Naruhashi, 2002; Yamaguchi, 2002; Fernandez-Teruel *et al.*, 2005), sarafloxacin (Fernandez-Teruel, 2005) and ofloxacin (Rabbaa *et al.*, 1996) and is highly suggested for other fluoroquinolones by an asymmetric transport directed to the luminal side in

Caco-2 cells (Griffiths, 1994; Cormet-Boyaka, 1998; Yamaguchi *et al.*, 2000; Naruhashi, 2001; Ruiz-GarcÍa *et al.*, 2002; Volpe, 2004). In *in vivo* studies, high concentrations of DM were found in the intestinal contents in healthy pigs (Lindecrona, 2000), cattle (von Traeder and Kleinhaus, 2002) and sheep (McKellar, 1998), and it has to be assumed that the underlying mechanism is an active secretion of danofloxacin by MRP2 and P-gp localized in the brush border membranes of epithelial cells.

It is worthwhile to recall that numerous factors affect the function of transporters, including genetic variation, gender, feed components, co-medication of substrate drugs, infection and inflammation. Expression of these transporters is partly co-regulated with phase I and II metabolising enzymes and typically depends on the activation of nuclear transcription factors such as CAR (constitutive androstane receptor) and PXR (pregnane X receptor) (Eloranta *et al.*, 2005). Activation of these nuclear receptors by physiological ligands, such as hormones (e.g. cortisol, estradiol, progesteron and thyroid hormone) or xenobiotics leads to changes in the rate of transcription of the drug-transporters. In turn, inflammatory mediators (including IL-6) are known to decrease the expression and function of P-gp and MRP2, similar to the decrease in the activity of various CYP450 isozymes in the liver and in the gastro-intestinal tract (Fernandez *et al.*, 2004; Kalitsky-Szirtes *et al.*, 2004). These mechanisms likely explain the previously observed decreases in the systemic clearance and secretion of DM into the intestinal lumen in the diseased animals (Lindecrona, 2000). Thus the disposition of fluoroquinolones into the target tissues may highly vary among individuals, with possible consequences for efficacy and resistance development in microbes.

In addition, certain plant derived polyphenolic compounds, structurally related to the quinolone antimicrobials, are substrates for P-gp, MRP2 and BCRP. High levels of these compounds, including the flavenoids genestin, quercetin, naringenin, hesperetin (for review see Morris and Zhang, 2006), may occur in food materials, especially in soybean products and (citrus-) fruits and hence may decrease the secretion of fluoroquinolones, including DM into the intestinal lumen.

In conclusion, Danofloxacin-Mesylate is a substrate for multiple transporters, including P-gp and MRP2, whereas the role of BCRP in Danofloxacin-Mesylate secretion remains elusive. The finding that DM is a substrate for the efflux transporters explains the previously observed secretion of Danofloxacin-Mesylate into the intestinal lumen and bronchial secretions.

Acknowledgements.
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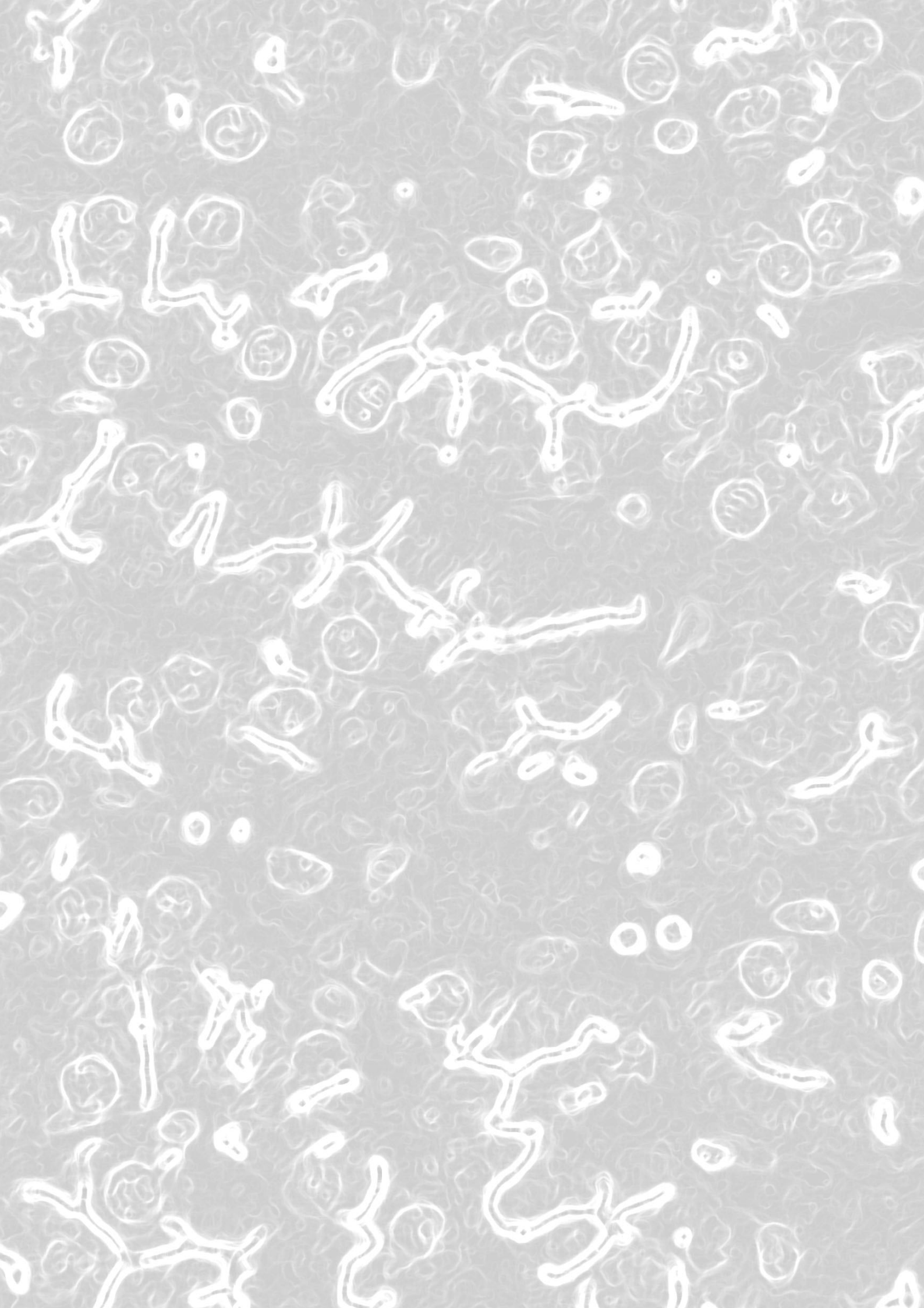

CHAPTER 4

Ochratoxin A secretion by ATP-dependent membrane transporters in Caco-2 cells

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Abstract

The ATP-dependent membrane transporters, P-gp, MRP2 and BCRP, localized in the luminal membranes of the intestines, liver and kidney, counteract absorption and increase excretion of xenobiotics and drugs. Previously, it has been suggested that the mycotoxin ochratoxin A (OTA) is a substrate for ATP-dependent transporters, and hence the absorption and secretion of OTA in the Caco-2 cell model was investigated. To this end Caco-2 cells were cultured as confluent monolayers in bicameral inserts and the transepithelial transport of the mycotoxin was assessed. Caco-2 cells secreted OTA to the luminal side in a concentration- dependent manner. This secretory permeability was higher than the absorptive permeability, while the absorptive permeability remained constant for all OTA concentrations tested. The secretion decreased and absorption increased in the presence of the MRP-inhibitor MK571, the P-gp and BCRP inhibitor GF120918, and the BCRP-inhibitor Ko143, suggesting that the secretion of OTA is mediated by MRP2 and BCRP. Cyclosporine A also decreased the secretory permeability, but did not affect absorptive permeability, while PSC833 did neither change absorption nor secretion of OTA. Hence it can be suggested that OTA is a substrate for MRP2 as well as BCRP. These findings are of interest in evaluating mycotoxin absorption after oral ingestion, tissue distribution and particularly excretion pathways, including renal, biliary and mammary gland excretion.

Introduction

Ochratoxin A (OTA), a major secondary metabolite formed by various fungal species of the genus Penicillum and Aspergillus, has been found throughout the world as a contaminant of many food commodities, among others in cereals, coffee, dried fruits, grapes, red wine and beer (e.g.(Speijers and van Egmond, 1993) OTA is considered to be primarily a nephrotoxin, but carcinogenic, embryotoxic and teratogenic properties have been demonstrated in laboratory animal species (Benford D et al., 2001; O'Brien and Dietrich, 2005). Long-term exposure to OTA has been implicated with a progressive kidney disease in humans, known as Balkan Endemic Nephropathy. This site-specific toxicity has been associated with an accumulation of OTA in the kidneys (Dahlmann et al., 1998; Schaaf et al., 2002; O'Brien and Dietrich, 2005).

Previous findings had indicated that OTA is a substrate for H+-dipeptide co-transporters and organic anion transporters belonging to the SLC15 (PEPT), SLC21(OATP) or SLC22(OAT)-family, facilitating cellular uptake or efflux of substrates (Sokol et al., 1988; Bahnemann et al., 1997; Schwerdt et al., 1997; Zingerle et al., 1997; Dahlmann, 1998; Schwerdt et al., 1998; Jung et al., 2001; Babu et al., 2002). In contrast to these transporters that are mainly directed towards cellular uptake, members of the ATP-Binding Cassette (ABC) transporter super-family facilitate secretion of xenobiotics out of the cell. Hence, these transporters counteract absorption of toxic compounds, contribute to protective tissue barriers like the blood brain barrier, and play an important role in elimination processes (Sparreboom et al., 1997; Borst and Elferink, 2002). The ABC efflux-proteins P-gp, MRP2 and BCRP are situated in the apical membranes of epithelial cells and their expression has been demonstrated in the main eliminating organs such as the intestines, liver and kidneys albeit at different levels (Langmann et al., 2003).

A common model to study the function of membrane transporters are monolayers of Caco-2 cells grown on permeable supports, as these cells express P-gp, MRP1-6 and BCRP (Taipalensuu et al., 2001; Prime-Chapman et al., 2004; Xia et al., 2005). When Berger et al. (2003) studied the transport of OTA in this Caco-2 cell model they found a higher secretion than absorption rate at physiological pH, whereas the rate of absorption exceeded the rate of secretion when the pH of the apical compartment was decreased to pH 6.0. Inhibition experiments using (non-)specific MRP inhibitors carried out under these acidic conditions, showed an inhibition of secretion and an increase of absorption, suggesting MRP2 mediated secretion of OTA (Berger et al., 2003). Based on these studies we hypothesized that various ABC-efflux proteins might be involved in OTA transport. Hence we studied with the Caco-2 cell model the transmembrane transport of OTA, also in the presence of the putative inhibitors MK571, Cyclosporine A, GF120918, Ko143 and PSC833 at physiological pH, with the aim to determine the individual contribution of ABC-efflux proteins to OTA transport.

Materials and methods

Chemicals

Alamar blue was purchased from Biosource (Camarillo, California, USA). Cyclosporine A and OTA were obtained from Sigma (St. Louis, MO, USA). [^{3}H]Ochratoxin A (7.2 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). MK-571 sodium salt was from Alexis Biochemicals (Grünberg, Germany). GF120918 was a generous gift of GlaxoSmithKline (Stevenage, Herts, UK), PSC833 was a generous gift of Novartis Pharma AG (Basel, Switzerland) and Ko143 was kindly provided by Prof. Koomen (University of Amsterdam, the Netherlands).
Foetal bovine serum, non-essential amino acids (100X) and trypsin-EDTA (1X) were purchased from Gibco (Grand Island, NY, USA). L-Glutamine (100X) and Penicilin-Streptomycin (100X) were from BioWhittaker (Maine, USA).

Cell Cultures

Caco-2 cells (American Type Culture Collection, ATCC HTB-37) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 4.5 g/l glucose, supplemented with 10% (v/v) foetal bovine serum (FBS), 1% (v/v) non-essential amino acids, 2mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained at 37^{o}C in a humidified atmosphere of 5% CO_2 in air. Cells were subcultured weekly by washing twice with PBS (without calcium and magnesium) and EDTA (2.2% w/v), and thereafter incubated with trypsin-EDTA until cells detached. Fresh culture medium was added and an aliquot of the cell suspension was plated in a new 75 cm^2 tissue culture flask with 25 ml culture medium.
For the experiments, Caco-2 cells, passages 90-95, were seeded on cell culture inserts (0.4 μm pore size, high pore density, polyethylene terephthalate (PET) microporous membranes, surface area 0.3 cm^2, BD Falcon NJ USA) at a density of 30,000 cells per insert and maintained for 18-21 days, with replacement of medium every 2-3 days.
Cell monolayer integrity was checked by measurement of the transepithelial electrical resistance (TEER, Millicell-ERS ohmmeter, Millipore Corporation, Bedford, MA) before and after the experiment. Transport studies, as described under 2.4., were carried out on cell-monolayers on inserts with a transepithelial electrical resistance value of more than 250 Ω.cm^2.

Cytotoxicity assay

Cells were seeded in 96-well plates at a density of 30.000 cells per well and after an incubation period of 48 hours, medium was replaced by transport medium consisting of DMEM without phenol red, supplemented with 1% (v/v) non-essential amino acids and 2mM L-glutamine. To this medium Alamar blue (10% v/v) and either solvent DMSO (0.2%), ochratoxin A solution, various inhibitors alone or the combinations of inhibitors together with OTA were added.
Alamar blue reduction was measured by fluorescence, using the Fluostar Optima from BMG labtechnologies (Offenburg, Germany) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm after 1, 2, 3 and 24 hours of incubation at 37^{o}C in humidified air (5%

CO_2). Viability was regarded as decreased when Alamar blue reduction was less than 90% of the control value.

Transport studies

Transport experiments were conducted in transport medium consisting of DMEM without FBS and phenol red, supplemented with 1% (v/v) non-essential amino acids and 2mM L-glutamine. For concentration-dependent studies, the donor solution containing 1, 10, 50 or 100 µM OTA, respectively, was composed of unlabelled and radiolabelled (2.3 x 10^6 dpm [^{3}H]-OTA/ml) mycotoxin. This solution was added either to the basolateral compartment in a volume of 700 µl, or to the apical compartment in a volume of 300 µl, for measuring secretion and absorption, respectively. After 1 hour of incubation at 37°C in humidified air with 5% CO_2, samples of 200 µl were taken from the receiver compartments. Radioactivity was measured in a liquid scintillation counter (Tri-Carb 1900CA, Packard) after dispersion of the sample in 4 ml scintillation fluid (Ultima Gold, Perkin Elmer). At least 10,000 counts were recorded, except for the background samples.

Inhibition experiments to assess the involvement of active transporters in OTA transport across Caco-2 cells were conducted with various concentrations of the inhibitors MK571, cyclosporine A, GF120918, Ko143 and PSC833. Inhibitors were added to both apical and basolateral compartment, whereas only the donor compartment contained 100 µM OTA as described above. The concentrations used were 125, 25, 5.0, 1.0 and 0.2 µM for MK571; 25, 5.0, 1.0 and 0.2 µM for cyclosporine A; 5.0, 1.0 and 0.2 µM for GF120918; 1.0, 0.25, 0.50 and 0.01 µM for Ko143 and 5 µM for PSC833. In each inhibition experiment transport was also assessed in the absence of inhibitors as control measure.
In all experiments the final concentration of the solvents in the transport medium was 0.2% for DMSO and 0.1% for EtOH.

Data analysis

All statistical analyses and curve fittings were done using Graph Pad Prism software (version 2.01; Graph Pad software Inc., San Diego, California). Group comparisons of more than two groups were made by analysis of variance ANOVA, followed by Bonferroni's post test. Comparisons of two groups were made by t-test analysis.

The apparent permeability (Papp) was calculated according to the following equation:

(1) $$Papp = (Vx\ dC/dt) / (AxC_0)\ \ [cm\ s^{-1}]$$

V Volume of receiver compartment [cm^3]
dC/dt Rate of time-dependent increase of the concentration in the receiver compartment mol dm^{-3} s^{-1}]
A surface area of microporous membrane of the inserts [cm^2]
C_0 Initial concentration of OTA in the donor compartment [mol dm^{-3}]

Results

Cytotoxicity and monolayer integrity

The viability of Caco-2 cells after exposure to the test compounds was determined by the alamar blue reduction assay. Neither the test compounds as such, nor the combinations thereof affected cell viability, with the exception of the lowest concentration of cyclosporine A which exerted a transient reduction of cell viability during the first 2 hours of incubation, after 3 and 24 hours no significant loss could be observed (data not shown).
To exclude an effect of OTA or inhibitors on the integrity of the Caco-cell monolayer, TEER was not only measured at the beginning of the experiments but also at the end, following the given exposure to OTA (and the inhibitors). No changes in membrane integrity could be observed, indicating that under the chosen experimental conditions, neither OTA nor the co-exposure to OTA and the inhibitors affected the tight junctions within the cell monolayer.

Concentration-dependent transport of Ochratoxin A.

The characteristics of OTA absorption and secretion were investigated after 1 hour of incubation at four concentrations: 1, 10, 50 and 100 μM OTA. For all concentrations tested, the rate of secretion of OTA was higher than the rate of absorption. Results expressed as apparent permeability (Papp) are presented in fig.1. Secretory permeability was concentration-dependent and saturable, and Papp decreased from 7.5 x 10^{-6} to 4.7 x 10^{-6} cm/s with increasing concentrations of OTA. Absorptive permeability was constant and independent from the concentration used.

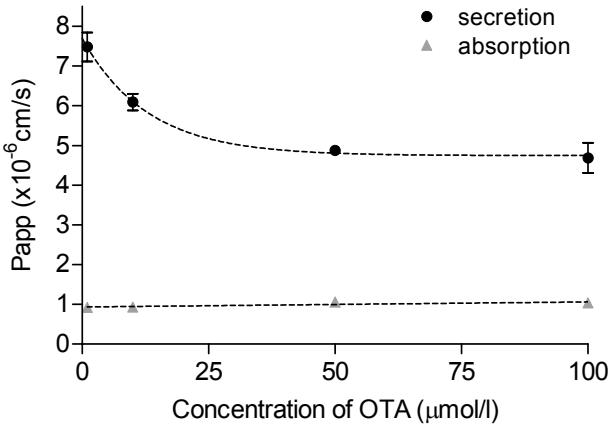

Figure 1. Concentration-dependent permeability of OTA across Caco-2 cell monolayers: Caco-2 cell monolayers on inserts were incubated for 1 hour with 1.0, 10, 50 or 100μM OTA, respectively, added to the apical or basolateral compartment. Each data point represents the mean ± standard deviation of three replicates.

Inhibition experiments

Inhibition experiments were performed with OTA at a concentration of 100 µM in the donor compartment and various concentrations of the inhibitors MK571, cyclosporine A, GF120918, Ko143 and PSC833, respectively, added to either compartment. Figure 2a shows that the MRP-specific inhibitor MK571 caused a concentration dependent decrease of OTA secretion, while absorption increased. Further analysis of these data using non-linear regression for curve-fitting (figure 2b), provided the best fit (R^2=1) for a two-site competition binding curve with EC_{50} values of 0.26 µM and 43.3 µM for secretion, with very wide confidence intervals for absorption in the presence of MK571.

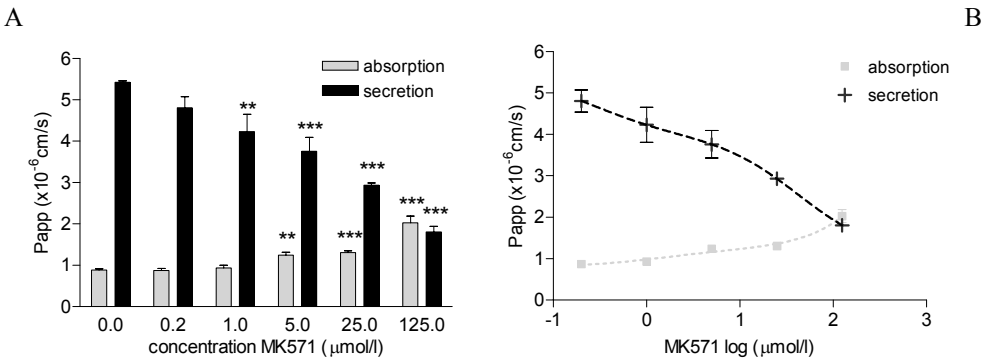

*Figure 2. Effect of MK571 on transmembrane-transport of OTA: Caco-2 cell monolayers on inserts were incubated for 1 hour with 100µM OTA, added to the apical or basolateral compartment, in the presence of various concentrations of MK571 in both compartments. Data represent means ± standard deviation of three replicates. Significant differences are marked ***, ** and * indicating P<0.001, P<0.01 and P< 0.05, respectively, as compared to the corresponding control values.*

The effect of cyclosporine A on OTA transport is demonstrated in figure 3. No effect could be observed with 0.2 µM cyclosporine A, but 1.0 and 5.0 µM cyclosporine A decreased secretory permeability, and 25 µM cyclosporine A caused a further decrease in secretion. No effect on the absorption of OTA was seen.

GF120918 decreased secretion of 100 µM OTA at a concentration of 1.0 µM GF120918, with a further decrease at 5 µM GF120918, whereas secretion was not changed at 0.20 µM (Figure 4). Absorption of OTA was increased at all three concentration of GF120918. When Ko143 was added, no effect was observed at 0.01 and 0.05 µM, but at higher concentrations of 0.25 µM and 1.00 µM Ko143, respectively, secretion decreased while absorption increased with increasing concentrations (Figure 5).

To test if the effects of cyclosporine A and GF120918 resulted from inhibition of P-gp, we used PSC833: At a concentration of 5 µM PSC833 did not significantly affect the absorption and secretion of 100 µM OTA (Figure 6).

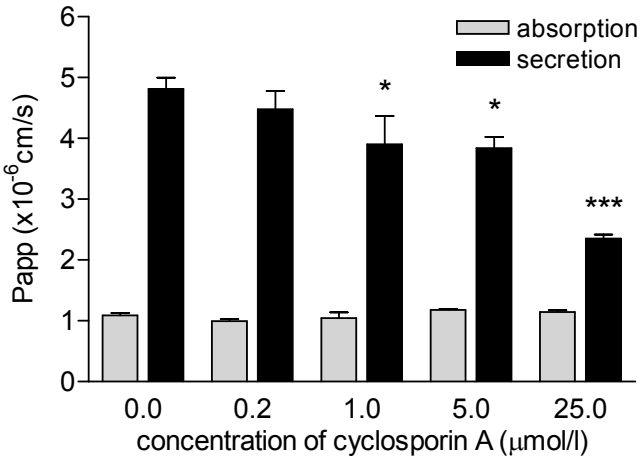

*Figure 3. Effect of Cyclosporine A on transmembrane-transport of OTA: Caco-2 cell monolayers on inserts were incubated for 1 hour with 100μM OTA, added to the apical or basolateral compartment, in the presence of various concentrations Cyclosporine A in both compartments. Data represent means ± standard deviation of three replicates. Significant differences are marked. ***, ** and * indicating P<0.001, P<0.01 and P< 0.05 , respectively, compared with controls.*

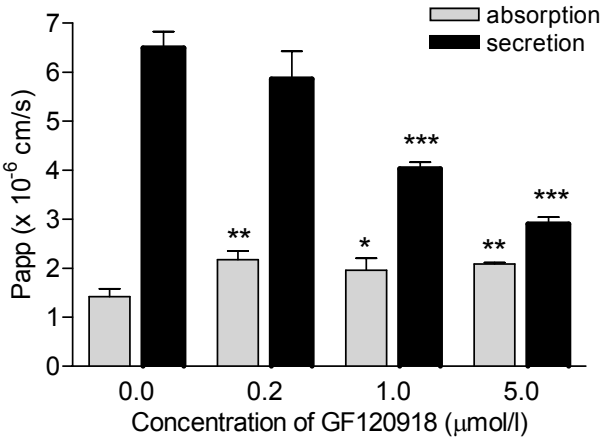

*Figure 4. Effect of GF120918 on transmembrane-transport of OTA. Caco-2 cell monolayers on inserts were incubated for 1 hour with 100μM OTA, added to the apical or basolateral compartment, in the presence of various concentrations GF120918 in both compartments. Data represent means ± standard deviation of three replicates. Significant differences are marked. ***, ** and * indicating P<0.001, P<0.01 and P< 0.05 , respectively, compared with controls.*

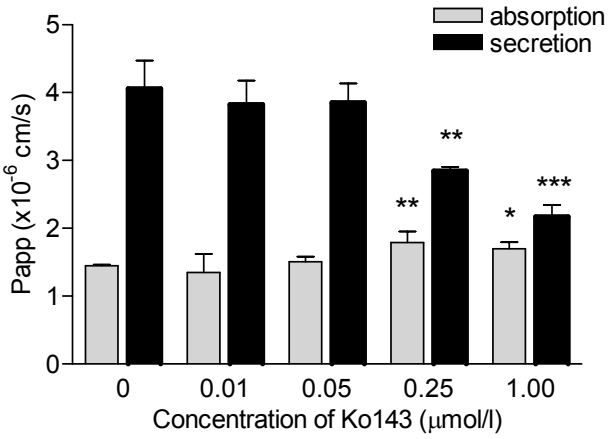

*Figure 5. Effect of Ko143 on transmembrane-transport of OTA: Caco-2 cell monolayers on inserts were incubated for 1 hour with 100μM OTA, added to the apical or basolateral compartment, in the presence of various concentrations Ko143 in both compartments. Data represent means ± standard deviation of three replicates. Significant differences are marked. ***, ** and * indicating P<0.001, P<0.01 and P< 0.05 , respectively, compared with controls.*

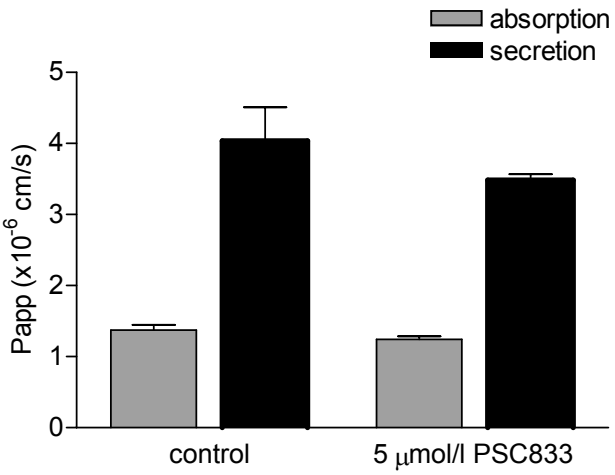

Figure 6. Effect of PSC833 on transmembrane-transport of OTA: Caco-2 cell monolayers on inserts were incubated for 1 hour with 100μM OTA, added to the apical or basolateral compartment, in the presence of 5μM PSC833 in both compartments. Data represent means ± standard deviation of three replicates. No significant differences were observed, compared with control values.

Discussion

Previous data had suggested that ochratoxin A (OTA) is a substrate for MRP2 (Leier *et al.*, 2000; Berger, 2003). However, reviewing these data we hypothesized that other ABC-transporters might be involved in OTA efflux as well. Hence it was the aim of this study to identify the contribution of individual ABC-transporters on OTA efflux from the apical side of polarized cells. Caco-2 cell monolayers were used as a model as these intestinal cells express multiple ABC drug efflux-proteins, including P-gp, BCRP and MRP1-6 (Prime-Chapman, 2004). P-gp, BCRP and MRP2 are localised to the apical membrane (Taipalensuu, 2001; Prime-Chapman, 2004). Initial experiments demonstrated that the permeability of OTA across Caco-2 cell monolayers in the basolateral to apical direction (secretory permeability) is concentration dependent and saturable, confirming that a carrier-mediated process is involved in the secretion of OTA. In contrast to secretion, the permeability of OTA absorption remained constant within the tested concentration range. Moreover, at all concentrations secretory permeability exceeded absorptive permeability. These findings are in agreement with previous results obtained with Caco-2 cell monolayers by Berger et al. (2003), who suggested that OTA is transported by MRP2.

For the assessment of the contribution of individual ABC transporters to the transmembrane transport of OTA at the saturating concentration of 100 μM, a set of selected inhibitors was used, including MK571 as an inhibitor of the MRP-family (Gekeler *et al.*, 1995), cyclosporine A as a dual inhibitor of P-gp and MRP2 (Chen *et al.*, 1999; Kamisako *et al.*, 1999), GF120918 as a dual inhibitor of P-gp and BCRP (de Bruin *et al.*, 1999), Ko143 as an inhibitor of BCRP (Allen *et al.*, 2002; Merino *et al.*, 2005; Xia, 2005) and PSC833 as an inhibitor of P-gp (Chen *et al.*, 1999; Bates *et al.*, 2001; Aouali *et al.*, 2005).

In the presence of MK571, secretory permeability for OTA decreased, while absorptive permeability increased. This inhibitory effect of MK571 on OTA secretion was expected, as Berger *et al.* (2003) already had described an inhibition of secretion by (non-)selective MRP2-inhibitors. Modelling our data, we found the best correlation with a two-site competition model, indicating two different binding sites for MK571 in the inhibition of OTA secretion. Therefore, the decreased OTA secretion might result from inhibition of more than one transport protein and hence different inhibitors were used to identify the other transporters involved in this process.

Cyclosporine A decreased secretion of OTA, which could result from either inhibition of MRP2, of P-gp or of both. Subsequently, the effect of GF120918 was tested. GF120918 was first identified as an inhibitor of P-gp and later as a BCRP inhibitor, at higher concentrations (de Bruin, 1999), whereas no effect can be observed on MRP2 mediated transport processes (Germann *et al.*, 1997; de Bruin, 1999; Wallstab *et al.*, 1999; Evers *et al.*, 2000; Tang *et al.*, 2002). A concentration-dependent inhibition of OTA secretion was observed in the presence of GF120918 at concentrations of 1 and 5 μM, with a corresponding concentration-independent

increase of the absorption. These findings suggested that BCRP contributes to OTA secretion. Since PSC833 did neither affect OTA absorption nor secretion, a contribution of P-gp in OTA transport can be excluded.

The contribution of BCRP was finally confirmed when we showed that Ko143 inhibited OTA secretion accompanied by an increase in OTA absorption. Our conclusion that BRCP is playing a significant role in OTA transport is indirectly supported by data from others: Berger *et al.* (2003) observed an inhibition of OTA efflux by genistein and CDNB (which could be attributed to non-specific inhibition of MRP2). Yet, genistein and DNP-SG, a glutathione conjugated metabolite of CDNB, were recently shown to be also substrates and inhibitors of BCRP (Suzuki *et al.*, 2003; Imai *et al.*, 2004). BCRP (ABCG2) was first described in 1998 (Allikmets *et al.*, 1998; Doyle *et al.*, 1998), and data on the inhibitory effect of substances on this transporter are still limited. As MK571 as well as inhibitors of BCRP decreased OTA secretion and in consideration of the finding that the concentration-dependent inhibition of OTA secretion by MK571 fitted to a two-site competition model, we attribute the effect of MK571 to the inhibition of MRP2 and BCRP. To the best of our knowledge, so far there are no other data available on the effect of MK571 on BCRP.
Very recently, another study was published which used Caco-2 cells for the evaluation of effects of polyphenols on OTA absorption (Sergent *et al.*, 2005). In the presence of selected flavonoids and resveratrol, an increase of absorption and cellular accumulation of OTA was found and attributed to inhibition of MRP2 efflux. The flavonoids (chrysin, quercetin, genistein, biochanim) and resveratrol are known to be inhibitors of BCRP (Cooray *et al.*, 2004; Zhang *et al.*, 2004; Zhang *et al.*, 2004a), whereas the flavenoid (-)-epigallocatechin-3-gallate (EGCG) that is not a BCRP inhibitor (Zhang, 2004a), did not affect OTA absorption in the studies by Sergent et al. (2005).

Identification of the role of individual transporters contribute to the understanding of absorption and elimination of OTA in individual organs, as differences in affinity and capacity of the individual transporters will influence the net result of intracellular uptake and efflux. Expression of BCRP in humans was found in various organs, including the intestines, liver, blood vessels, placenta (placental syncytiotrophoblast cells) (Maliepaard *et al.*, 2001), lactating mammary gland (Jonker *et al.*, 2005), 'side population' stem cells (Zhou *et al.*, 2001) and kidneys (Maliepaard, 2001; Langmann, 2003). The finding that OTA is a substrate for BCRP could explain its moderate oral bioavailability. Yet, bioavailability of OTA may vary in the presence of food components, many of which are BCRP substrates as well as inhibitors (*vide supra*). Considering the broad tissue distribution of BCRP, it can be assumed that this transporter facilitates also the biliary excretion of OTA and its secretion with breast milk, as demonstrated in humans (Turconi *et al.*, 2004). In the kidneys, considered as primary target sites of OTA toxicity, uptake of OTA *via* the basolateral membrane of the proximal tubule cells is facilitated by organic anion transporters (Sokol, 1988; Bahnemann, 1997; Jung, 2001), while re-absorption along the nephron is facilitated by organic anion transporters and by H^+-dipeptide co-transporter(s) (Schwerdt, 1997; Zingerle, 1997; Dahlmann, 1998; Schwerdt, 1998; Babu,

2002). The luminal efflux of OTA in proximal tubule cells has been attributed to OAT4, mediating re-absorption as well as efflux (Babu, 2002) and OATK1 (Dahlmann, 1998). Our new findings suggest that BCRP and MRP2 contribute to this process as well and seem to determine the intracellular concentration, and subsequently the site-specific toxicity of OTA. Since expression of MRP2 in the proximal tubule cell has been demonstrated previously by RT-PCR and immunological methods (Schaub *et al.*, 1999; Scheffer *et al.*, 2000; Sandusky *et al.*, 2002) and more recently also expression of BCRP in the kidney (Maliepaard, 2001; Langmann, 2003), it will be of interest to further unravel their role in OTA transport in this tissue in future studies.

In conclusion, MK571, cyclosporine A, GF120918 and Ko143 inhibit OTA secretion across Caco-2 cell monolayers. These findings suggest that OTA is not only a substrate for MRP2 but also for BCRP. Given the broad distribution of BCRP in different organs, the net absorption, distribution and excretion of OTA is likely to be modulated by substrates for this transporter that are abundantly present in food commodities.

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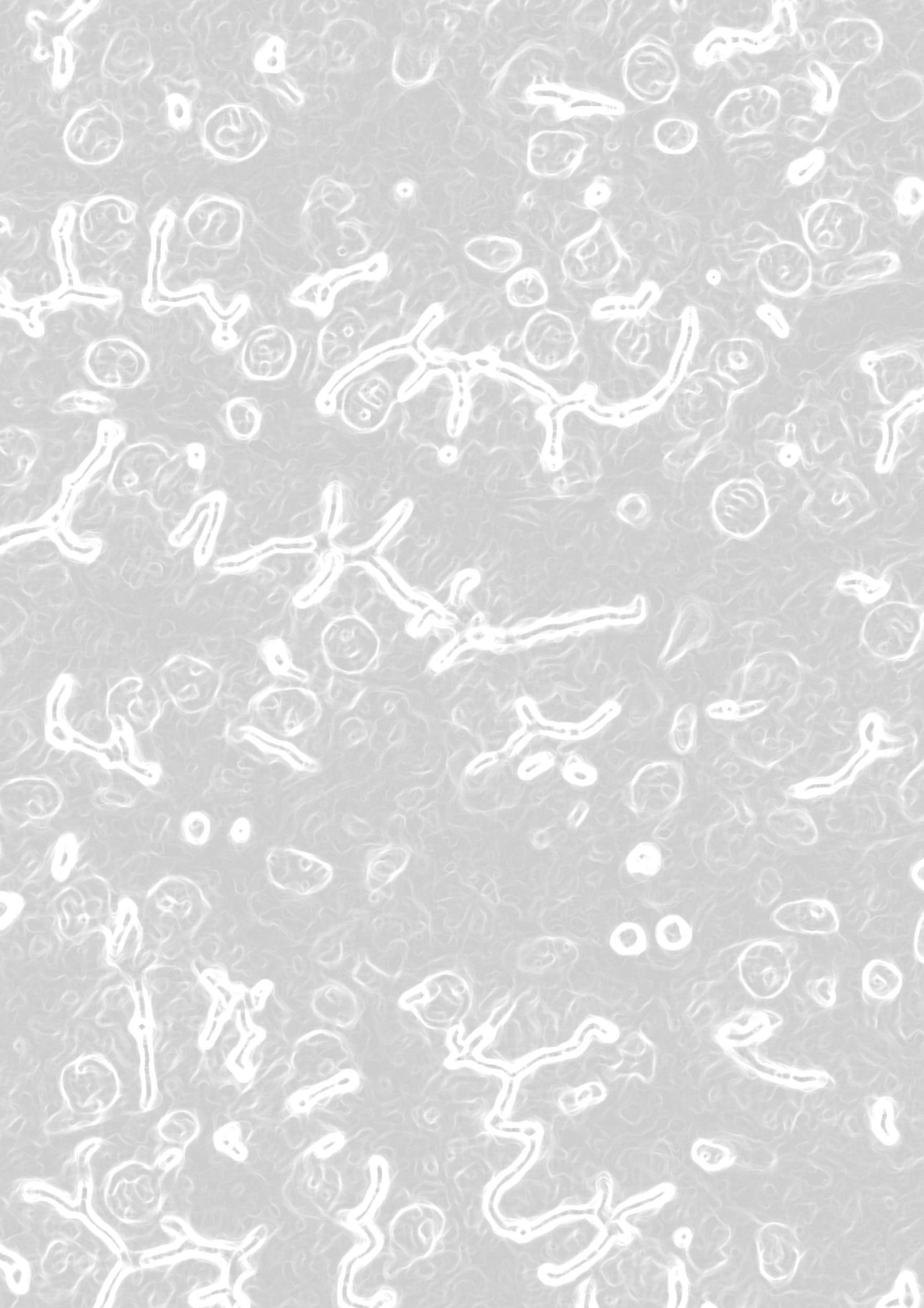

CHAPTER 5

Immunohistochemical detection of BCRP, P-gp and MRP2 in porcine tissues

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submitted

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Abstract

The membrane-bound efflux transporters BCRP, P-gp and MRP2 are significant determining factors in the kinetics of drugs and toxins. We used a panel of monoclonal antibodies directed against human BCRP, P-gp and MRP2 to study the distribution of these transporters in the porcine jejunum, colon, liver, kidneys and lung. Human tissues served as positive controls. The presence of BCRP, detected by BXP-21, was found at apical membranes of the intestinal epithelium, bile ducts, bronchi, bronchioles and the vascular endothelium. The monoclonal antibody, MM4.17 directed against P-gp, stained intracellular structures reflecting the Golgi system, with a limited membrane staining of epithelial cells of the intestines, hepatic bile ducts and bronchi. Using a second antibody against P-gp, C494, a similar distribution pattern was observed, however, with the typical membrane staining and with staining of bronchioles, the endothelium of vasculature and lymph vessels. No immuno-reactivity was detected for BCRP and P-gp in the apical membranes of porcine hepatocytes and kidney tubule cells. The antibody M2III-6 typically stained the membranes of the bile canalicular ducts, proximal tubules and the jejunum epithelial cells, indicating the presence of MRP2. In conclusion, the staining patterns strongly suggest that porcine BCRP and P-gp limit the absorption and facilitate the secretion of substrates into the intestines, bronchi and bronchioles but have a less prominent role in the excretion of substrates by the liver and kidneys. In contrast, porcine MRP2 seems to play a major role in the excretion of substrates by the liver and kidneys, with a limited role in the jejunum.

Introduction

Cellular membrane transporters have been recognized to play an important role in the disposition of xenobiotics. Generally, a distinction is made between uptake and efflux transporters. Cellular uptake transporters belong to the family of solute carriers (SLC), while most efflux transporters are members of the ATP-Binding Cassette (ABC) superfamily of transmembrane proteins (for review see Ho and Kim, 2005). ABC transporters are integrated in the cellular membrane to function as a primary active exporter of structurally diverse substrates from the intracellular to the extracellular space. For three ABC-efflux transporters, P-gp (ABCB1), BCRP (ABCG2) and MRP2 (ABCC2) it has been shown that they are expressed in the apical membranes of secretory cells in organs of various species, relevant for xenobiotic absorption and elimination (for review see Dietrich et al., 2003). The location in the intestines, liver and kidneys determines their role in pharmaco- and toxico-kinetics, as they limit absorption and facilitate excretion. In addition, the lung is a target for drugs either delivered via the pulmonary route (for review see Gonda, 2006) or systemically. Moreover, it also forms a first line of defense against potential toxic constituents of inhaled air and as these transporters have an overlap in their substrate recognition, they may therefore serve as a parallel protection line for potentially harmful xenobiotics.

The individual organ and (sub-)cellular distribution of P-gp, BCRP and MRP2 has been well-studied in human tissues (Thiebaut et al., 1987; Cordon-Cardo et al., 1990; Konig et al., 1999; Maliepaard et al., 2001), but only limited data are available about the cellular expression, apart from brain tissues, in animal species.

For example, MRP2 has been detected in the rat liver, kidney and intestines by immunohistochemistry (Mayer et al., 1995; Buchler et al., 1996; Schaub et al., 1997; Mottino et al., 2000; Scheffer et al., 2000; Rost et al., 2002; Cizkova et al., 2005; Villanueva et al., 2005), the rabbit small intestine (Van Aubel et al., 2000) and in the liver and kidney of the guinea pig, while no reactivity was observed in the murine liver and kidney by a panel of monoclonal antibodies (Scheffer, 2000). The subcellular expression of P-gp was demonstrated in the rat intestine (Omae et al., 2005) and lung (Campbell et al., 2003) and in various tissues in the dog (Ginn, 1996). Although, reactivity of the antibody C219 was observed in the liver of the mouse, rat and guinea pig (Scheffer, 2000), it does not definitely demonstrate the presence of P-gp, since it is not a specific antibody, also recognizing P-gp homologues (Georges et al., 1990), including the phospholipid transporter, MDR3 and the bile salt efflux protein, BSEP (Childs et al., 1995), that are highly expressed in the biliary canalicular membrane. BCRP was demonstrated in the mouse kidney (Jonker et al., 2002), liver (Aleksunes et al., 2006) and mammary gland (Jonker et al., 2005).

Despite the relevance of P-gp, BCRP and MRP2 in pharmaco- and toxicokinetics (for review see Leslie et al., 2005), comparative data are lacking for the (sub-) cellular distribution of these transporters in the intestines, liver, kidneys and lung for animals species, including the pig.

Hence, we studied the protein distribution of P-gp, BCRP and MRP2 in porcine tissues using immunohistochemistry.

Materials and methods

Animals

Crossbred pigs (Large white x Finnish landrace x Yorkshire) of approximately 10 weeks of age with an average body weight of 27 kg were used. The pigs were housed inside at the pig farm of the Faculty of Veterinary Medicine, Utrecht University and were fed a standard growing diet. The ethical committee of the Faculty approved the use of these animals as organ donors.

Tissue collection and storage

Tissues were collected from three healthy female pigs and of each sample, preparations were made with the following fixatives: Formalin (neutral buffered 4% formaldehyde), Bouin's fixative: saturated aqueous solution of picric acid (150 ml), 40% formaldehyde (50 ml) and glacial acetic acid (5 ml) and Carnoy's fixative: ethanol (60 ml), chloroform (30 ml) and glacial acetic acid (10 ml). After fixation, specimens were embedded in paraffin.

Antibodies

Monoclonal mouse antibodies of the same isotype (IgG2a) were used for the detection of the transporter proteins. BXP-21 directed against human BCRP was purchased from Abcam (Cambridge, U.K.); MM4.17 directed against human P-gp was obtained from Chemicon International (Hampshire, U.K.); C494 directed against human P-gp was purchased from Alexis (Lausen, Switzerland); M_2-III-6 directed against rat MRP2 was obtained from Monosan (Uden, the Netherlands).

Immunohistochemistry

Immunostaining was performed on 4μm paraffin tissue sections mounted on poly-L-lysine-coated slides. The tissue-sections were processed according to a standardized protocol and the different tissues of human and porcine origin were run in one session per antibody. After the slides were deparaffinized in xylene and rehydrated, they were immersed in 10 mmol/L preheated citric acid, pH 6.0 and boiled in a microwave oven for 10 minutes for antigen retrieval.

Endogenous peroxidase activity was blocked using 1% (v/v) H_2O_2 in methanol. The slides were than incubated with 10% normal equine serum for 30 minutes. This blocking serum was drained off and the slides were incubated with the primary antibodies overnight at 4^{o}C. Initially, a dilution series of the primary antibodies was tested and the preferred dilution was than applied to the tissue slides (liver, kidney, jejunum, colon and lung) of three animals. A biotin-conjugated horse anti-mouse antibody (1:125 for 30 min) followed by 30 min incubation with avidin-biotin peroxidase complex (ABC) (Vector laboratories, Ca, USA) was used for staining. As a chromogen 3,3'-diaminobenzidine (DAB, Sigma, St. Louis, MO, USA) was

applied. Washing between the treatments was performed with 0.01 mol PBS/1% Tween. The incubations were performed at room temperature, except for the incubation with the primary antibody, in a humidified chamber. Sections were counterstained with haematoxylin, dehydrated and cleared with xylene and mounted with Eukitt (Electron Microscopy Sciences, Hatfields, USA).
As positive controls, formalin fixed paraffin embedded human tissues (liver, kidney and colon) were stained parallel to the porcine sections and using the same procedure.

Results

T*he effect of three different fixatives and a dilution series of the primary antibodies on the staining results*

The dependency of the staining results on the fixative could be clearly demonstrated in our porcine tissue sections. A diffuse intracellular staining was observed for the tissue sections fixed in formalin and stained with a dilution series of either BXP-21, MM4.17 or C494. BXP-21 was not reactive in tissue sections fixed in Carnoy's fixative. Positive staining by BXP-21 was observed in sections fixed in Bouin's fixative, while a 1:10 dilution showed a dense specific staining of the brush border membranes of the jejunal epithelial cells, a cytoplasmic staining was also observed together with staining of nerve tissue. Although this latter staining might have been specific, a 1:40 dilution was preferred, specifically staining the brush border membranes with a minimal cytoplasmic staining in the epithelial cells. The diffuse intracellular staining by MM4.17 was also observed in tissue sections fixed in Bouin's and Carnoy's fixative, but this was minimal in Carnoy's fixative when the antibody was diluted 1:80, that was further used for analysis of all slides. The staining results with C494 applied on tissue slides fixed in Carnoy's fixative were weak, but the tissue slides fixed in Bouin's fixative had a dense positive staining with a minimal intracellular staining when diluted 1:160 that was further used for the analysis of all tissue sections. The monoclonal M2III-6 demonstrated a positive staining in all fixatives with absent nonspecific staining patterns. The sections fixed in Bouin's fixative demonstrated the strongest signal and were further used for analysis with a dilution of M_2III-6 of 1:40.

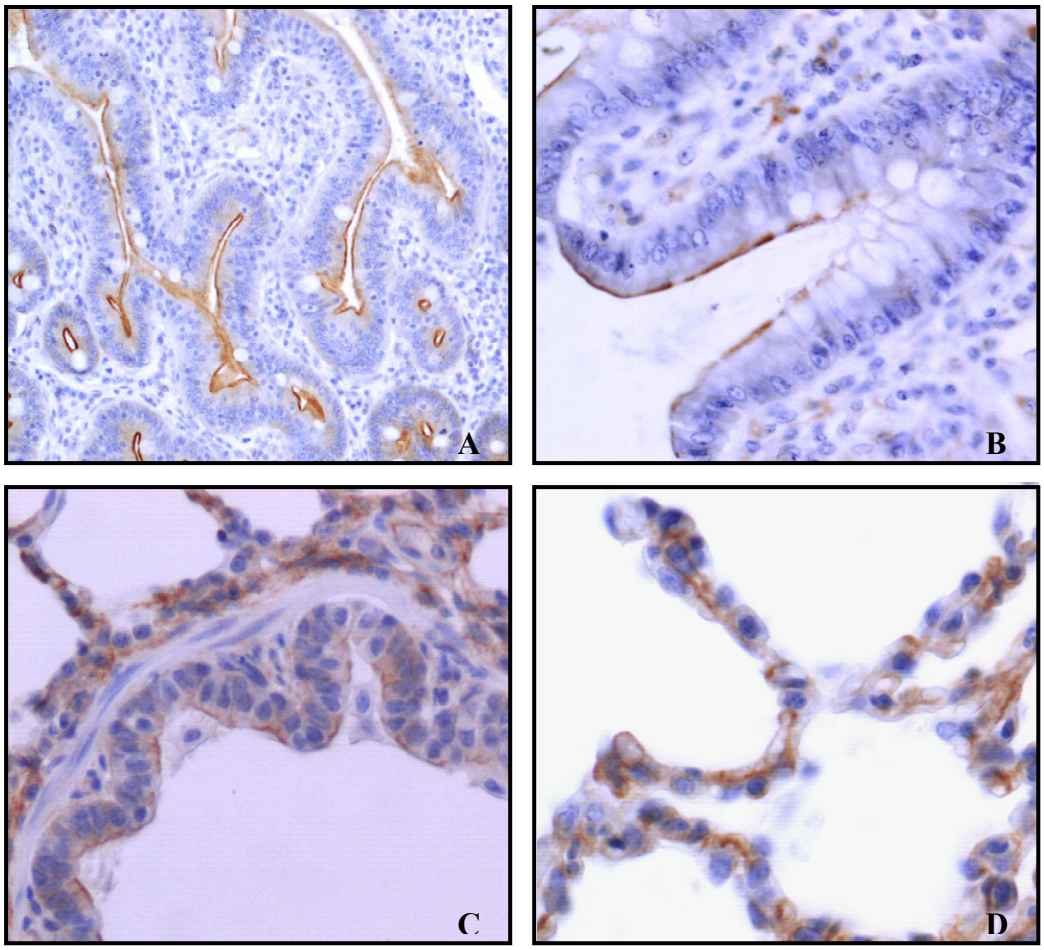


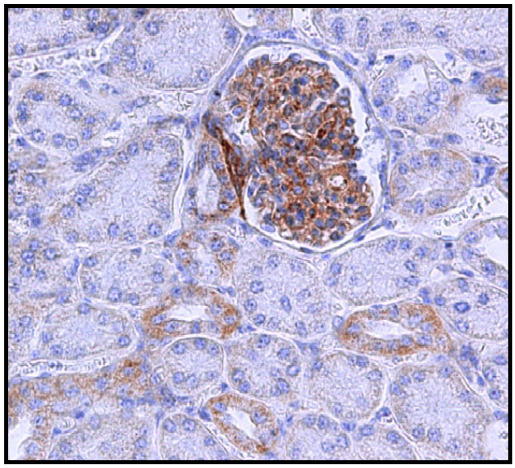
Figure 1. Immunostaining of porcine tissue sections by BXP-21. A. Jejunum, B. Colon, C. lung bronchus, D. Lung alveolus

BCRP detection by BXP-21 in porcine tissue sections

Immunohistochemical analysis of BCRP expression in the tissue sections (see Fig. 1.) demonstrated a strong but in intensity decreasing staining of the brush border membranes along the crypt-villus axis in the jejunum (Fig. 1A) and in the colon (Fig. 1B) as well as of fibroblasts in the lamina propria. Staining of the luminal side of the endothelial layer of large venules and lymph vessels was detected in the jejunum of one animal.

In the porcine liver only the apical membrane of the epithelial cells in the bile ducts were positive. The distal tubules of the kidney showed an intracellular basal staining with a rod shaped aspect that remained positive in the medulla; this was considered a non-specific

reactivity (Fig. 2.). Endothelium in the glomerulus and the vascular pool stained positive, while the proximal tubules, collecting ducts and large vessels were negative (Fig. 2.).



A moderate to strong staining was observed in the lung, lining the bronchial and bronchiolar epithelium and the alveolar wall highly suggestive for the presence of BCRP in type I pneumocytes on the abluminal side (Fig. 1 C and D). A slight and inconsistent staining was detected on the luminal side of endothelium of veins, arteries and lymph vessels, while venules and capillaries were mostly negative. The tissue slides of one animal were stained minimally or staining was completely absent.

Figure 2.Immunostaining of porcine kidney by BXP-21

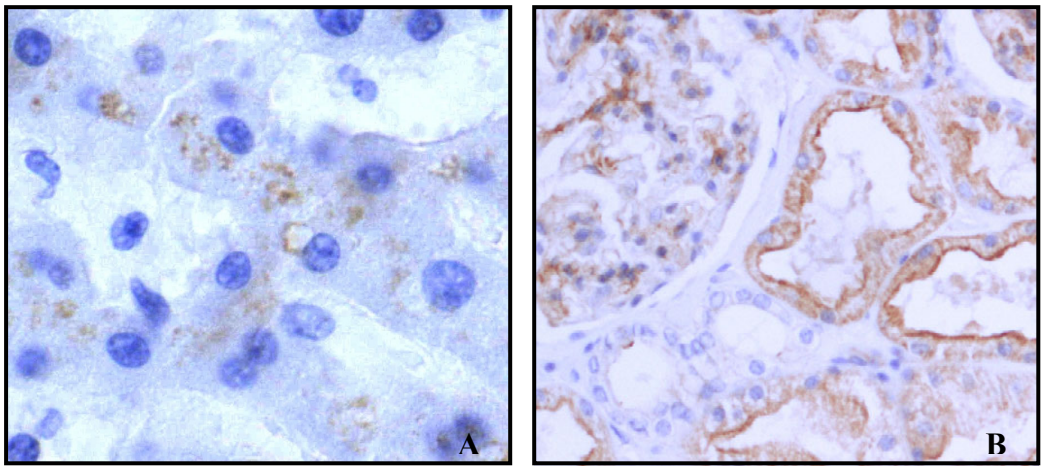


Figure 3. Immunostaining of human tissue sections by BXP-21. A. Liver, B. Kidney

BCRP detection by BXP-21 in human tissue sections

In the human control slides a specific staining was observed of the brush border membranes of the colon epithelial cells, the bile canalicular membrane (Fig. 3A), the luminal membrane of bile duct epithelial cells, glomerular endothelium and the brush border membranes of kidney proximal tubule cells (Fig. 3B). The proximal tubule cells and the intestinal cells showed a diffuse intracellular staining, that was weaker than the membrane staining and may be caused

by the fixation method since this was also observed in our porcine intestinal slides fixed in formalin as well as previously in formalin-fixed tissues (Julio E. Diestra, 2002).

P-gp detection by MM4.17 in porcine tissue sections

The results of the tissue sections incubated with MM4.17 are presented in Figure 2. A strong positive reaction was detected in the epithelial cells lining the intestinal tract (Fig.4A), the distal tubules of the kidney and the epithelial cells of the larger bronchi in the lung. Fibroblasts in the intestinal lamina propria stained mildly positive. Incubation of the liver slides demonstrated a strong staining of the bile ducts, but no staining could be detected in the liver parenchymal or supporting tissue. Although a slightly diffuse intracellular staining was noted in the positive cells, the greatest intensity was observed in the perinuclear region for the intestines (Fig. 4B), bile ducts and bronchi, while a cytosolic granular staining on the apical side of the distal tubule epithelium was observed. The jejunum and liver sections showed an irregular staining of the brush border membranes, and an apical membrane staining was seen in one of the two positive lung sections. Endothelium of vasculature and lymph vessels stained slightly to moderately positive, although it was an inconsistent observation.

P-gp detection by C494 in porcine tissue sections

We next evaluated the presence of P-gp by the use of the monoclonal antibody C494 (Fig. 4 C-F). A positive staining was then detected of the same cell-types as for MM4.17, but also of additional cell types. A dense staining was observed of the brush border membranes of the epithelial cells in the jejunum (Fig. 4C) and colon (Fig. 4D), but also on the luminal side of the lymph vessels and blood capillaries and venules. The latter two findings were also observed in the liver and in the kidneys. The brush border membranes of the bile duct epithelium demonstrated a strong staining and the sinusoidal endothelium in the liver stained moderately positive (Fig. 4E), but no staining could be detected in the hepatocytes. In the kidneys, the glomerular mesangium was stained and a strong rod shaped staining was observed intracellular, basolateral in the distal tubule epithelial cells that remained positive in the medulla, while the proximal tubules stained moderately positive on the basolateral membrane in two animals. These latter two atypical staining patterns in the kidneys were considered to be non-specific.
In the lung, the brush border membranes of the bronchi and bronchioles stained moderate to strong as did the capillary endothelium in the alveolar septa (Fig. 4F). Endothelium of veins, venules and arteries stained slightly to moderately positive.
Again, the tissue slides of one pig were stained minimal or staining was completely absent for both MM4.17 and C494, however it was a different animal than for BCRP.

P-gp detection by MM4.17 human tissue sections

Human tissues incubated with MM4.17 resulted in a weak to moderate diffuse intracellular staining in all slides. In the liver (Fig 5A), a dense granular staining of the biliary canalicular membrane of the hepatocytes, the bile duct epithelium and a moderate staining of the

sinusoidal lining was detected. The epithelial lining in the colon stained slightly positive. The proximal tubules in the kidneys stained negative, while a non-specific moderate to strong intracellular granular staining was observed in the distal tubules and the thick walled parts of Henle's loop, the mesangium stained moderately positive. A positive, though inconsistent staining was detected in the endothelium of the vasculature (veins, venules and arterioles) in all tissues (Fig 5B). Finally, a non-specific staining of muscle layers was observed.

P-gp detection by C494 in human tissue sections

Incubation of the human tissues with C494 showed a moderate reaction at the canalicular membranes of the hepatocytes (Fig. 5C) and apical membranes of the epithelial cells lining the bile ducts. A strong reaction was detected at the brush border membranes of epithelial cells in the colon (Fig. 5D) and proximal tubule cells, both with a slight to moderate intracellular staining. A moderate but inconsistent staining was detected in the mesangium. Endothelium of the vasculature stained moderately positive, although the pattern was inconsistent.
A nonspecific reaction was noted by reactivity of muscle layers.

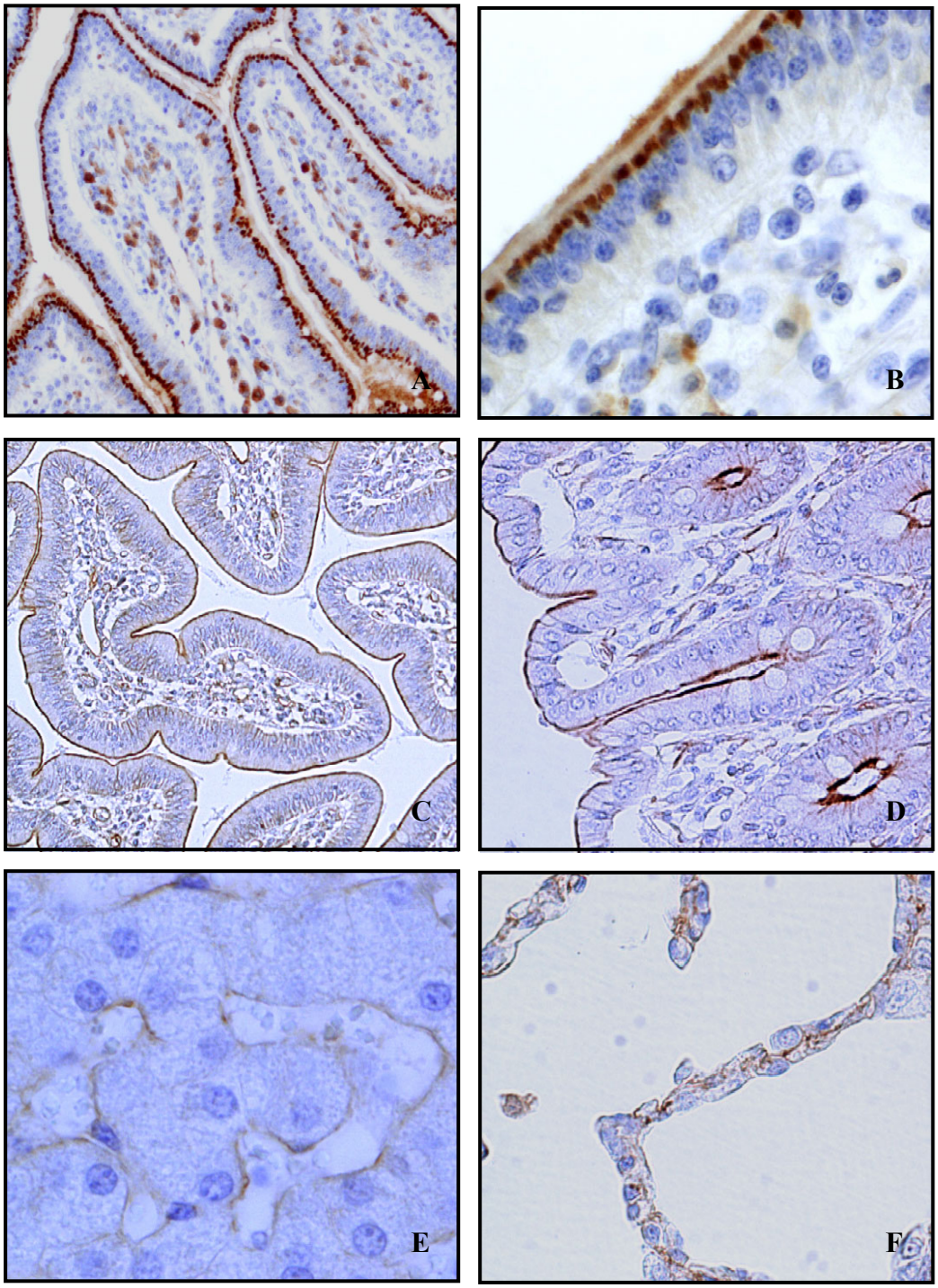
MRP2 detection by M_2III-6 in porcine tissue sections

Analysis of the five different tissues demonstrated a slight to moderate staining in the jejunum brush border membranes of the villi (Fig. 6A.), while no staining was observed in the colon. A strong staining was detected in the liver bile canalicular membranes (Fig. 4B). No staining could be detected in the structures of the portal areas. A specific staining of the brush border membranes of the proximal tubules in the kidney remaining positive in the medulla was detected (Fig. 4C). Lung tissue was negative.

MRP2 detection by M_2III-6 in human tissue sections

The specimens of human tissue demonstrated a specific staining of the biliary canalicular membrane and the brush border membranes of the proximal tubule cells, with a negative staining of the colon.

A summary of the staining results in the porcine tissue sections is presented in table 1.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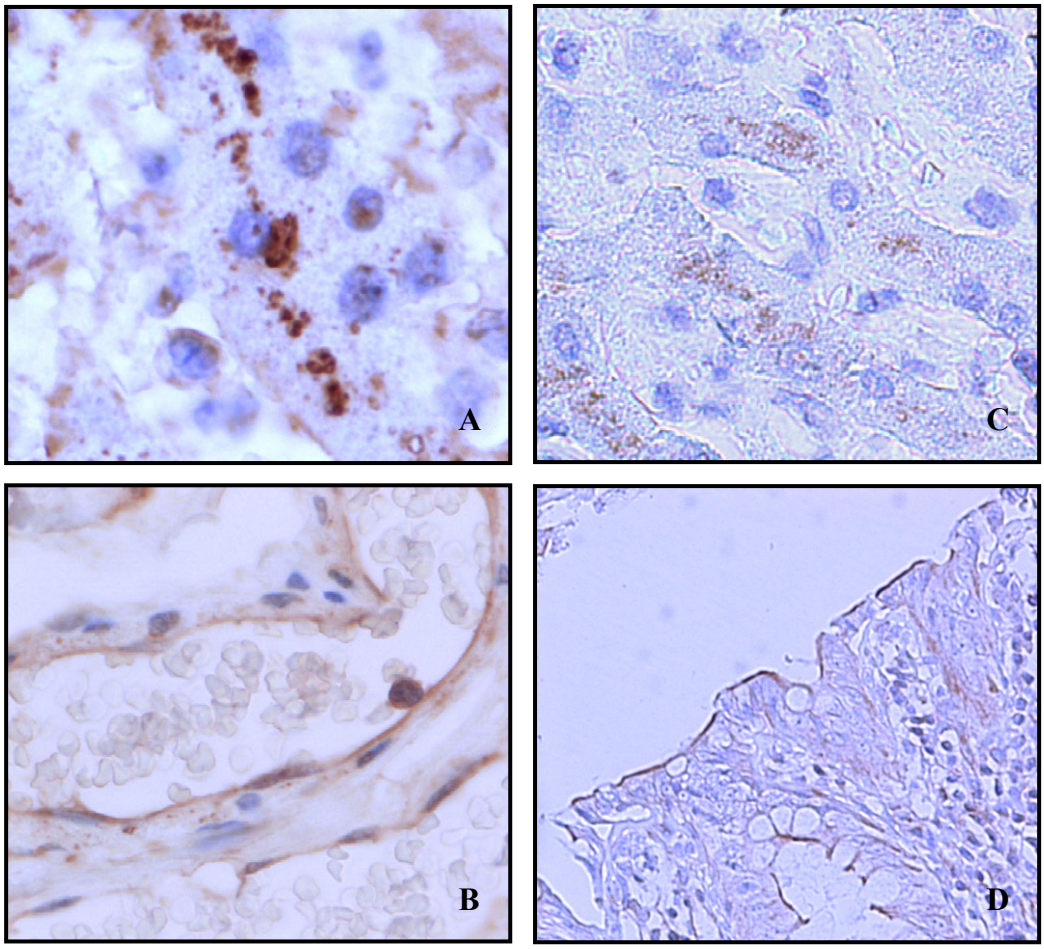

Figure 5. Immuno-staining of human tissue sections. A-B MM4.17, A. Liver, B. vaculature colon mucosal layer. C-D C494. C. liver, D. colon.

Left page: Figure 4. Immunostaining of porcine tissue sections by MM4.17 (A and B) and C494 (C-F). A. jejunum, B. jejunum magnified view, C. jejunum, D. colon, E. liver, F. lung alveolus.

Table 1. Summary of results

Porcine tissues	BCRP (BXP-21)	P-gp (MM4.17)	P-gp (C494)	MRP2 (M_2III-6)
Liver				
Bile canaliculi	-	-	-	++
Sinusoidal endothelium	-	-	+	-
Bile duct epithelium	++	++	++	-
Intestines				
Epithelium	++	++	++	+ [1)]
Endothelium vasculature	+	+-	+	-
Endothelium lymph vessels	-	-	+	-
Fibroblasts	+	+	+	-
Kidney				
Glomerulus				
Mesangium	-	-	+	-
Endothelium	+	-	-	-
Proximal tubule epithelium	-	-	-	++
Distal tubule epithelium	+ (n.s.)	++ (n.s.)	++ (n.s.)	-
Lung				
Bronchioles	+ / ++	-	+	-
Bronchi	+ /++	++	+	-
Endothelium vasculature	+-	+-	+	-
Pneumocytes type I	+ / ++	-	-	-
Endothelium lymph vessels	+-	+-	+	-

[1)] jejunum only
(n.s.) non specific
++ strong staining
+ moderate staining
+- slight staining

Discussion

We have analyzed the cellular distribution of BCRP, P-gp and MRP2 in porcine paraffin-embedded tissues using the monoclonal mouse antibodies MM4.17, C494, BXP-21 and M_2III-6. At first, the effect of three different fixatives on the staining results was assessed and major differences in reactivity were observed. Tissues fixed in Bouin's fixative were subsequently used for the analysis of protein expression incubated with BXP-21, C494 and M_2III-6, while tissues fixed in Carnoy's fixative were incubated with MM4.17.

In several porcine tissues incubated with BXP-21, a strong reactivity was observed that is subcellular specific, and similar to the location of BCRP in humans (Maliepaard, 2001), providing evidence that BXP-21 recognizes the porcine BCRP homologue. BCRP was highly expressed in the intestinal epithelium of both the jejunum and colon indicating a role similar to

human and rodent species in the regulation of absorption and secretion of substrates. The observed positive staining of the venous and capillary endothelial cells and only sporadic staining in arterial endothelium is in line with human data (Fetsch *et al.*, 2006), where BCRP has also been localized to the endothelium (Maliepaard, 2001). A protective role under hypoxia has been suggested to account for its presence (Krishnamurthy *et al.*, 2004). The presence of BCRP in the bronchiolar epithelium, identical to human (Scheffer *et al.*, 2002), indicates a role in the secretion of substrates into the lumen, whereas the suggested expression of BCRP on the abluminal side of type I pneumocytes in our porcine lung sections is evocative for a facilitated alveolar uptake of substrates.

In contrast to P-gp (Thiebaut, 1987; Ernest *et al.*, 1997) and MRP2 (Schaub *et al.*, 1999), expression of BCRP protein in the brush border membranes of proximal tubule cells in the human kidney had not yet been demonstrated (Maliepaard, 2001), but here it is demonstrated for the first time in our human control slides. Reflecting inter-individual variation in expression that was also noted in our tissue sections from the individual animals.

For the immunological detection of P-gp we have used two antibodies, MM4.17 and C494. The monoclonal antibody MM4.17 markedly stained the epithelial cells in the jejunum, colon, the bile ducts, the distal tubules of the kidney and the bronchi. A cellular staining pattern that could be expected based on data in humans, however no staining was observed in the hepatocytes and in the kidney proximal tubule cells. The subcellular localization in the intestinal tract, bile ducts and bronchi appeared to be perinuclear and in the brush border membrane, although the latter was inconsistent. MM4.17 has been previously used to detect the expression of P-gp on apical cell-surfaces in tissues of human origin (Cianfriglia *et al.*, 1994). In human cancer cells, MM4.17 stained the Golgi apparatus (Molinari *et al.*, 1994; Molinari *et al.*, 1998) and it was suggested that P-gp was responsible for the accumulation of doxorubicin in the Golgi apparatus, however without evidence of a direct relationship. And although this accumulation could have resulted from uptake by other transporters such as MRP1 (Rajagopal and Simon, 2003), it is noteworthy that the Golgi localization has been reported recurrently using different methods (Willingham *et al.*, 1987; Ernest, 1997; Lala *et al.*, 2000; De Rosa *et al.*, 2004; Petriz *et al.*, 2004). Nevertheless staining of the Golgi apparatus could have resulted from positive staining of newly synthesized P-gp (Sai *et al.*, 1999) and a stronger affinity of MM4.17 to immature P-gp in the pig as well as in human cancer cells. The intracellular identification of P-gp by MM4.17 is supported by the overlapping cellular localization pattern of MM4.17 and C494, the latter staining the typical luminal membrane localization of P-gp (Thiebaut *et al.*, 1987; Cordon-Cardo *et al.*, 1990; Ernest, 1997).

Although various reports describe an intracellular localization of P-gp different from the Golgi system, such as caveolae and cytoplasmic vesicles (Adam B. Shapiro, 1998; Lavie *et al.*, 1998; Demeule *et al.*, 2000) and both antibodies directed against P-gp in the present study stained the kidney distal tubule cells intracellular, we considered these findings to be non-specific. Not only the cellular localization is atypical, also the intracellular localizations for the reactivity by

MM4.17 and C494 were different, staining by MM4.17 was mainly on the apical side, but without the typical Golgi-shape, and staining by C494 was mainly on the basolateral side.

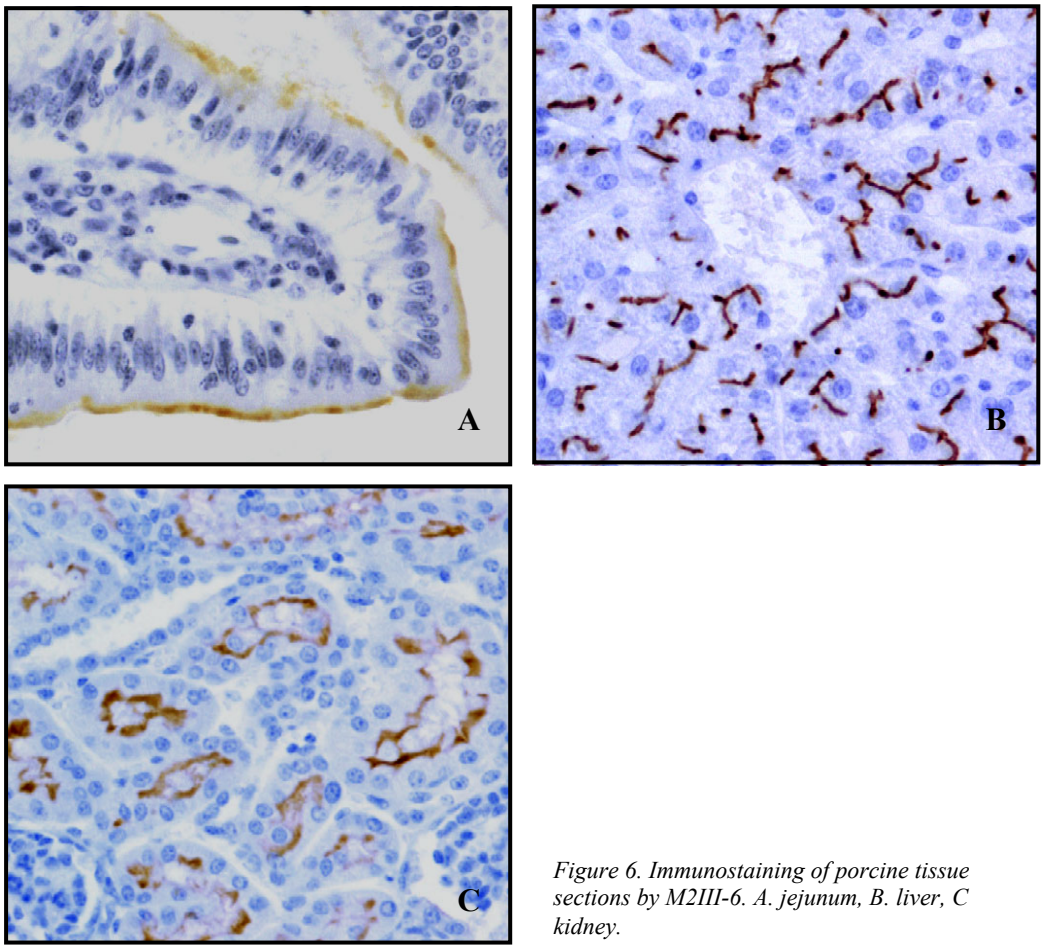

Figure 6. Immunostaining of porcine tissue sections by M2III-6. A. jejunum, B. liver, C kidney.

The presence of P-gp in the lung conducting airways is similar to humans (Lechapt-Zalcman *et al.*, 1997), but the alveolar wall staining in our slides was highly suggestive for P-gp in the capillary endothelium in contrast to the previously observed expression of P-gp on the luminal side of alveolar type I cells in the human lung (Campbell, 2003). Our results insinuate that the alveolar wall does not form a barrier mediated by P-gp (and BCRP) against inhaled drugs and toxicants, but might even facilitate the absorption.
Whereas a major role of P-gp and BCRP in the blood-brain barrier is attributed to their expression in the endothelial cells of the cerebral vasculature (for review see Loscher and Potschka, 2005) where the paracellular passage is limited by tight junctions and a continuous basal membrane, data about the effect of these transporters in the vasculature of tissues

different from those protected by a similar barrier (Bazzoni and Dejana, 2004) on xenobiotic disposition are absent and needs further evaluation.

We also have observed the expression of P-gp and to a lesser extend BCRP on the luminal side of lymph vessels in the intestines and lungs, which may enhance the absorption of lipophilic drugs and toxins via the lymph.

The monoclonal antibody M_2III-6 highly reacts with porcine MRP2, as demonstrated by the staining of the typical structures for expression (for review see Schaub, 1997; Konig, 1999; Schaub *et al.*, 1999; Scheffer, 2000; Villanueva, 2005), the biliary canalicular membrane, the brush border membrane of the jejunum and the brush border membrane of proximal tubule cells.

We have thus found expressions of porcine P-gp, BCRP and MRP2 overlapping those in human tissues. BCRP and P-gp demonstrate an overlapping tissue expression with the typical localizations in the intestines, lung bronchi (-oles) and vascular endothelium, however we have also noted major species differences for P-gp and BCRP. While in human liver P-gp and BCRP are expressed in the canalicular membrane of hepatocytes (Thiebaut, 1987; Maliepaard, 2001), which also stained positive in our human control slides, we did not observe this in the porcine liver slides. We observed the expression of P-gp in the liver sinusoidal endothelium, however, further extending the vascular localization of P-gp. The absence of specific staining in the porcine kidneys, the brush border membranes of proximal tubule cells, indicate a low expression level, suggesting that P-gp and BCRP have a limited role in the biliary and urinary excretion of substrates in the pig.

Considering the above results it is thus very probable that porcine P-gp and BCRP limit the absorption and facilitate the secretion of substrates in the intestines, bronchi and bronchioli and have a less prominent role in the excretion by the liver and kidneys. The absorption of lipophilic compounds may be enhanced by P-gp and BCRP in the lymph vessels, while the effect of P-gp and BCRP in the vasculature on substrate disposition remains a challenging subject for further research as well as a possible role of BCRP in the alveolar type I cells. Porcine MRP2 was detected at the typical localizations suggesting a role similar to other species in the elimination of mainly conjugates of endogenous and exogenous substances.

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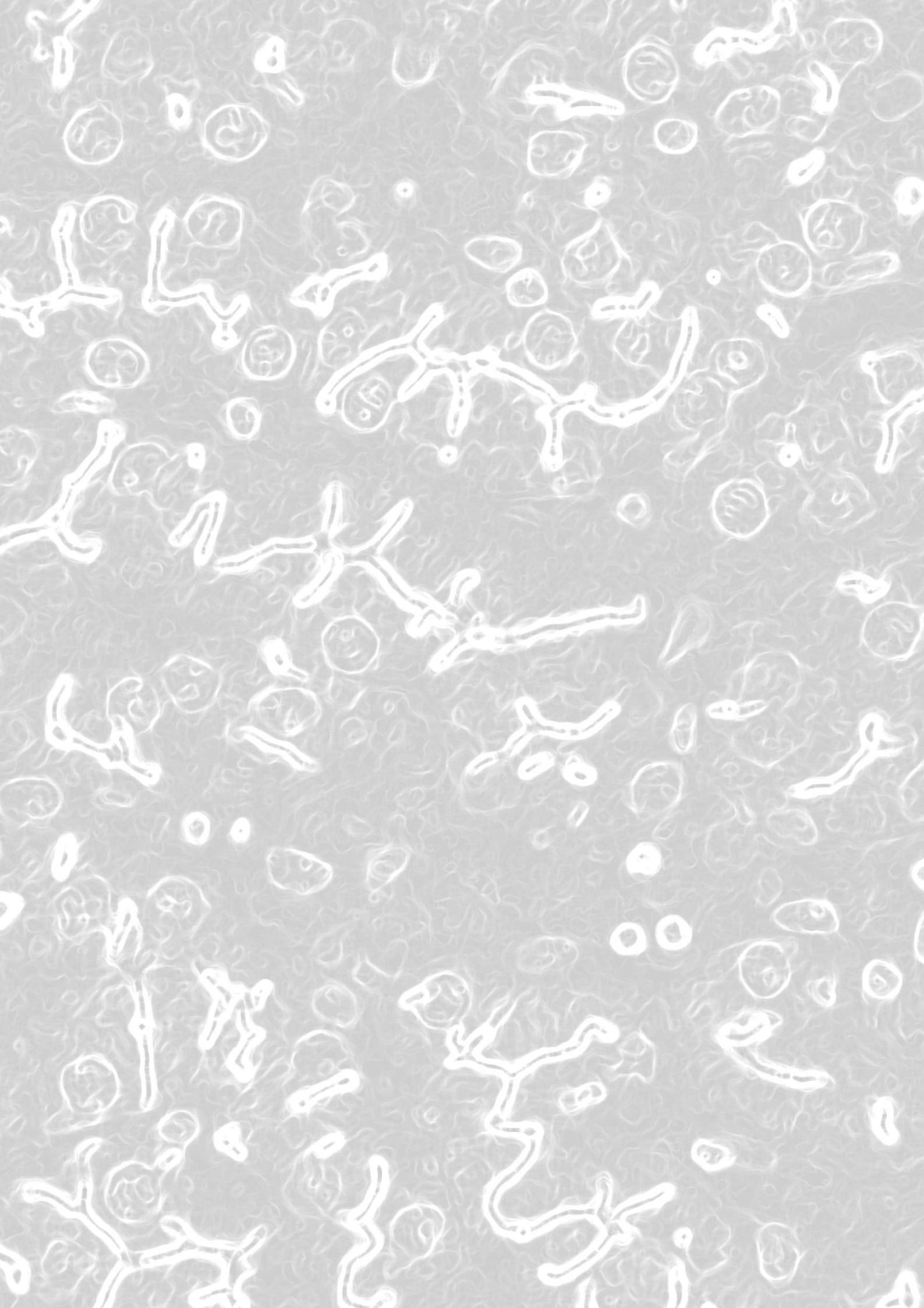

CHAPTER 6

Expression of ABCB1, ABCC2 and ABCG2 in porcine tissues

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Submitted

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Abstract

The ATP-Binding Cassette transporters, P-gp, MRP2 and BCRP, have a considerable role in the absorption, distribution and elimination of xenobiotics. The expression of these transporters has been mainly studied in humans and rodents, however, a comprehensive and comparable set of expression data for the pig is lacking. Hence, by quantitative RT-PCR analysis, the expression of P-gp, MRP2 and BCRP in porcine tissues was measured. The expression of P-gp and BCRP was detected in all tested tissues, while MRP2 expression was detected in the small intestines, liver and kidneys. These data are highly similar to those previously reported in human studies. However, differences in expression were observed for the colon and the kidneys with a low expression of P-gp and for the liver with a low expression of BCRP. These data provide relevant information regarding the interpretation of drug and toxin handling in the pig.

Introduction

The ATP-Binding Cassette (ABC) proteins comprise a large family of membrane bound ATP-dependent transporters, translocating a wide range of substrates across biological membranes. To date, 49 transporters have been identified in humans and have been phylogenetically classified in 7 subfamilies of transporters (ABCA-ABCG). ABC transporters play important roles in diverse physiological processes (Borst and Elferink, 2002) and in the transport of xenobiotics and their metabolites. P-glycoprotein (P-gp), encoded by the ABCB1 gene was the first mammalian ABC transporter to be discovered (Juliano and Ling, 1976). This finding led to the understanding of multi-drug resistance in cancer therapy and stimulated the interest in the role of ABC transporters in pharmacokinetics. Multi-drug resistance protein 2 (MRP2), encoded by the ABCC2 gene was formerly called canalicular Multi-specific Organic Anion Transporter (cMOAT) (Mayer *et al.*, 1995; Paulusma *et al.*, 1996). MRP2 is primarily an organic anion transporter, recognizing endogenous metabolites, xenobiotics and conjugates (Konig *et al.*, 1999). Breast Cancer Resistance Protein (BCRP), the ABCG2 gene product, is one of the latest discovered members of the ABC transporter family (Allikmets *et al.*, 1998; Doyle *et al.*, 1998). Expression of BCRP is associated with drug resistance to chemotherapeutics and with the protection of the body from xenobiotics (for review see Doyle and Ross, 2003). A common feature of P-gp, BCRP and MRP2 is that they are localized in the apical membrane of epithelial cells and that they recognize a wide range of drugs and toxins, among the latter 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (Dietrich *et al.*, 2001; van Herwaarden *et al.*, 2003), arsenic (Kala *et al.*, 2000), diazinon (Lecoeur *et al.*, 2006), benzopyrene (BP) conjugates (Ebert 2005), ochratoxin A (OTA) (Berger *et al.*, 2003; Schrickx *et al.*, 2006), aflatoxin B1, Ilimaquinone (IQ) and 3-amino-1,4-dimethyl-5H-pyrido[4,3-b]indole (Trp-P-1) (Herwaarden *et al.*, 2006). Due to the broad substrate specificity of these transporters and their affinity to gluthathione, glucuronide and sulphate conjugates, it can be expected that an increasing number of toxins and drugs will be recognized as transported compounds in the near future.

The tissue-specific expression determines the significant impact of ABC-transporters on the disposition of xenobiotics and variations in expression results in variations in organ exposure. Interspecies variations in expression of ABC-transporters have previously been observed in rodents, but only little data are available about the expression of these transporters in the pig. Expression of P-gp has been demonstrated by RT-PCR analysis in the porcine liver and brain capillary endothelial cells (pBCECs) (Childs and Ling, 1996), and in the kidneys (Goh *et al.*, 2002). BCRP expression has been demonstrated in various porcine tissues including brain tissues (Eisenblatter and Galla, 2002), whereas MRP2 was detected in the porcine kidney and liver (Goh, 2002), primary proximal tubule cells (Schlatter *et al.*, 2006) and brain capillaries (Miller *et al.*, 2000).

In consideration of the increasingly recognized role of P-gp, MRP2 and BCRP in xenobiotic disposition (for review see Leslie *et al.*, 2005) and with the aim to complete the former findings we have studied the expression in porcine tissues by quantitative RT-PCR.

Materials and methods

Animals

Six crossbred pigs (Large white x Finnish landrace x Yorkshire), approximately 10 weeks of age and with an average body weight of 27 kg served as experimental animals. The pigs were housed in the animal unit of the Faculty of Veterinary Medicine, Utrecht University, and were fed a standard growing diet. The ethical committee of the Faculty approved the use of these animals as organ donors.

Tissue samples

Tissue samples from stomach, duodenum, jejunum (proximal-1 and middle part-2), ileum, caecum, colon (ascendens), rectum, liver, pancreas, kidney, lung, heart, brain, cerebral cortex, superficial vasculature of the brain, choroidal plexus, adrenal gland, uterus, juvenile mammary gland, skeletal muscle, salivary gland (Parotid) and lymphocytes, were collected from healthy pigs. The pigs were sacrificed by an high dose of pentobarbital (Euthesate, Seva Santa Animale, France) and the tissue was immediately collected and snap frozen in liquid nitrogen. Samples of the choroidal plexus were collected by careful scraping of the upper cell layer. Lymphocytes were isolated from peripheral blood on a Ficoll density gradient and further purified by washing twice in phosphate buffered saline and a subsequent macrophage attachment step by selective plating. The samples were stored at –70^{o}C until isolation of RNA.

RNA isolation

Tissue samples (30-50 mg) were mechanically homogenized (ultra-turrax, IKA, Staufen, Germany) in lysis buffer and total RNA was isolated using the SV-total RNA isolation columns (Promega), according to manufactures' descriptions including a DNA-ase treatment step. The RNA was quantified spectrophotometrically at 260nm (ND-1000, Nanodrop technologies) and stored at –70^{o}C.

cDNA synthesis

First strand cDNA synthesis was performed with the iScript^{TM}cDNAsynthesis kit from Bio-Rad (Hercules, CA, USA). A quantity of 2 μg total RNA was added to the mixture containing both oligo(dT) and random hexamer primers in a final volume of 40 μl. The reaction mixture was incubated at 25^{o}C for 5 minutes and 42^{o}C for 45 minutes, followed by heat inactivation of the enzyme at 85 ^{o}C for 5 minutes and a subsequent fast cool-step to 4^{o}C. The cDNA was stored at –70^{o}C.

Primer development

We have first analysed a part of the cDNA sequence of the porcine homologue to MRP2 (data not shown). The obtained cDNA sequences were assembled and a construct of 1449 base pairs with an homology of 87.5% to human MRP2 was submitted to the NCBI database (accession number DQ530510). Previously we have analysed a 2866 basepair cDNA sequence for porcine

P-gp and found a homology of 90.8 % with human P-gp [Schrickx and Fink-Gremmels, Chapter 7].
Gene specific primers were developed assessed by blast analysis against human and porcine cDNA sequences (NCBI BLAST program) and were manufactured commercially (Isogen, IJsselstein, the Netherlands). The final primers were selected (table I.) based on specificity and efficiency when tested by real-time quantitative PCR analysis of a dilution series of pooled cDNA at a temperature gradient for primer-annealing and subsequent melting curve analysis, agarose gel-electrophoresis and nucleotide sequence analysis (data not shown).

Real-Time Quantitative PCR analysis of ABCB1, ABCC2 and ABCG2 expression

The Quantitative PCR analysis was performed with 25ng reverse-transcribed RNA in a 25µl reaction using the IQ^{TM} SybrGreen Supermix (Biorad, Hercules, CA) containing SybrGreen I as an intercalating dye for the real-time detection of double stranded DNA, fluorescein as an internal standard (20nM), iTaq DNA polymerase (50 units/ml), 6mM $MgCl_2$ and 0,4mM of each dNTP. The reaction-mixture contained 7.5 pmol of each specific primer-set and was run on a MyIQ single-colour real-time PCR detection system (Bio-rad, Hercules, CA). Following an initial hot-start for 3 minutes, each of the reactions went through a PCR cycle with a denaturation step at 95^{o}C for 20 seconds, an annealing step specific for each set of primers for 30 seconds and an elongation step at 72^{o}C for 30 seconds, after 35 cycles a melting curve was obtained by increasing the temperature with 0.5 ^{o}C every 10 seconds from 65^{o}C to 95^{o}C.

Table I. Primer sets used for the quantitative PCR analysis.

Gene	NCBI accession number	Forward primer 5' → 3'	Reverse primer 5' → 3'	Nucleotide location	Ta (^{o}C)
ABCB1	AY825267	TGGCAGTGGGACAGGTTAGTTC	CACGGTGCTTGAGCTGTCAATC	2155-2270	65
ABCC2	DQ530510	GTGGCTGTTGAGCGAATAAATGAATAC	TGCTGGGCCAACCGTCTG	798-888	65
ABCG2	NM_214010	GATCTTTTCGGGGCTGTTCCTCA	TGAGTCCCGGGCAGAAGTTTTGT	1753-1874	61,5
GAPDH	AF017079	GGCAAATTCCACGGCACAGTCA	CTGGCTCCTGGAAGATGGTGAT	495-576	65
ACTB	AY550069	GCAAATGCTTCTAGGCGGACTGT	CCAAATAAAGCCATGCCAATCTCA	1202-1301	64
HPRT	NM_001032376	ATCATTATGCCGAGGATTTGGA	CCTCCCATCTCTTTCATCACATCT	84-183	63

Data Analysis

The expression of ABCB1, ABCC2 and ABCG2 was normalized to the geometric mean expression of the internal control genes Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), beta actin (ACTB) and hypoxanthine phosphoribosyltransferase (HPRT), according to Vandesompele *et al.* (2002), eq. 1.

$$\textit{Relative expression level of GOI} = Q_{GOI} / \textit{GeoMean}\,(Q_{GAPDH}; Q_{beta\ actin}; Q_{HPRT}) \quad \text{(eq. 1)}$$

GOI: gene of interest
relative quantity, $Q = 2^{EXP\Delta Ct}$
$\Delta Ct = Ct_{min} - Ct_{sample}$
Ct = threshold cycle
Ct_{min} *= sample having the lowest Ct value*
Ct sample = threshold cycle of sample

Arbitrary limits were set for the comparison of the expression data for P-gp and BCRP.
Low expression: an expression level that is equal to or lower than the level of expression in the stomach. Intermediate expression: an expression level between the arbitrary limits for low and high. High expression: an expression level that is equal to or higher than the level of expression in the duodenum**.**

Results and discussion

The role of the ABC-transporters BCRP, P-gp and MRP2 in xenobiotic disposition is increasingly recognized, not at last due to the growing list of substrates. Previous studies have demonstrated that inter-species variation in expression of these transporters results in variation in kinetic handling of substrates. Thus, for the interpretation of xenobiotic absorption and disposition, detailed information on the tissue distribution of these transporters is needed. We have studied the expression of three transporters relevant for xenobiotic disposition in the pig, as a major target species in veterinary medicine and as a commonly used animal-model for toxicological and pharmacological studies.

Our quantitative RT-PCR data demonstrate that porcine BCRP and P-gp are expressed in all tested tissues (fig. 1 and 2B). The expression of BCRP in several of these tissues confirmed and extended the results from Eisenblätter *et al*. (2002), analysing the expression of porcine BCRP in various tissues by RT-PCR and Northern blot analysis. The expression of P-gp was highest in the adrenal gland and lowest in skeletal muscle tissue, corresponding to previous findings in humans (Sugawara, 1990; Langmann *et al.*, 2003). The expression pattern of MRP2 in porcine samples (fig. 2A) is in line with previous results in humans and dogs, with a high expression in the liver, kidneys and small intestines (Konig, 1999; Conrad *et al.*, 2001; Langmann, 2003; Nishimura and Naito, 2005; Zimmermann *et al.*, 2005). Moreover, as a

transporter of endogenous and exogenous glutathione conjugates, the distribution of MRP2 shows an expression pattern that matches the expression of glutathione-S-transferases in human tissues (Coles *et al.*, 2002). In more detail, in an attempt to compare the expression profiles of ABC transporters, differences and similarities between species are discussed.

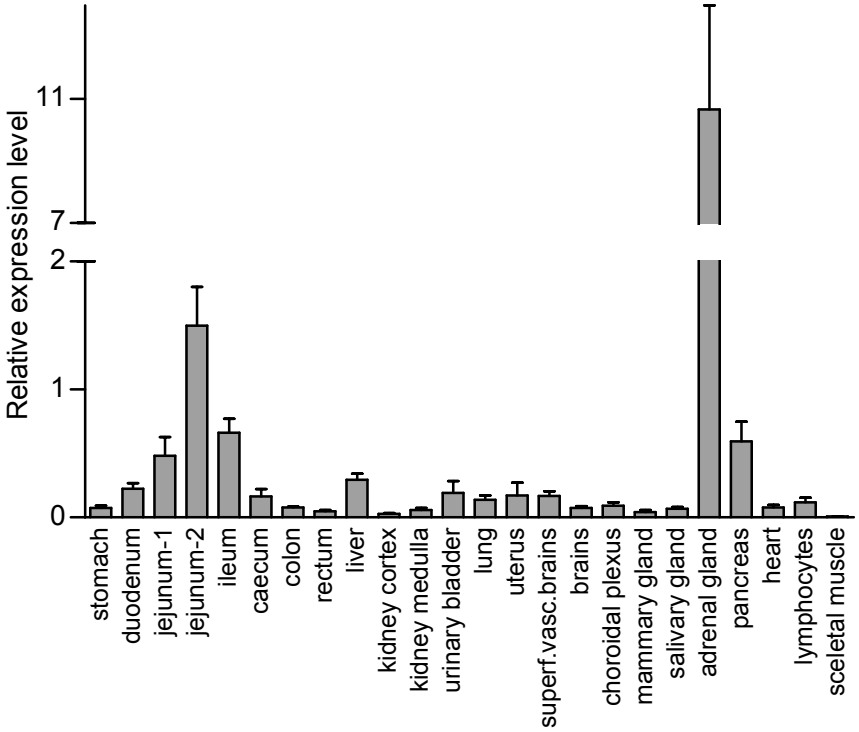

Figure 1. The expression profile of ABCB1 (P-gp) in porcine tissues. Data are presented as means +/- SEM of each tissue from n=3-6.

Intestinal tract

The intestines are the primary site for the absorption of orally administered drugs and ingested toxins. The presence of P-gp, MRP2 and BCRP in the intestinal epithelial cells decrease the absorption of their substrates, moreover they are the major determinants in the excretion of xenobiotics into the faeces (Takano *et al.*, 2006). In pigs, the expression of BCRP and P-gp was found to increase along the small intestinal tract with the highest expression level in the mid jejunum and decreasing levels in the ileum. BCRP appears to be moderately expressed in the colon and rectum, where expression of P-gp is relatively low. A higher mRNA-expression for P-gp in the small intestines than in the colon may be expected, because of a higher fraction

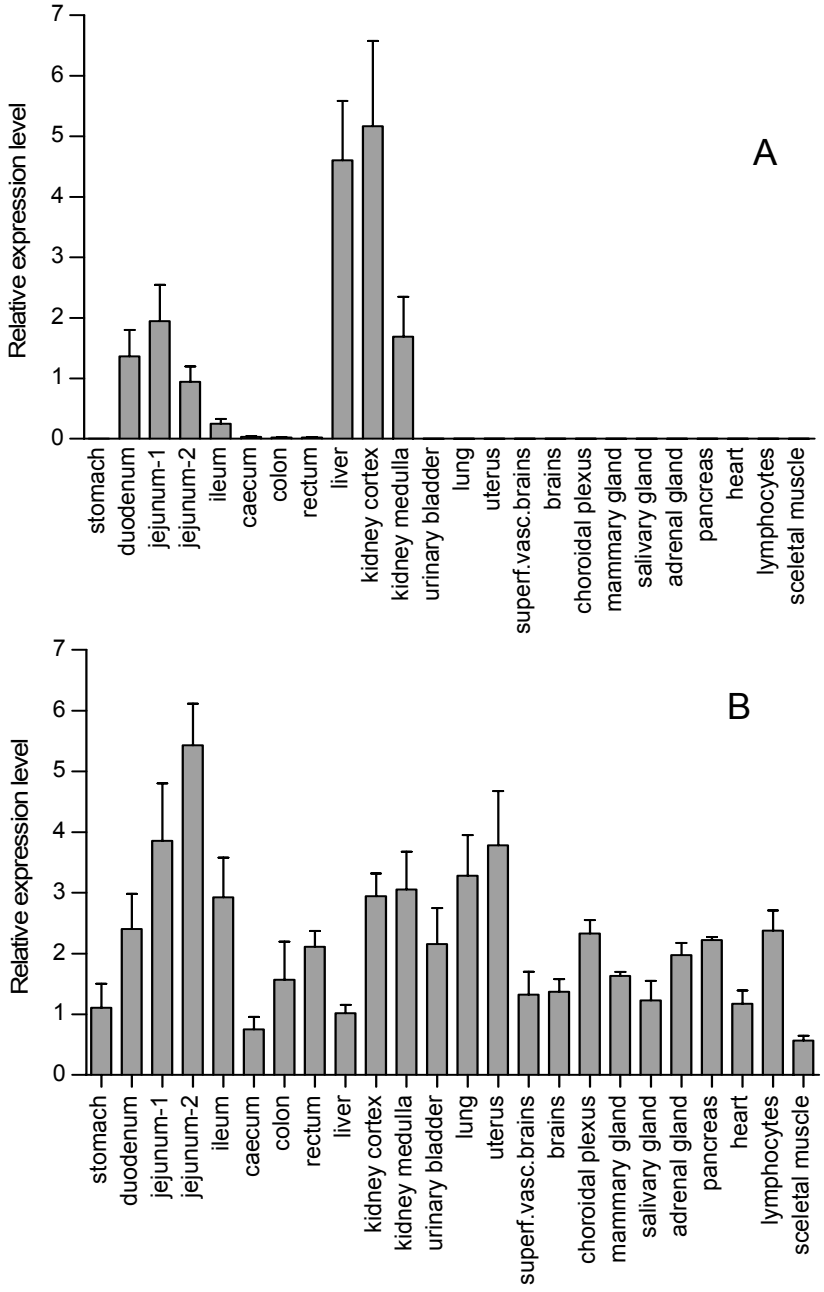
A
B
Relative expression level
0
1
2
3
4
5
6
7
stomach
duodenum
jejunum-1
jejunum-2
ileum
caecum
colon
rectum
liver
kidney cortex
kidney medulla
urinary bladder
lung
uterus
superf.vasc.brains
brains
choroidal plexus
mammary gland
salivary gland
adrenal gland
pancreas
heart
lymphocytes
sceletal muscle

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Figure 2. A The expression profile of ABCC2 (MRP2) in porcine tissues. Data are presented as means +/- SEM of each tissue from n=3-6.
Figure 2. B. The expression profile of ABCG2 (BCRP) in porcine tissues. Data are presented as means +/- SEM of each tissue from n=3-6.

of columnar cells, with P-gp present in the brush border membranes, in the jejunum (Hazel Cheng, 1982). Moreover, western blot analysis of intestinal samples from the Yucatan micropig showed a lower P-gp content in the colon and rectum than in the small intestines (Tang *et al.*, 2004). Although, various reports have suggested that the mRNA levels for P-gp in humans increase from the stomach to the colon (Fricker *et al.*, 1996; Thorn *et al.*, 2005; Zimmermann, 2005), other data indicated a lower expression of P-gp in the human colon than in the small intestines (Nakamura *et al.*, 2002; Langmann, 2003; Nishimura and Naito, 2005). BCRP mRNA expression in humans was previously found to be lower in the colon when compared to the small intestines, comparable to our findings (Maliepaard *et al.*, 2001; Gutmann *et al.*, 2005). The expression of MRP2 was highest in the middle of the jejunum and decreased towards the ileum, while only a low expression could be detected in the more distal parts of the intestinal tract, that is comparable to data obtained in human and mice (Maher *et al.*, 2005; Zimmermann, 2005).

Liver

In the liver, the major organ for metabolism, P-gp, BCRP and MRP2 are important for the biliary excretion of drugs, toxins and metabolites (for review see Chandra and Brouwer, 2004). The pig liver expresses MRP2 and P-gp at high levels, comparable to previous human and canine data (Conrad, 2001; Langmann, 2003; Nishimura and Naito, 2005), whereas expression of BCRP in the liver is low. Human data suggest a moderate expression of BCRP in the liver when compared to the colon and small intestines (Doyle, 1998; Maliepaard, 2001; Langmann, 2003; Nishimura and Naito, 2005), whereas mice highly express BCRP in the liver as compared to the intestines and rats have a low expression (Tanaka *et al.*, 2004). This points to the need for a careful interpretation of functional data obtained in mice for the extrapolation to other species, as human and pigs have an apparently lower capacity for hepatic excretion of BCRP substrates.

Kidneys

The kidney is involved in the excretion of endogenous metabolic waste products, xenobiotics and their metabolites, however the specific role for P-gp and BCRP needs to be further established. Expression of MRP2 and BCRP in the kidneys is relatively high, while the levels of P-gp mRNA in the kidneys are relatively low. The level of expression of MRP2 is comparable to previous human data (Langmann, 2003). Human data about the relative expression of BCRP in the kidneys vary from low to moderate, but data obtained in rodent species demonstrated a high expression of BCRP in the kidneys (Tanaka, 2004). The expression of P-gp is remarkably low in our porcine samples compared to the expression in the

human kidney that is much higher than in the liver and intestines (Langmann, 2003; Nishimura and Naito, 2005), but data about its specific role in the kidneys are controversial.

BCRP and P-gp expression was detected in the urinary bladder and while immunostaining of BCRP has previously stained the transitional epithelium in the human bladder (Fetsch *et al.*, 2006), it suggests that these transporters also form a barrier against the absorption of eliminated xenobiotics.

Central nervous system

The superficial vasculature on the brains and brain tissue show an intermediate expression for both BCRP and P-gp. As a part of the blood-brain barrier (BBB), P-gp and BCRP are predominantly expressed on the luminal side of capillary endothelium, and although expression of P-gp was observed in astrocytes (Golden and Pardridge, 1999; Ronaldson *et al.*, 2004) and microglia (Lee *et al.*, 2001) it seems to be low under physiological conditions but increases under pathological conditions (for review see Loscher and Potschka, 2005). Moreover, capillary enriched fraction expressed BCRP (mRNA, pig/mice) and P-gp (protein, rat) in a much higher amount than total brain homogenates (Beaulieu *et al.*, 1997; Eisenblatter *et al.*, 2003; Cisternino *et al.*, 2004). Therefore the observed expressions of BCRP and P-gp in brain tissue may reflect their (high) expression in the brain capillaries and their functional contribution to the BBB in the pig.
Although MRP2 expression has been detected by immunohistochemical methods and RT-PCR in isolated porcine and rat brain capillary endothelial cells (Miller, 2000), various groups could not demonstrate this (for review see Loscher and Potschka, 2005). In the presented studies, we detected a low expression in the brain superficial vasculature in two out of six animals.
The homeostasis of the brain depends on both the endothelial BBB and the epithelial barrier at the choroid plexus. The choroidal plexus produces the cerebrospinal fluid (CSF), and both BCRP and P-gp are expressed on the apical side op the choroidal epithelial cells in humans, thus contributing to the transport in the direction of the CSF (Rao *et al.*, 1999; Maliepaard, 2001). The porcine choroïdal plexus expresses BCRP at a moderate and P-gp at a low level. Since these transporters exhibit an opposite transport direction than in the BBB, they may increase the concentration of substrates into the CSF.

Lung

BCRP is relatively highly expressed in the lung, while the expression of P-gp is intermediate. In humans P-gp and BCRP are expressed in the epithelium of the bronchi and bronchioles, with additional expression of BCRP in the endothelium (Cordon-Cardo *et al.*, 1990; Lechapt-Zalcman *et al.*, 1997). Similarly, various cytochrome P450 enzymes, that are co-regulated at a transcriptional level, have previously been detected in the lung, mostly those belonging to the CYP2 family (Ding and Kaminsky, 2003). The function of the transporters in the lung with respect to physiology and xenobiotic disposition is still not well understood (for review see: van der Deen *et al.*, 2005), but given the expression levels of BCRP and P-gp it can be

assumed that they may have a considerable impact on the kinetics of their substrates. In turn, ABC transporters will have a protective role against inhaled toxins.
Although a low expression of MRP2 has been detected in the human lung by Langmann *et al.* (2003), in another study expression in lung tissue appeared to be negative (Kool *et al.*, 1997). We found a low expression in two out of six animals indicating that MRP2 does not play a role in the normal lung, or at most a very limited one.

Heart

Couture *et al.* (2006) recently reviewed the current state of knowledge about ABC-transporters in the heart. P-gp and BCRP were found to be mainly present in the endothelium of the vasculature, with conflicting data about the presence in normal cardiomyocytes. However, P-gp-protein was detected in cardiomyocytes of chronically ischemic porcine myocardium (Lazarowski *et al.*, 2005). It therefore seems that the observed low expressions of BCRP and P-gp in the porcine heart under normal conditions, is mainly a result of endothelial expression and to a lesser extent of myocardial expression.

Uterus

We detected the expression of P-gp and BCRP in the uterus that was intermediate for P-gp and high for BCRP. P-gp and BCRP are present in the normal endometrium of human and rodents and the expression of P-gp increases during pregnancy (Axiotis *et al.*, 1991). Our data suggest that both transporters already have a function in the juvenile endometrium.

Mammary gland

A moderate expression of BCRP was detected in the juvenile mammary gland. BCRP has not been detected in the non-lactating mammary gland of human, mouse and cow by immunological methods (Jonker *et al.*, 2005), but P-gp is expressed in non-lactating human mammary epithelial cells (Alcorn *et al.*, 2002). Since the RT-PCR is highly sensitive, the observed levels of BCRP and P-gp in our porcine tissue may have resulted from the expression in a combination of cell-types: epithelial cells, endothelial cells and progenitor cells (Clayton *et al.*, 2004; Jonker *et al.*, 2005). The elucidation of the role of both transporters in relation to the latter cell-type and its role in the development of the mammary gland requires further research.

Salivary gland

The expression of drug-transporters in the salivary gland is essential for the prediction of the secretion of drugs and toxins into the saliva. This might be of clinical relevance either beneficial for odontogenic infections (Malizia *et al.*, 1997) or causing undesirable side effects in the intestinal tract, as following salivary excretion of drugs and toxins follows the oral route, which has been associated with dysbacterioses, as demonstrated for various macrolide antimicrobials (Heimdahl and Nord, 1982; van Miert, 1995). Expression of P-gp was previously observed in human salivary duct cells (van der Valk *et al.*, 1990), and our data suggest that both P-gp and BCRP contribute to the active secretion of substrates into the saliva.

Adrenal gland

The expression of P-gp was by far the highest in the adrenal gland, where BCRP was moderately expressed, pointing towards a role in steroid-hormone transport in the pig. Mineralo- and glucocorticoids are the major steroid hormones synthesised in the adrenal cortex, and previously it was already demonstrated that cortisol and aldosterone are substrates for P-gp (Ueda *et al.*, 1992). DHEAS, the sulphated form of DHEA also formed in the adrenal gland, however, is a substrate for BCRP (Suzuki *et al.*, 2003).

Pancreas

P-gp is highly expressed in the pancreas, while BCRP has a moderate expression. Whether there is any function of these transporters has not been studied in this tissue, but epithelial cells of small pancreatic ductules express P-gp at their luminal surface suggesting an excretory role, at least for P-gp (Thiebaut *et al.*, 1987).

Lymphocytes

A low expression was detected in porcine lymphocytes for P-gp, but a moderate expression was detected for BCRP. P-gp and BCRP are expressed in various subsets of lymphocytes, e.g. in natural killer cells (Klimecki *et al.*, 1995; Zhou *et al.*, 2001). Functional characteristics of porcine P-gp have been obtained by FACS analysis of the P-gp substrate rhodamine 123 retained in lymphocytes isolated from the peripheral blood (Chapter 7) indicating that even a low expression has functional consequences.

The detection of gene expression by quantitative PCR requires the normalisation of the results to the amount of input material, usually by internal control genes (housekeeping genes). However, it cannot be excluded that the described species differences are partly related to differences in the normalization methods used; for the present studies we used three individual housekeeping genes for the normalization of the PCR data, whereas other studies often used only one housekeeping gene. The relative expressions of the housekeeping genes are presented in figure 3 and demonstrate that the use of a single housekeeping gene has consequences for the calculated tissue expression levels.

In conclusion, we here present the first compilation of data of the expression of P-gp, MRP2 and BCRP in porcine tissues. The presented data show that these ABC-transporters have a tissue expression pattern in the pig that is very similar to that in man, suggesting a same role in physiology and xenobiotic defence and an overlapping regulation of tissue specific gene expression. However, some differences were observed that potentially affect the elimination-routes of substrates. For example, the expression of P-gp is low in the colon and particularly low in the kidneys of the pig, and the expression of BCRP is rather low in the porcine liver. These data might explain species-differences in the kinetics of BCRP, P-gp and MRP2 substrates, including drugs and toxins.

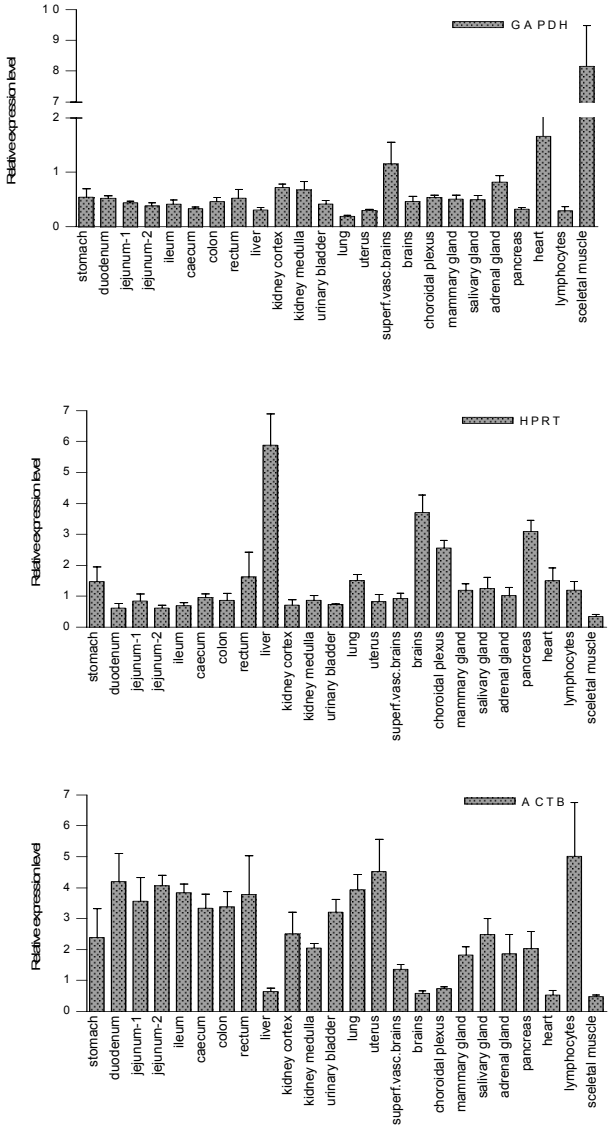

Figure 3. The relative expression of housekeeping genes in porcine tissues, calculated according to eq. 1 in the materials and methods section. Data are presented as means +/- SEM of each tissue from n=3-6.

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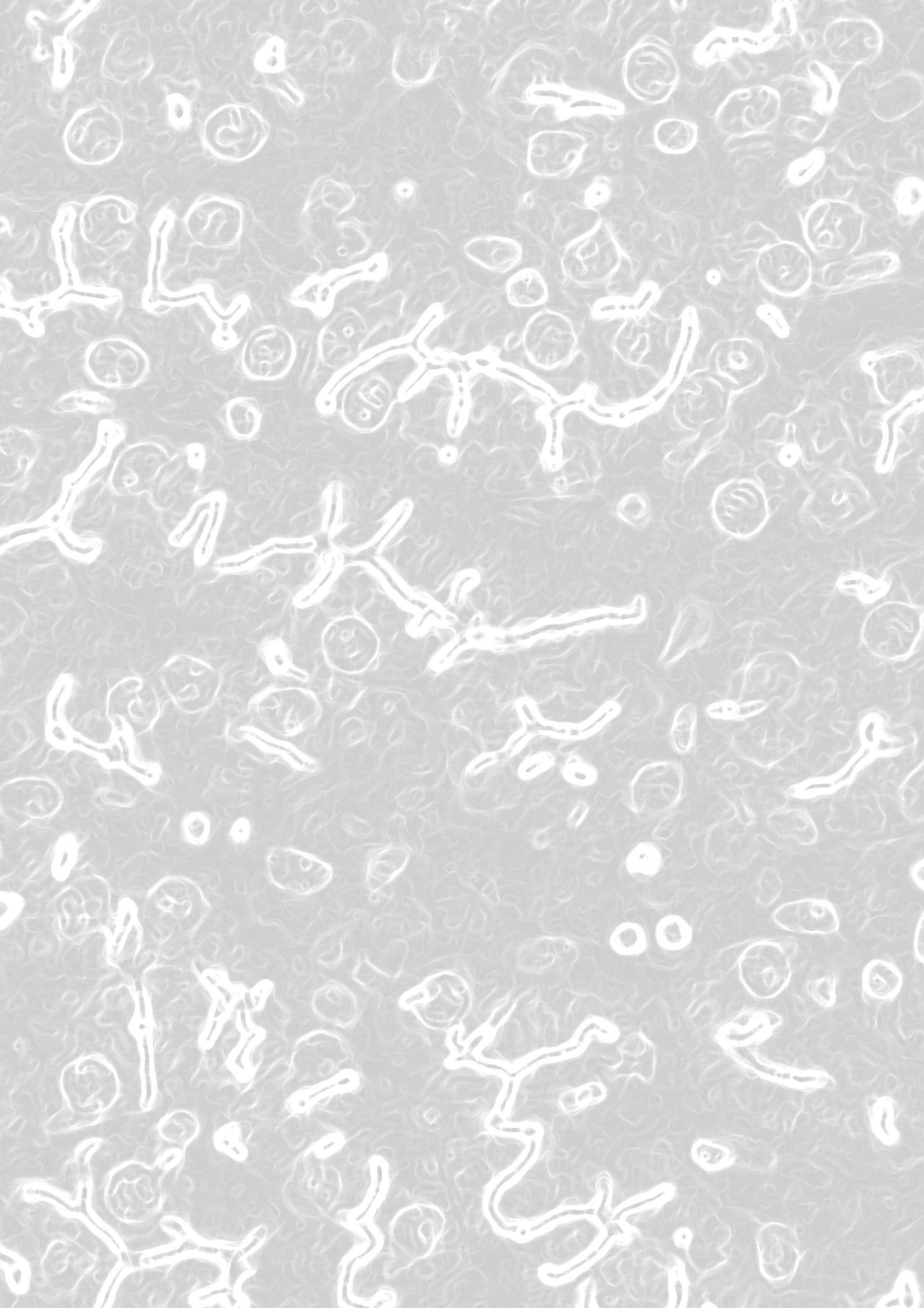

CHAPTER 7

Characterization of porcine MDR1 and validation of a lymphocyte model for functional studies

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Abstract

The clinical relevance of the organ-specific expression of P-gp (MDR1, ABCB1), as a modulator of drug absorption and distribution is increasingly recognized. At the same time species differences in P-gp expression have been noted and it has been demonstrated that rodents have two P-gp genes that differ in function and expression, while other mammalian species including humans seem to have only one gene. As yet, little is known about the porcine P-gp homologues and their function, despite the frequent use of porcine tissues and cells as a model for human P-gp functions. We analysed the partial cDNA sequence of porcine P-gp and found a relatively high homology with human P-gp (90.8%). Peripheral blood lymphocytes are known to express P-gp and hence we validated the usefulness of isolated porcine lymphocytes to study P-gp function in an ex vivo model. Using the prototypical P-gp inhibitors PSC833, GF120918, ketoconazole, cyclosporine A and verapamil, as well as a range of other model substrates, an inhibitory profile of porcine P-gp that is highly comparable to human P-gp was established. As porcine lymphocytes are easily available and considering the high homology of porcine and human P-gp, this model can be recommended as a rapid high-throughput system to identify P-gp inhibitors and putative substrates in the early screening of new pharmaceutical entities for both, human and animal therapy.

Introduction

The ABCB1 gene encodes the 1280 amino acid P-glycoprotein (P-gp), which belongs to the ATP-binding cassette (ABC) superfamily of transmembrane transporters. P-gp acts as an efflux transporter that uses ATP for translocating its substrates from the intracellular to the extracellular space. Due to its expression in the intestinal tract and at tissue barriers, P-gp is considered to significantly determine drug absorption and distribution in humans and animals. Potent inhibitors of P-gp have been developed to overcome multidrug resistance of cancer cells that depends on the up-regulation of P-gp in the course of a therapy with many cytostatic agents (Hyafil et al., 1993; Aouali et al., 2005). Next to cytostatics, an increasing number of other drugs have been shown to be substrates for P-gp. Hence, insight into P-gp expression, function and modulation is of clinical importance and may serve strategies to improve the oral bioavailability and effective drug concentrations at target sites, including the distribution into barrier protected tissues, such as the central nervous system (Imbert et al., 2003; Kemper et al., 2003; Anderson et al., 2006) and the placenta (Ceckova-Novotna et al., 2006).

Despite the fact that porcine P-gp was the first identified eukaryotic ABC-transporter (Juliano and Ling, 1976) and porcine tissues and cells are widely used in functional studies related to P-gp dependent transport processes, only very limited data are available about structure and function of P-gp and related transporters in pigs (Miller et al., 2000; Schwab et al., 2003; Schlatter et al., 2006). In 1996, Childs and Ling cloned the 3' terminal genomic fragments for the porcine P-gp homologue (Childs and Ling, 1996). Based on their results the authors suggested that pigs might have four P-gp (ABCB1) gene homologues. In contrast, rodent species have two P-gp isoforms that differ in function and expression pattern (Croop et al., 1989; Gruol et al., 1999). As further data about the existence of multiple P-gp genes in pigs are not available, we here describe the partial analysis of the cDNA sequence of the porcine P-gp homologue, and the results of a search for related EST sequences available at the NCBI database.

In vitro screening assays for the identification of potential drug interactions at the level of P-gp, mainly consist of cell-monolayers cultured on permeable supports with the use of radio-labelled or fluorescent probe-substrates. Functional data regarding modulation of P-gp were previously obtained by the use of porcine brain capillary endothelial cells (pBCECs) and calcein-AM as a probe substrate, serving as a model for the blood-brain barrier (Bauer et al., 2003; Schwab, 2003; Weiss et al., 2003; Weiss and Haefeli, 2006). An alternative cell-population that can be easily obtained and physiologically express P-gp are peripheral blood lymphocytes (PBLs). PBLs have been widely used to study the functional activity of P-gp by the retention or efflux of Rhodamine 123 (Rh123), a P-gp substrate, in the presence or absence of potent P-gp inhibitors (Parasrampuria *et al.*, 2001). As P-gp expression in PBLs can serve as surrogate marker to assess ex vivo changes in P-gp function at different stages of life, during disease and long-term treatments, we explored the use of porcine peripheral blood lymphocytes as an ex vivo assay to identify substrates and inhibitors of P-gp. To this end, we selected a

group of drugs known to be substrates and/or inhibitors for human P-gp and tested these for their inhibitory potency on porcine P-gp.

Materials and Methods

Chemicals and media

GF120918 (Elacridar) was a kind gift of GlaxoSmithKline (Stevenage, Herts, UK) and PSC833 (Valspodar) was a kind gift of Novartis Pharma AG (Basel, Switzerland). MK-571 sodium salt was obtained from Alexis Biochemicals (Grünberg, Germany). Ko143 was a kind gift of Prof. Koomen (University of Amsterdam, the Netherlands). Azithromycin, levofloxacin, ciprofloxacin, oxytetracycline HCl, tetracycline HCl and doxycycline hyclate were purchased from Biochemica/Fluka (Buchs, Switzerland). Gentamicin pentahemisulfate hydrate and loperamide HCl were purchased from Riedel de Haen (Seelze, Germany). Rhodamine 123, digoxin, cyclosporine A, colchicine, ketoconazole, verapamil-HCl, streptomycin-sulfate, erythromycin, minocycline-hydrochloride and ivermectin were purchased from Sigma-Aldrich (St Louis, MO).

RPMI1640 cell culture medium, phosphate buffered saline (PBS), Hanks balanced salt solution (HBSS, Ca^{2+}/Mg^{2+} free) and foetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY, USA). L-Glutamine was from BioWhittaker (Maine, USA). Ficoll Hypaque (Ficoll Paque Plus Research, Pharmacia Biotech, Sweden) was purchased from Amersham (Diegem, Belgium).

Tissue collection, RNA isolation and cDNA synthesis

Porcine tissues (liver, kidney, adrenal gland and jejunum) were collected from healthy crossbred pigs (25 kg body weight) and snap frozen in liquid nitrogen; samples were stored at –70^{o}C until isolation of RNA. Total RNA was isolated using the RNeasy mini kit (Qiagen), according to manufactures' descriptions with an additional DNAase step incorporated in the procedure. The RNA was quantified spectrophotometrically and stored at –70^{o}C. First-strand cDNA synthesis was performed with the Reverse Transcription System from Promega (Madison, USA). The reaction mixture containing oligo(dT) primers was prepared according to manufactures' descriptions and incubated at 42^{o}C for 45 minutes, followed by heat inactivation of the enzyme at 95^{o}C for 5 minutes and a subsequent fast cooling step to 4 ^{o}C. The samples were stored at –20^{o}C until use.

Primer design

The mRNA sequences for porcine (AF403245, 364 basepares) and human P-gp (NM_00927) and the EST sequences were derived from the NCBI GenBank (for accession numbers see Table 1.). The EST sequences were selected based on their homology to human P-gp (≥85%)

by using the NCBI BLAST-software. Primers (Table 1) were designed that are complementary to porcine P-gp or EST-sequences and producing overlapping amplicons.

Table 1. Primer-sets used for the sequence analysis of porcine P-gp.

forward primer 5' → 3'	reverse primer 5' → 3'	NCBI GenBank accession number	Position 5' on NM_00927
GCTTAACACCCGGCTCACAGATG		CF176979	929-
	ACGCCGATAATTTCCCGCAGATA	CF175635	-1837
CGGAGGGCGTGGTCAGTATT		CF175635	1762-
	CCGTGGTGTTTTTAGGGTCATC	BX673636	-2859
GGGACCACAAGGCCAAGACAGAA		AF403245	2442-
	AACCGGAAACAGGCAGCGTAAGA	BX673636	-3301
CACATTTTTCCTTCAGGCTTCACG		BX673636	2729-
	CTTCTTCACCCCCAGGCTCAG	BI344244	-3611
TGTTTCCGGTTTGGTGCCTACTTG		BX673636	3291-
	TCTGCGTTCTGGATGGTGGACA	AW316442	-4147

PCR-protocols

Pooled cDNA from the tissues was amplified in a PCR reaction. The PCR reaction mixture contained 2.5 U platinum Taq polymerase, 0.8 pmol/µl of each primer, 200µM dNTP's and 1.5 mM $MgCl_2$ (Promega). PCR reactions were run in a MJ thermal cycler (Biorad, Hercules, CA, USA). The cycling conditions comprised a denaturation step at 94°C for 1 min, an annealing step at a temperature gradient for 30 sec and an extension step at 72°C for 1 min. The PCR-products were analysed by gel-electrophoresis and visualized by ethidium bromide staining and subsequently used for sequence analysis.

Sequence analysis

The ABI PRISM Big Dye Terminator v3.0 ready reaction cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with fluorochrome labelled dideoxynucleotides was used for the preparation of terminated DNA chains in a thermal cycler according to the manufactures instructions and subsequently purified using sephadex G-50. The DNA sequences were than analysed in an ABI PRISM 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

Data analysis and assembly

The sequences obtained were assembled by alignment with human P-gp by the Clustral W method (MegAlign, DNA Star, Madison, USA) and the predicted protein sequence was derived from the open reading frame of human P-gp.

Isolation of Peripheral Blood Lymphocytes (PBLs)

Whole blood was collected from adult crossbreed sows into a heparinised syringe. The blood was diluted 1:1 with HBSS, layered onto Ficoll Hypaque and centrifuged at 500*g* for 20 minutes. The mononuclear cell layer was withdrawn and washed twice with supplemented RPMI (RPMI with 1% L-Glutamine and 10% FBS) by centrifugation at 260*g* for 5 minutes. The remaining erythrocytes were lysed with ice-cold erylysis buffer (0.155 M ammonium chloride, 10 mM potassium bicarbonate and 0.1 mM disodium EDTA in distilled water, pH 7.2) and washed in HBSS. The cell-pellet was re-suspended in RPMI supplemented with 1% L-Glutamine and 10% FBS. For further purification of the lymphocytes, the cell-suspension was plated onto dishes and incubated at 37^{o}C and 5% CO_2 to allow attachment of monocytes. After four hours, the medium containing the PBLs was collected and stored at 4^{o}C overnight.

Rhodamine123 accumulation/efflux assay

Cell viability was assessed by trypan blue exclusion and the PBLs were counted in Türk solution using a hematocytometer. Medium was washed off and the cells were re-suspended in HBSS containing 0.25 μM Rh123. A total number of 0.5 million cells per sample were incubated at 37^{o}C, 5 % CO_2 in 96-well plates. After 30 min the cells were washed twice in PBS (4^{o}C) by centrifugation at 200*g* and the cell pellets were re-suspended in HBSS without Rh123, but containing the test compounds or vehicles. After incubation at 37^{o}C, 5 % CO_2 for the indicated period, cell-suspensions were collected and added to tubes pre-filled with ice-cold PBS and propidium iodide (PI), resulting in a final concentration of 1 μg/ml PI, and kept on ice. A flow cytometer (FACS Calibur, Biorad, The Netherlands) equipped with an argon 488 nm laser was used to analyse the samples. Cell-associated Rh123 fluorescence was measured using a 530-nm bandpass filter and cell-associated PI fluorescence was measured using a 610-nm longpass filter. The samples were gated on forward scatter versus side scatter to exclude non-lymphocyte populations, clumps and cell debris. PI staining was used to exclude the dead cells from the measures. Data were collected for a minimum of 10,000 gated events per sample.

Data analysis

The data were collected as geometric mean fluorescence intensity for all samples. Efflux was calculated according to equation 1 (eq. 1) and is expressed as a percentage.

$$Efflux = [\ (FL_0-FL_B) - (FL_S-FL_{BS})\ ] / (FL_0-FL_B)\ \ [\%] \qquad \text{(eq. 1.)}$$

FL_0 : Fluorescence intensity at t=0
FL_B : Fluorescence intensity of the background of control samples
FL_S : Fluorescence intensity of the samples exposed to the test compounds
FL_{BS} : Fluorescence intensity of the background of samples exposed to the test compounds

The area under curve (AUC) was calculated by integration of the function efflux-*versus*-time, using Graph Pad Prism software (version 2.01, Graph Pad software Inc., San Diego, California.). Inhibition of Rh123 efflux by the test compounds was than calculated from the AUC's as a percentage of the control value (eq. 2).

$$Inhibition = 1 - AUC_S / AUC_C \ [\%] \quad \text{(Eq. 2)}$$

AUC_S: : Area under curve [% x min] of the samples exposed to the test compounds
AUC_C : Area under curve [% x min] of the control

The percentage of inhibition of Rh123 efflux by the test compounds, cyclosporine A, verapamil, loperamide, ketoconazole, colchicine, digoxin, erythromycin, azithromycin, gentamicin, streptomycin, levofloxacin, minocycline, doxycycline, tetracycline and oxytetracycline, was than calculated according to eq. 3 at t=120 minutes. The level of no-effect was set at two times the standard deviation of the inhibition calculated for multiple controls, and was 7%.

$$I_t : Inhibition_t = 1 - Efflux_{t,S} / Efflux_{t,C} \ [\%] \quad \text{(Eq. 3)}$$

t : time-point of the sample collected
$Efflux_{t,S}$: Efflux of the samples exposed to the test compounds at time t
$Efflux_{t,C}$: Efflux of control samples at time t

The correlation between inhibition calculated by the AUC method and the inhibition at the indicated time-points (30, 60, 120 and 180 minutes), calculated according to eq. 3 (I_t), was analysed by linear regression analysis, using Graph Pad Prism software.

For the calculation of inhibitor effects, a sigmoïd-shaped curve was fitted (eq. 4) to the inhibition data either obtained by eq. 2 (GF120918, PSC833 and ivermectin) or those obtained by eq. 3 (cyclosporine A, verapamil, loperamide, ketoconazole), using Graph Pad Prism software.

$$Y = I_{min} + (I_{max} - I_{min}) / (1 + 10^{((logIC50-X) \times h)}) \quad \text{(Eq. 4)}$$

I_{min} : minimum effect
I_{max} : maximum effect
IC_{50} : the concentration leading to 50% of Imax
h : hill's equation

The results for the compounds that did not reach their maximum inhibitory effect at the concentrations used are presented as the percentage of inhibition according to eq. 3.

Results

Sequence analysis of porcine P-gp

Sequences of the PCR products were assembled and the obtained sequence was entered into the NCBI GenBank database (accession number AY825267) and is publicly accessible.

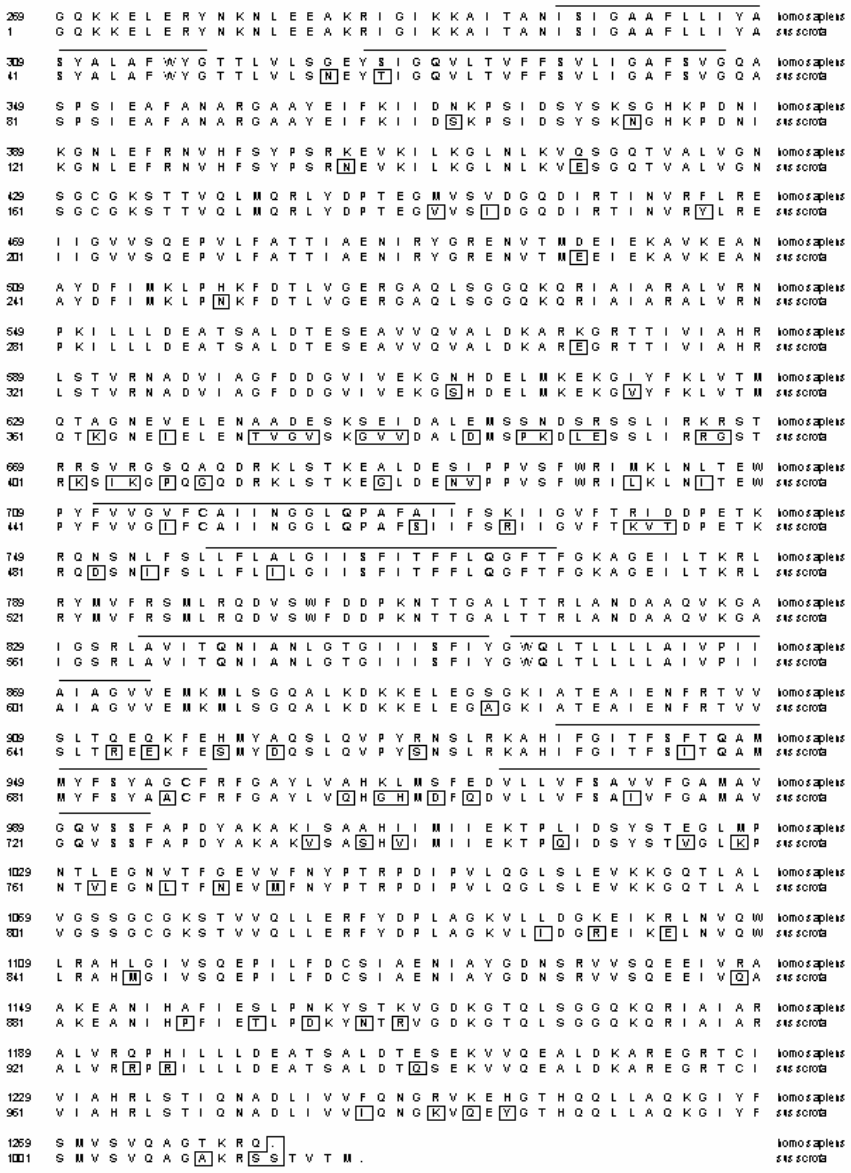

Figure 1. D*educed partial amino-acid sequence of porcine MDR1, the amino-acids letters in bold and over-lined represent the predicted membrane-spanning domains that were derived from (Chen, 1986).*

A construct covering the 3' end of the porcine P-gp cDNA was made by alignment with the previously sequenced partial porcine DNA (NCBI accession number U27704), demonstrating a homology of 90.8 % to human P-gp. The deduced amino-acid sequence is presented in figure 1 and the predicted transmembrane domains according to Chen et al. (1986) are marked.

Time-dependent Rhodamine 123 efflux from porcine peripheral blood lymphocytes

After incubation of the PBLs in the presence of 0.25 μmol/L Rh123 for thirty minutes, the subsequent efflux into the dye free medium was time dependent and was decreased in the presence of PSC833 and GF120918. Figure 2 shows the percentage of Rh123 efflux over a period of 180 minutes for PBLs in the absence (control) and in the presence of 1 μMol/L GF120918 and PSC833.

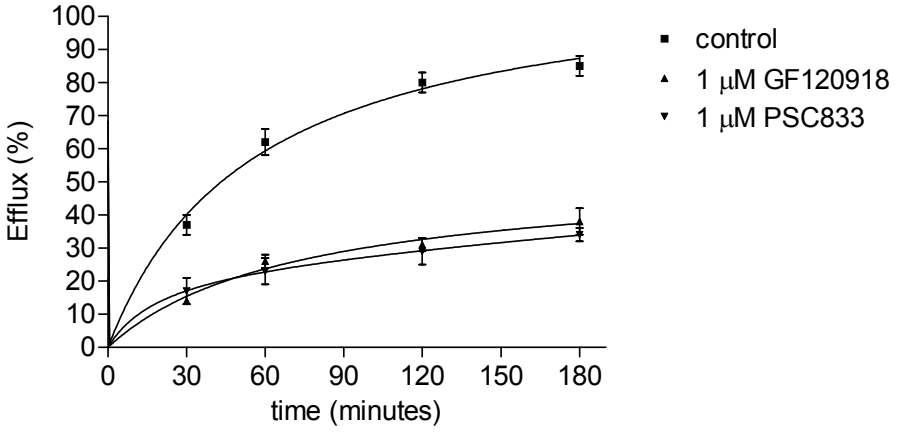

Figure 2. Time-dependent efflux of Rh123 in the absence or presence of the prototypic inhibitors GF120918 and PSC833. Data represent means +/- SD (n=3) from a representative experiment.

Concentration dependent inhibition of Rh123 efflux by GF120918, PSC833 and ivermectin

The inhibitory effect of a range of concentrations of GF120918, PSC833 and ivermectin on Rh123 efflux was then measured over a period of 180 minutes and samples were collected immediately following the Rh123 uptake period of thirty minutes and at the time-points 30, 60, 120 and 180 minutes of incubation in dye free medium. The percentage of inhibition was calculated by the use of AUC's, according to equation 2 as given in the Materials and Methods section, and fitted to a sigmoid concentration - effect curve. These sigmoid curves for the inhibition of Rh123 efflux by the prototypical P-gp inhibitors GF120918, PSC833 and ivermectin are presented in figure 3, and the calculated IC_{50} values are presented in table 2.

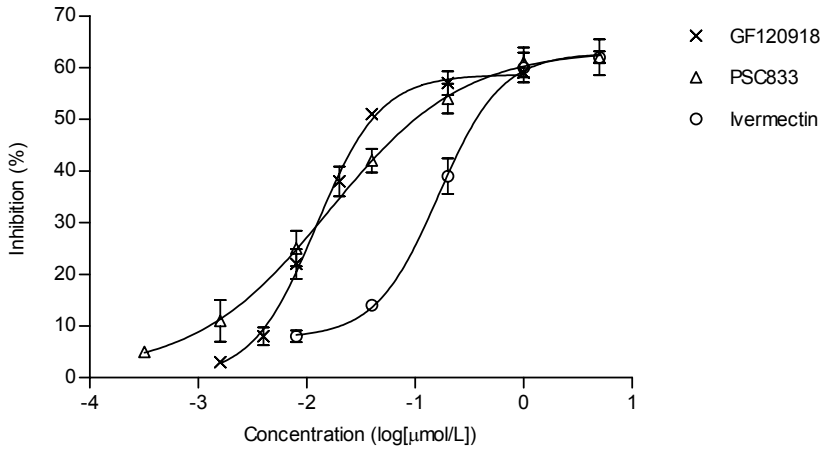

Figure 3. Sigmoïdal concentration-inhibition curves of the proto-typical P-gp inhibitor GF120918, PSC833 and the P-gp substrate ivermectin. Data represent means ± SEM of three independent experiments.

Analysis of the data revealed that the correlation between the inhibition calculated by the AUC method and the inhibition at the individual time-points (30, 60, 120 and 180 minutes), was highest for the samples collected after 120 minutes (r^2 = 0.996). Therefore, in the following experiments, samples were only collected immediately after the loading of the PBLs with 0.25 μmol/L Rh123 for 30 minutes (t=0) and following incubation in dye-free medium for 120 minutes in the presence or absence of the test compounds. The variability of the cellular fluorescence detection and the calculated percentage of inhibition of multiple samples were assessed. The intra-assay variation in fluorescence was 2.8% ± 2.7% and the intra-assay variation of inhibition (calculated after 120 minutes) was 1.8% ± 1.3%.

Inhibitory effects of selected drugs on Rhodamine 123 efflux.

Cyclosporine A, verapamil, loperamide and ketoconazole were selected based on their previously documented inhibitory effect on P-gp function. All four compounds potently inhibited Rh123 efflux by the PBLs as presented in Table 2. The macrolide antibiotics, erythromicin and azithromycin, the fluoroquinolone levofloxacin, the glycoside digoxin, and the alkaloid colchicine were selected, as these also have been described to be substrates for human P-gp. Erythromycin and azithromycin inhibited P-gp mediated Rh123 efflux in porcine PBLs, but a maximal inhibition could not be reached in the concentration range tested (with a

maximum of 125 μM). Digoxin only exhibited an inhibitory effect at the highest concentration tested and thus no IC_{50} value could be calculated for these compounds. Colchicine, levofloxacin, ciprofloxacin, gentamicin and streptomycin did not exert any inhibitory effect. The tetracyclines minocycline, doxycycline and tetracycline decreased Rh123 efflux, albeit at different levels. Oxytetracycline, did not significantly inhibit Rh123 efflux (Table 2).

Table 2. Inhibitory effects of selected test compounds on Rh123 efflux. S: substrate for human P-gp; I: inhibitors for human P-gp; N.E. means no effect (i.e. the inhibitory effect was equal to or less than 7%). Data represent means of at least three independent measurements.

Compound	**S/I**	**IC_{50} (μmol/L)**	**95% confidence intervals**
GF120918	I	0.012	0.011-0.013
PSC833	I	0.016	0.015-0.017
IVM	S	0.16	0.16-0.17
Cyclosporine A	S/I	0.31	0.27-0.35
Verapamil	S/I	1.4	1.0-2.0
Loperamide	S	1.8	1.7-2.0
Ketoconazole	S/I	7.0	7.0-7.2
		Inhibition of Rh123 efflux (%) at	
		25 μM	**125 μM**
Colchicine	S	N.E.	N.E.
Digoxin	S	N.E.	12 +/- 2
Erythromycin	S	22 +/- 2	46 +/- 8
Azithromycin	S	13 +/- 2	39 +/- 3
Levofloxacin	S	N.E.	N.E.
Ciprofloxacin		N.E.	N.E.
Gentamicin		N.E.	N.E.
Streptomycin		N.E.	N.E.
Minocycline		10 +/- 0.4	25 +/- 3
Doxycycline		N.E	17 +/- 3
Tetracycline	S	N.E	12 +/- 2
Oxytetracycline	S	N.E	7 +/- 2 (N.E.)

Discussion

We have analysed a part of 2866 base pairs of the porcine P-gp cDNA. This sequence was obtained following the development of primer-sets complementary to porcine EST sequences deposed at the NCBI database, highly homologues (≥85%) to human P-gp (ABCB1/MDR1) and producing overlapping amplicons. This sequence partly overlaps the previously analysed sequence by Childs and Ling , completing the sequence of porcine P-gp at the 3'end. Alignment with human P-gp provided a homology of 90.8% and demonstrates an additional 5

amino acid fragment at the COOH terminal end. The small part of the porcine gene coding for P-gp that was previously cloned (Childs and Ling, 1996) had already indicated a high homology of porcine P-gp with human P-gp for the final exon nearest to the 3'untranslated region (3'UTR). The authors proposed at that time, that pigs would have four P-gp gene homologues, which were named P-gp1A, B, C en D, with P-gp 1A sharing the highest homology to human ABCB1. Moreover, a SINE element was found in the 3'UTR in three out of the four P-gp variants. The extended data presented here, provide no evidence for the expression of more than one porcine P-gp gene or for an insertion following a search on the NCBI database for highly identical EST sequences. Previously, the expression of P-gp 1B, C and D, carrying an insertion of a SINE element, could neither be demonstrated in porcine livers nor brain endothelial cells obtained from pigs (Childs and Ling, 1996). Taken together these results suggest that only one P-gp (ABCB1) is expressed in the pig, similar to humans. In contrast, rodents have two P-gp homologues displaying marked differences in substrate recognition and tissue expression (Croop, 1989; Schinkel *et al.*, 1997). Comparison of the rodent P-gp homologues with human P-gp over the equivalent nucleotide range (2866 base pares) showed that mouse and rat abcb1a shares an identity with human of 89.4% and 89.5 % respectively, while mouse and rat abcb1b shares an identity with human P-gp of 82.7 and 83.3 %, respectively.

As it is known that transmembrane regions of P-gp are important for substrate binding (Srinivas *et al.*, 2006), with the presentation of the deduced partial amino acid sequence of porcine P-gp the high homology between porcine and human P-gp in these regions could be demonstrated, suggesting also overlaps in functions. This allows the conclusion that the pig, expressing only one P-gp homologue with a high identity, seems to be a better model for humans as compared to rodent species.

Table 3. Comparison between several described human single nucleotide polymorphisms (SNIPs) and the porcine P-gp, nucleotide positions at NCBI acc. number AY825267.

Human SNPs	**Amino acid**	**Porcine P-gp**	**Amino acid**
G1199A	Ser400Ala	397G	Ser
C1236T	Non coding	434G	No difference
Gct2677Tct	Ala893Ser	1875Gcc	Ala
T3421A	Ser1141Thr	2619T	Ser
C3435T	Non coding	2633C	No difference

Recently, variations in activity and substrate specificity of ABC-transporters have been associated with single nucleotide polymorphisms in the P-gp gene (Kerb, 2006). A clinically relevant mutation in P-gp was reported in certain dog breeds in which a deletion of four base pairs (frameshift) leads to premature stop codons, resulting in a dysfunctional protein (Mealey

et al., 2001). Subsequently, these animals show a functional impairment of the blood-brain barrier and are highly sensitive to potentially neurotoxic compounds, including ivermectin and loperamide, which may pass the blood-brain barrier in these animals (Neff et al., 2004; Sartor et al., 2004).

The discussion on the impact of P-gp polymorphisms on the pharmacokinetics of P-gp substrates in humans is yet controversial. A comparison between several described human single nucleotide polymorphisms (SNPs) and the deduced amino-acid sequence of porcine P-gp indicates both, similarities and differences (Table 3). Most reports have suggested consequences of the above-mentioned SNPs on either function or expression of P-gp, while other previously reported SNPs appear to be as yet of limited relevance.

The second aim of this study was the evaluation of a functional P-gp assay, using porcine peripheral lymphocytes and Rh123 as a model P-gp substrate. For this validation, known potent inhibitors of human P-gp such as GF120918 and PSC833 were used, which both significantly inhibited Rh123 efflux, confirming that Rh123 efflux is mediated by P-gp in this porcine model. Rhodamine123 was also found to be a substrate for a mutant of human BCRP, and GF120918 has also been identified as an inhibitor of BCRP. Therefore we measured also the effect of Ko143, a potent inhibitor of human and rodent BCRP. At concentrations up to 1 μM, Ko143 did not have any inhibitory effect on Rh123 efflux, indicating the absence of a functional role for BCRP (data not shown). With the aim to assess the potential contribution of members of the multidrug-resistance related transporters (MRP-family) in Rh123 efflux, we measured the effect of MK571. As up to 1 μM, MK571 did not have any inhibitory effect on Rh123 efflux it can be concluded that Rh123 efflux by porcine lymphocytes seems not to be associated with MRPs. Subsequently it was demonstrated that ivermectin, a common model compound, was found to be a potent inhibitor of P-gp with an IC_{50} value of 0.17 μMol/L. Taken together, these findings allow the conclusion that Rh123 is a reliable model substrate to quantify P-gp function in porcine peripheral lymphocytes.

To quantify an inhibitory effect, the percentage of inhibition was calculated based on the AUC-method which requires the collection of samples at multiple time-points. Analysis of the correlation between the inhibition calculated by the AUC method, and the inhibition calculated at individual time-points, demonstrated a high correlation for all measurements after 120 minutes of incubation. Hence this time frame could be considered as being representative and was used in all other experiments. These additional data demonstrated that the substrates and inhibitors of human P-gp, cyclosporine A, verapamil, loperamide and ketoconazole are potent inhibitors of porcine P-gp as well, although remaining less potent than the proto-typical P-gp inhibitors, GF120918 and PSC833, and ivermectin. All calculated IC_{50} values were in the same order of magnitude as those obtained previously in MDCKII-MDR1 and LLC-PK1-MDR1 cells (Schwab, 2003; Rautio *et al.*, 2006). The macrolide antibiotics, erythromycin and azithromycin, known substrates for human P-gp (Pachot *et al.*, 2003) and digoxin, again a P-gp model substrate, exhibited a lesser inhibitory effect. In contrast, colchicine and levofloxacin,

although classified as substrates for human P-gp (Ito *et al.*, 1997), did not exert any inhibitory effect in porcine lymphocytes.

The inhibition of Rh123 efflux by cyclosporine A, verapamil, loperamide, ketoconazole and erythromycin, suggest a role of P-gp in drug-drug interactions. Indeed, erythromycin has been demonstrated to alter the pharmacokinetics of various P-gp substrates *in vivo* (Rodin and Johnson, 1988; Schwarz *et al.*, 2000; Eriksson *et al.*, 2006), whereas in cell-lines (LLC-PK1 and MDCK) expressing human MDR1, erythromycin was not identified as a potential candidate for P-gp mediated drug interactions (Schwab, 2003; Keogh and Kunta, 2006; Rautio, 2006). An inhibitory effect was previously found in porcine Brain Capillary Endothelial Cells (pBCECs) (Schwab, 2003), while inconsistent results have been reported in Caco-2 cells (Kim *et al.*, 1999; Schwab, 2003). These data indicate that individual *in vitro* models vary in their predictive value and that Rh123 efflux by lymphocytes as a surrogate marker for P-gp activity seems to be reliable and unique, being exclusively dependent on P-gp function as discussed above.

The aminoglycoside antibiotics, gentamicin and streptomycin, and the fluoroquinolone ciprofloxacin, have not been described as substrates or modulators of P-go before, and did also not have any inhibitory effect on Rh123 efflux in the presented model. Due to their relevance in veterinary medicine, we have also tested four congeners of the tetracycline group of antibiotics. These amphoteric compounds differ in their lipophilicity, suggesting differences in membrane permeability. This applies also to tetracycline (Grandi and Giuliani, 1988; Kavallaris M. *et al.*, 1993; George *et al.*, 1996) and oxytetracycline (Chapter 2) that are weak substrates for human P-gp. Although the various tetracyclines may be different in their intrinsic inhibitory potency, our data strongly suggest that the observed differences result from differences in lipophilicity and membrane permeability of individual compounds, rather than being related to a distinct P-gp inhibition.

In conclusion, the presented data demonstrate a high homology between human and porcine P-gp, both at the transcriptional, protein and functional level. A lymphocyte-based *ex vivo* model measuring functional P-gp in porcine lymphocytes could be validated by the use of proto-typical P-gp inhibitors and various substrates demonstrating the good agreement between the obtained results of this rapid assay and previously published data. The fact that Rh123 efflux by porcine lymphocytes depends on P-gp, and obviously no other efflux transporters, makes this model recommendable for the rapid identification of P-gp substrates in the course of drug development, as well as for the identification of drug-drug interactions at the level of P-gp.

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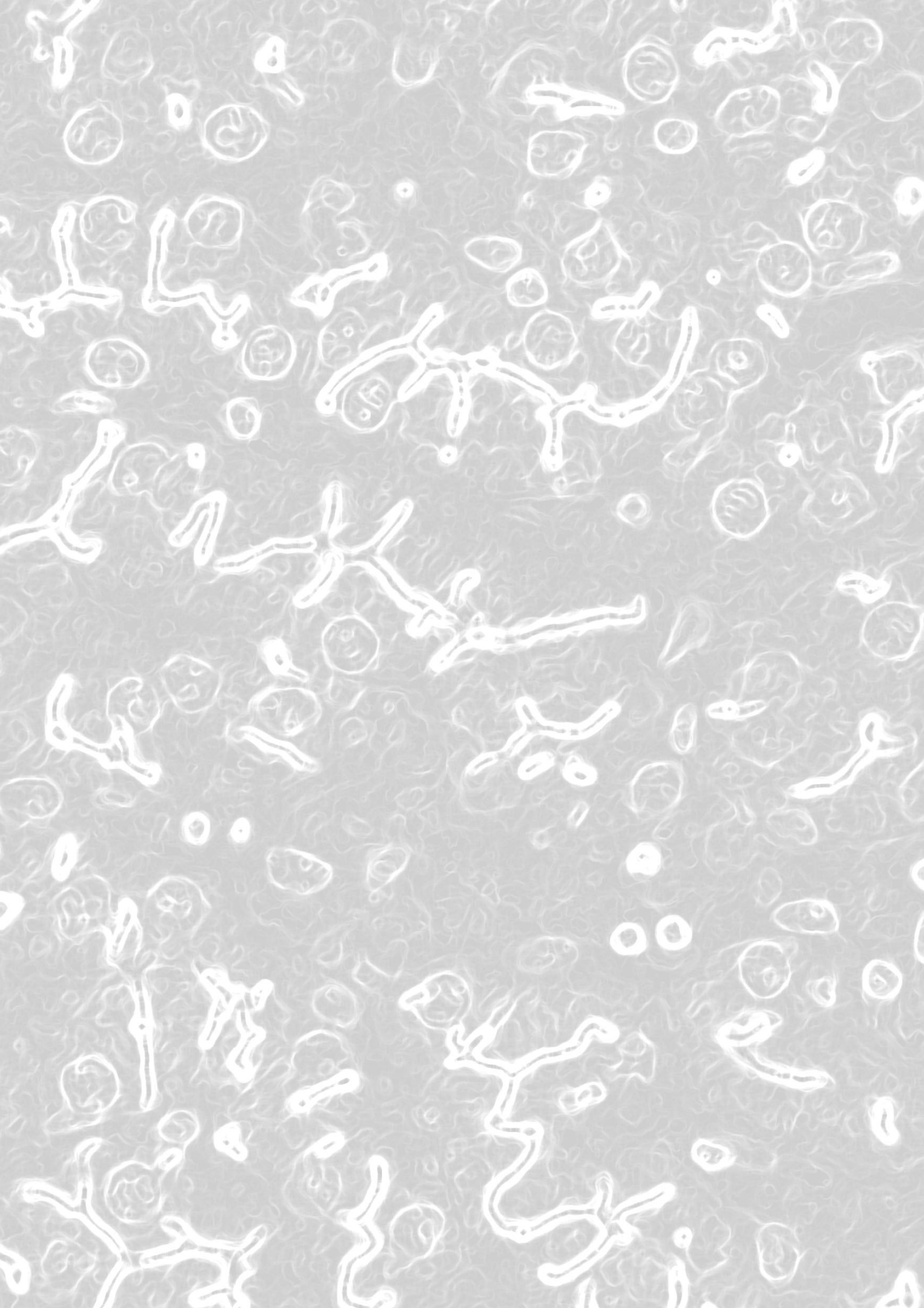


CHAPTER 8

General discussion

General discussion

ABC-transporters, and the role they play as modulators of the kinetics of drugs and toxins, are an area of intense research. Previously, drug-metabolising enzymes were considered as key players in drug absorption, disposition and elimination. Recent data, however, suggest that ABC-transporters are at least of equal importance in these processes. They recognize endo- and xenobiotics, control their absorption and excretion and often prevent the distribution of toxins into vulnerable compartments such as the central nervous system, reproductive cells or the unborn foetus. Among the group of ABC-transporters, P-gp, MRP2 and BCRP are important efflux transporters involved the kinetics of drugs and toxins.

Historically, the role of P-gp in the disposition of drugs has initially been demonstrated for the veterinary endectocidal drug ivermectin, which caused fatal intoxications in mdr1a knock-out mice. In these experiments the functional absence of P-gp resulted in neurotoxicity, and shortly thereafter the same function was confirmed for the gut barrier, as in these knock-out mice the absorption of orally administered digoxin was significantly increased (Sparreboom *et al.*, 1997). In turn it was postulated that in intact animals, the P-gp expression at the intestinal barrier is one of the major limiting factor in the oral bioavailability of its substrates. Later studies showed that oral absorption is apparently not reduced for all P-gp substrates, but mainly for those substrates that have a low diffusion rate (or permeability) and a low solubility. Subsequently, the bioavailability seems to be minimally affected by P-gp when the administered drug has a high solubility and high permeability, mainly because P-gp is than readily saturated. (Wu and Benet, 2005).

In the years following the first incident with ivermectine, other members of the ABC-transporter family were characterized, among other MRP2, being the most prominent exporter of conjugates out of hepatocytes, hence determining biliary excretion of drugs and toxins, but also the generic liver function, as also conjugated bile acids need to be exported out of the hepatocyte by this active transporter. Even more recently, BCRP, first identified as breast cancer resistance gene, conveying resistance to cycostatic agents in breast cancer cells, was shown to share many features including the expression at biological barriers with P-gp (Jonker et al., 2000; Sesink et al., 2005). Both P-gp and BCRP contribute to the barrier function of the intestinal wall and the blood-brain barrier that allows only 5% of all drugs to penetrate the brain tissue (Pardridge, 2003). BCRP plays a similar role in the human placenta, protecting via reduction of its permeability the exposure of the foetus to drugs and toxins (Jonker, 2000). Of veterinary interest is the high expression of BCRP in the lactating mammary gland (Jonker et al., 2005), where it facilitates the excretion of substrates, including various toxins, into the milk.

The nomenclature of P-gp, MRP2, BCRP and other transporters was based on historical findings. More recently, all transporters with an ATP binding domain that is essential for their function, are collectively called ABC-transporters, and these have been allocated to diverse gene families which seem to be highly conserved in animal species, including humans (Tab. 1).

Table 1: Nomenclature of important drug transporters (human gene names are in upper cases / rodent genes names in lower cases).

gene	protein	initially denoted as	Major Function
ABCB1	MDR1	P-gp	Steroid and drug transport
ABCB4	MDR3		phosphatidylcholine transport
Abcb1a	mdr1a	mdr3	Drug transport
Abcb1b	mdr1b	mdr1	Steroid and drug transport
Abcb4	Mdr2		phosphatidylcholine transport
ABCB11	BSEP	sPgp	Bile salt transport
ABCC2	MRP2	cMOAT	Transport of conjugates
ABCG2	BCRP	MXR	Transport of heme metabolites, xenobiotics and conjugates

Identification of substrates for ABC-transporters: examples from pig husbandry

While the importance of ABC transporters in human medicine is well perceived, drugs or toxins that are relevant in veterinary medicine often remain neglected (with the well known exception of ivermectin). One the first aims of this thesis was to demonstrate that structurally diverse veterinary drugs are likely to be substrates for more than one ABC-transporter as well. To this end, we applied the most commonly used model for the identification of substrates for drug transporters, which are Caco-2 cells. These cells grow to complete confluency and form a tight mono-layer of polarized cells resembling an intact tissue barrier. Caco-2 cells have been originally derived from colon carcinoma cells of human origin, but they exhibit features of small intestinal epithelial cells expressing P-gp, MRP2 and BCRP in their apical membrane (Seithel *et al.*, 2006). These characteristics make them suitable to be grown on microporous membranes, allowing to study the passage from one extra-cellular compartment to the other (Artursson and Karlsson, 1991; Balimane *et al.*, 2006). Moreover, the expression of multiple efflux proteins in one cell type make them an appreciated model also for a parallel screening of the characteristics of various transporters.

Transmembrane transport of Oxytetracycline

In Chapter 2 we describe various aspects of the transmembrane transport of Oxytetracycline (OTC). OTC is a generic antimicrobial and is still widely used in pig husbandry due to its broad antibacterial spectrum and attractive price, allowing oral mass medication of pig herds in the metaphylaxis and treatment of respiratory tract infections. High doses are needed to achieve clinical efficacy, as the oral bioavailability in pigs is only 3-9 %, and hence more than 90% of the given dose is excreted unused with the faeces. We hypothesized that OTC, like other tetracyclines, might be a substrate for efflux transporters in the intestinal tract. Our results indicated, however, a minor role for P-gp in the trans-cellular passage of OTC. Moreover, it seems that P-gp is readily saturated at low concentrations when related to the estimated intestinal concentrations of OTC achieved after common oral dosing regimens, even when the low solubility is taken into account. It is therefore not likely that P-gp has a significant effect on the oral bioavailability of OTC and in turn, the co-administration of P-gp inhibitors will not improve significantly the oral bioavailability.

Danofloxacin-mesylate, a veterinary fluoroquinolone with a large volume of distribution

Another class of antimicrobials, the fluoroquinolones, has been identified in the past as substrates for one or more ABC-transporters, albeit with significant differences in the affinity of individual fluoroquinolones for individual drug transporters. In veterinary medicine, Danofloxacin-mesylate is an important representative of the class of fluoroquinolone, used in the treatment of acute infections in relatively high dosages according to the principles of concentration-dependent dosing. Danofloxacin-mesylate is commonly applied parenterally. Kinetic analysis revealed, however, high concentration in the lumen of the gastro-intestinal tract, suggesting an active secretion from the circulation into the gut lumen. Our data show (**Chapter 3**) that danofloxacin-mesylate is a substrate for multiple efflux transporters, including P-gp, MRP2 and likely BCRP, providing mechanistic evidence for the observed drug distribution into the intestinal lumen, as P-gp and BCRP are both highly expressed in the apical membrane of intestinal epithelial cells. Hence, the high levels of Danofloxacin-mesylate as reported in the intestines of calves, for example, may be attributable to the secretory mechanisms that support the clinical efficacy against enteric pathogens. Other published findings, like the increased Rhodamine retention in the rabbit lung in the presence of P-gp inhibitors (Roerig *et al.*, 2004) and the secretion of Rhodamine in the apical direction by human bronchial epithelial cell-lines and primary human bronchial epithelial cells (Karen O. Hamilton, 2001; Lehmann *et al.*, 2001; Ehrhardt *et al.*, 2003) suggest that the high danofloxacin mesylate concentrations in the bronchial fluid can be also explained by the excretory function of ABC transporters. These extracellular levels are of therapeutic relevance as they may eliminate susceptible pathogens before these are able to invade bronchial cells.

Danofloxacin-mesylate was also found to be excreted with dairy milk. In dairy cattle husbandry, the secretion of drugs into milk can be regarded as either desirable or non-desirable. High antibiotic levels in the mammary gland and the alveolar lumen are favourable in cases of severe and recurrent mastitis that require systemic treatment. On the other hand, excretion of drugs into the milk, given for other indications than mastitis, requires a long withholding period for milk, which is undesirable under practical conditions as it results in considerable economic losses. Recently, the role of BCRP in the excretion of important veterinary drugs, including other fluoroquinolones as well as benzimidazoles into the milk has been confirmed in *in vivo* experiments with sheep (Merino *et al.*, 2005; Merino *et al.*, 2006).

Ochratoxin A, the most prominent renal toxin in pigs

The pig has been described as a sensitive species for ochratoxin A (OTA) induced nephrotoxicity, and toxicological data from pigs have driven recent risk assessments of this mycotoxin that is present in cereals and grains, coffee and nuts, as well as resins and wines (see EFSA, 2006). A typical feature of OTA is its long half-life in pigs and humans, as well as its accumulation in the kidneys. As not only pharmaceuticals, but also various toxins are substrates for ABC-transporters, we studied the role of ABC efflux transporters in OTA secretion in the Caco-2 cell model (**Chapter 4**). Applying this model, we demonstrated for the first time that OTA is not only a substrate of MRP2 but is also a BCRP-substrate. MRP2 and BCRP may thus contribute to the renal excretion of OTA, determining indirectly the degree of intracellular accumulation. Human pathologies associated with OTA have been described particularly in distinct geographic areas (see Balkan Endemic Nephropathy) and hence it would thus be interesting to study genetic instability in MRP2 and BCRP expression and function in the affected population. Moreover, epidemiological data repetitively suggested a link between OTA exposure and nutritional preferences in the local population of the affected districts on the Balkan Peninsula. It can be speculated that their diets contain natural compounds that act as inhibitors of ABC-transporters, resulting in a reduced capacity to eliminate the toxin (including a subsequent increased oral absorption). The finding that BCRP is involved in the cellular transport is in line with the observation that OTA is excreted with breast milk in humans (and most likely also in pigs) following nutritional exposure. The subsequent exposure of the neonates that might be related to differences in expression or function of BCRP, needs further attention (Ishikawa *et al.*, 2005; Yoshihito Korenaga, 2005; Tamura *et al.*, 2006).

In conclusion, these three examples further illustrate that knowledge of the interaction between ABC-transporters and a given drug or toxin is of importance for both pharmacology and clinical toxicology. These examples also indicate that efflux transporters may have to be considered as major determinants of drug distribution and elimination, with consequences for their efficacy and for target organs of toxicity in case of natural toxins and contaminants.

ABC-transporters in pigs: species differences

Species differences in the expression of both ABC transporters and biotransformation enzymes complicate the extrapolation of rodent data to humans and veterinary relevant species, and also between individual veterinary target species. The issue of species differences remains important, as extrapolation of kinetic data between animal species would be helpful in drug development, and remains necessary in the therapy of minor species, for which many drugs have not been tested and licensed yet. Thus, the species-specific knowledge of the expression level and the specific localization of ABC-transporters could contribute to our understanding of the kinetics of drugs and toxins in veterinary target animal species, and increase the accuracy of extrapolations between animal species.

At present, the knowledge about ABC-transporters in animals species others than rodents is very limited. Some data are available for dogs, particularly with regard to the expression of P-gp, associated with the intolerance to ivermectin and related avermectines. That is related to a spontaneous gene knock out in a subpopulation of Collie dogs that is extremely sensitive to the neurological side effects of P-gp substrate drugs, including ivermectin and loperamide. A 4-bp deletion (frame shift) generates several stop codons that prematurely terminate P-gp synthesis. Homozygous dogs are hypersensitive, while those that are homozygous normal or heterozygous do not display increased sensitivity to P-gp substrates (Mealey *et al.*, 2001; Mealey *et al.*, 2003; Roulet *et al.*, 2003; Sartor *et al.*, 2004). These findings elegantly explain the long-standing clinical observation that certain dog breeds are frequently involved in ivermectin intoxications.

Only very limited data were available in other target animal species, including the pig. The pig is of interest for two reasons: firstly, it is a major farm animal species and the intensive production systems require an intensive medication of pigs at nearly all stages of life. The subsequent high use of antimicrobial agents, however, forms a risk for environmental contamination with excreted antibiotics (as discussed for OTC), and requires the use of modern compounds (such as danofloxacin-mesylate) for which optimal dose regimens need to be established for all animal species to avoid the occurrence of antimicrobial resistance.
Secondly, pigs are a well recognized model species in transition medicine, i.e. serve as surrogate species in the development of new therapies intended for use in human medicine (Miller *et al.*, 2000; Myers *et al.*, 2001; Anzenbacher *et al.*, 2002; Fricker *et al.*, 2002; Bauer *et al.*, 2003; Schwab *et al.*, 2003; Weiss *et al.*, 2003; Angelow *et al.*, 2004; Petri *et al.*, 2006; Schlatter *et al.*, 2006; Skaanild, 2006).

In **Chapters 5 and 6** we report the tissue distribution of P-gp, MRP2 and BCRP in the pig as analysed by quantitative RT-PCR analysis (qPCR), and provide an overview on their cellular and subcellular localization as demonstrated by immunohistochemistry. Since only limited sequences for transporters and enzymes were available at the NCBI database, we had to analyse the cDNA sequences for the porcine homologues of the transporters P-gp and MRP2.

In addition, we analysed the sequences for the partial cDNA's of porcine ABCC4 (MRP4) and UGT1A and developed primers for quantitative PCR analysis of ABCC4, ABCB4 (MDR3), ABCB11 (BSEP), UGT1A and CYP2C to be applied in forthcoming studies.

Porcine ABCB1, ABCC2 and ABCG2

The presented results (**Chapter 5 and 6**) indicate an overlapping and highly comparable expression pattern of ABCB1, ABCC2 and ABCG2 transporters in pigs as compared to humans, strengthening the assumption that pigs are a good model in (human) drug development. The compilation of the qPCR data and immuno-histochemical analysis indicate a limited expression of P-gp and BCRP in the canalicular membranes of hepatocytes and the proximal tubule cells of the pig. In contrast, in mice a high hepatic and renal expression of BCRP has been reported, whereas the low hepatic expression in rats resembles more closely the expression in other species (Tanaka *et al.*, 2004). Mice excrete BCRP substrates via the renal and hepatic route, but this has not been demonstrated yet in humans, and due to the high expression in mice, an extrapolation of data from mice to humans and other animal species might not be useful (and justifiable). The expression of P-gp in the porcine kidney is apparently lower when compared to previous findings in humans, but the clinical impact of this difference remains to be elucidated as the role of P-gp in the urinary elimination of endo- and xenobiotics seems to be limited (Inui *et al.*, 2000).

An interesting finding was the positive staining for BCRP in the human kidney specimen in the presented immuno-histochemistry studies, as this was not demonstrated before. This finding indicates inter-individual variability in protein expression. Comparable differences in protein expression were also observed in pig tissues, with an absence of staining for P-gp and BCRP in two individual animals. Taken together these findings demonstrate not only the need for the use of a representative number of samples when evaluating protein expression, but also point towards potential differences in transport capacity and subsequently inter-individual variation in pharmaco- and toxicokinetics.

In consideration of the various similarities between pigs and humans in the tissue expression of ABC-transporters and the relation to drug-elimination, an attempt was made to compare the expression levels of the individual transporters in the major excretory organs. To this end we prepared a calibration curve containing known amounts of cDNA templates with large cDNA products for subsequent qPCR analysis (similar to nested PCR). With this method, the absolute expression in porcine livers, small intestines and kidneys could be ranked as follows: **liver**: MRP2 >> BCRP > P-gp; **kidneys:** MRP2 > BCRP >>> P-gp; **small intestines:** BCRP > P-gp > MRP2. The abundance of BCRP-transcripts in the small intestines was 2-5 times higher than that for P-gp, and in the liver 2 times higher than P-gp. In the interpretation of these data it needs to be considered, however, that BCRP is a so-called half transporter, with six membrane spanning domains, that needs the formation of homo-dimers or homo-tetramers for its function

(Liu *et al.*, 1999; Ozvegy *et al.*, 2002). Hence it remains to be elucidated to what extend these expression levels reflect quantitatively the actual capacity of these transport proteins.

The described findings indicate clinical and pharmacokinetic consequences of the studied transporters in the pig: the high expression of MRP2 in the liver and kidney can be associated with the competent handling of conjugates, including glucuronides and glutathion conjugates of endo- and xenobiotics, including drugs, which might compensate for the low activity of sulfotransferases in pigs. P-gp seems to play a minor role in urinary excretion, but both P-gp and BCRP are prominent efflux transporters at biological barriers, including the intestinal barrier, protecting the animal against environmental toxins. The data also indicate a prominent role for BCRP in organs others than the mammary gland, which might have been underestimated in previous studies, as BCRP was among the ABC-transporters discovered first in the late 90-ies.

ABCB1 and Cytochrome P450 3A – a faithful couple

We also aimed to compare the levels of expression of P-gp and CYP3A in porcine liver and intestines, these data are still incomplete and have not been published yet. Expression of CYP3A was found predominantly in the proximal intestines, but remained low as compared to the liver which is the major organ expressing CYP3A in all animals species tested yet – including humans. The expression in the pig liver was 14-fold the expression in the small intestines. P-gp and CYP3A share common transcription factors, such as PXR (pregnane X receptor), CAR (constitutive androstane receptor) and other heterodimers with retinoid receptors (RXR). The qPCR data from porcine tissues confirmed the co-regulation, regarding the expression pattern in the intestines and the liver. The expression of CYP3A in the porcine liver is very high, compared to its expression in the intestines and to the absolute hepatic expression of P-gp. Controversially, intestinal expression of P-gp was very high as compared to its hepatic level. This inverse relationship between liver P-gp and CYP3A has previously been suggested to occur in humans as well (Schuetz *et al.*, 1995), and it has been hypothesized that a decreased exposure of CYP3A inducers due to P-gp mediated efflux of these compounds contribute to this inverse relationship (Schuetz *et al.*, 1996). In this respect, it was demonstrated that hepatic CYP3A was increased in *abcb1* knock out mice, but also other Cytochrome P450 isoforms (Schuetz *et al.* 2000). This regulatory response may therefore be considered as an adaptive mechanism to protect the individual against toxic compounds present in foods and feeds and other environmental contaminants. Thus, since there is an extensive overlap in drugs and (endogenous) steroids that are both substrates for P-gp and modulators of CYP3A expression, their regulation at the transcriptional (and possible post-transcriptional) level deserves further attention.

Porcine P-gp drug-interactions

Functional characteristics for porcine P-gp were assessed in **Chapter 7** with the help of an *ex vivo* inhibition assay using porcine peripheral blood lymphocytes. This model that has been used previously for the monitoring of transporter functions in the course of diseases or during drug application. A range of structurally diverse compounds that are substrates or inhibitors for human P-gp were tested for their potency to inhibit porcine P-gp in lymphocytes. A potent inhibition was observed for the compounds that also potently (competitively) inhibit human P-gp, and although inhibitors are not necessarily substrates, these data are indicative for a functional homology between human and porcine P-gp.
We also observed that oxytetracycline does not inhibit P-gp mediated Rhodamine 123 efflux, while tetracycline had a week inhibitory activity. We hypothesized that inhibition of P-gp by tetracyclines depends on their lipophilicity driving membrane permeability and subsequently the inhibitory potency in lymphocytes. Subsequently two other tetracycline congeners with differences in lipophylicity were tested. Minocycline with the highest lipophilicity was the most potent inhibitor of P-gp activity, and OTC the least potent, whereas doxycyline and tetracycline were intermediates. With reference to our previous data in Caco-2 cells, we suggest that specific uptake transporters are missing in the lymphocytes (in contrast to Caco-2 cells) and that therefore the extends of inhibition solely depend on the passive diffusion rate of the tetracycline congeners.

Drug-drug interactions have been documented in humans for those compounds that inhibited P-gp mediated Rh123 efflux by porcine lymphocytes at the concentrations less than 25 μM. This indicates that this rapid method can be used for the early recognition of P-gp inhibitors in pigs and also human drug development. The lymphocyte-Rh123 assay is less time consuming (and less expensive) than the Caco-2 cell assay, conducted in permeable inserts, and can be easily extended to other species of veterinary interest. Preliminary experiments indicate the possible use of a comparable assay for BCRP, and will be further developed.

In conclusion, veterinary medicinal products and naturally occurring toxins in feeds are substrates for ABC-transporters that imply that the dynamic effects are related to the *in vivo* functionality of these transporters as determinants of xenobiotic absorption, distribution and elimination. The tissue-specific transport capacity of these proteins highly depend on the amount of transporters and the presence of (competitive) inhibitors. We demonstrated here the functional inhibition of porcine P-gp by structurally diverse drugs that exemplify the risk for drug interactions that could lead to an increased absorption by barrier-tissues and/or a decreased excretion and consequently an increased organ exposure. The amount and cellular localization of the transporters were assessed by qPCR and immunohistochemistry and it was demonstrated that the expression of the major ABC-transporters relevant for the secretion and excretion of these xenobiotics display a highly similar tissue distribution pattern in the pig when compared to humans. We also found a high level of homology in transporter cDNA sequences and the inhibitory activity of drugs on porcine P-gp that suggest comparable roles in

kinetics, however further studies should aim to study the functional characteristics of P-gp and the other transporters in the pig (and other animal species) in order to establish the species-specific knowledge of the functions. In addition, we have observed inter-individual differences in the mRNA and protein expression levels that are potential factors in the inter-individual variation in pharmacokinetics and subsequent efficacy of therapeutics. A more detailed knowledge of the complex regulation of transporter gene expression (and genetic polymorphisms) would help the clinician or practitioner in his decision for more individualized (including animal units) therapies.

Future perspectives:

ABC-transporters are known to translocate a wide range of chemically unrelated compounds across extra- and intracellular membranes, and substrates of these transporters also include metabolic products such as bile salts, lipids and sterols. The level of expression of ABC transporters in individual organs and cells has been associated with major transcription factors that also regulate the expression of various biotransformation enzymes. Hence originally, research focussed on the identification of the role of these efflux transporters in pharmacotherapy. With the increasing insight in the diverse functions of these transporters also in the compartmentalization of endogenous substrates, it becomes evident that these efflux transporters play not only a protective role in modulating absorption, distribution and excretion of drugs and toxins, but are also involved in the pathogenesis of various diseases.

ABC-transporters as regulators of liver function and diseases

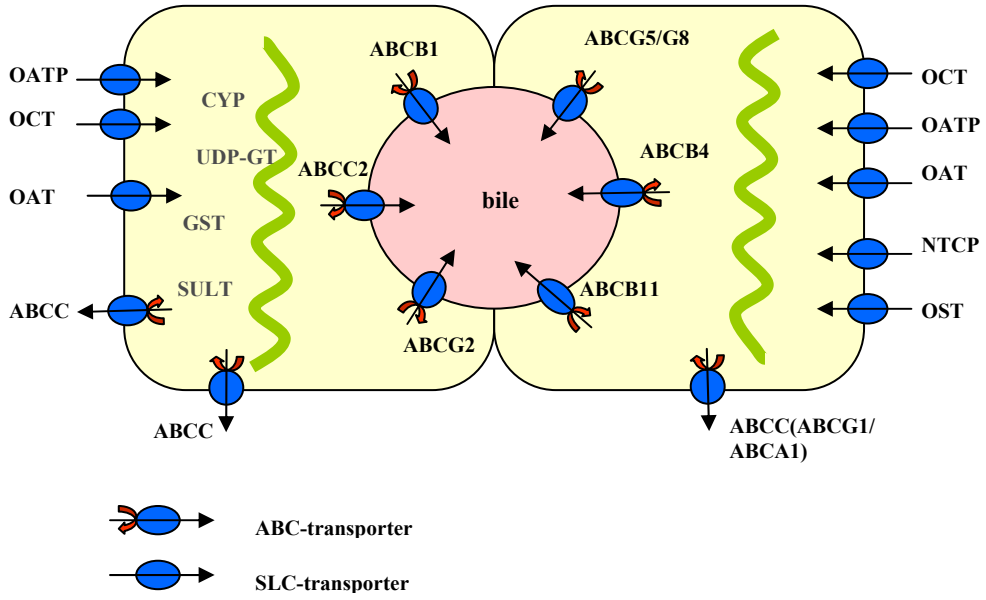
Endogenous and exogenous substances that enter the hepatocyte are subjected to oxidative (Phase I) and conjugation (Phase II) reactions, to improve their excretability by increasing their water solubility. However, in particular Phase II conjugates formed in the hepatocyte are normally too hydrophobic to passively diffuse across the canalicular membrane or sinusoidal membrane, and thus rely on active transport mechanisms. Transporters are, however, saturable and thus sensitive to substrate overload and competition (for example due to concomitant application of more than one drug, or concomitant exposure to drugs and environmental compounds (incl. feed components) resulting in decreased capacity (Lin and Yamazaki, 2003; Morris and Zhang, 2006). A decreased capacity leads to increased intracellular levels (liver) of drugs on one hand but also on the other hand of bile acids (and other conjugated metabolites). In cases of inherited or acquired dysfunctions of hepatic efflux transporters, accumulation of bile acids (, xenobiotics) and their metabolites may induce severe hepatocytic toxicity. Subsequently, the adaptive response of the hepatocytes during cholestasis aims at limiting the accumulation of potentially toxic biliary constituents within the cell: basolateral uptake transporters (OATP and NTCP) and metabolising enzymes are down regulated and apical and/or basolateral transporters are up-regulated, including members of the ABCC (MRP)

family (Scheffer *et al.*, 2002). In addition, compensatory upregulation of renal transporters has been observed (Chen *et al.*, 2005).

These compensatory mechanisms are also activated during the treatment of cholestasis with ursodeoxycholic acid (Zollner *et al.*, 2003), although the exact mechanism of induction is still incompletely understood.. Therefore, understanding of the underlying (dys-)regulatory factors in cholestasis is a key for understanding its molecular mechanisms and may identify potential therapeutic targets.

Various (inherited) diseases have been related to dysfunctions or genetic instability of ABC-transporters. Cholestasis in humans, such as the progressive familial intrahepatic cholestasis (PFIC-3), has been associated with dysfunction of MDR3 (ABCB4) and BSEP (ABCB11). MDR3 has been recognized as a major phophatidylcholine translocator, where BSEP is the main bile salt transporter (Pauli-Magnus *et al.*, 2005; Oude Elferink and Paulusma, 2006). It is worthwhile to mention here that BSEP (also denoted sister Pgp) has originally been identified in the porcine liver (Childs *et al.*, 1995). The pathophysiological role of these transporters in animal liver diseases is a challenging area, and could describe dysfunctions as well as hereditary diseases.

Figure1: Enzymes and transporters involved in the handling of drugs, xenobiotics and (endogenous) metabolites by hepatocytes



ABC transporters: ABCB1, ABCB4, ABCB11, ABCC2, ABCG2, ABCG5/8, ABCG1 and ABCA1; SLC transporters: OATP, OCT, OAT, NRCP, OST
Biotransformation enzymes: CYP; UDP-GT, GST, SULT

Novel targets for therapeutic intervention

As mentioned in chapter 1, FXR increases the expression of BSEP, MDR3, MRP2 and ligand treatment induced, in an animal model of intrahepatic cholestasis, the expression of genes encoding proteins involved in the secretion of biliary constituents into the canalicular system and decreasing bile acid uptake and *de novo* bile acid synthesis. At the same time, FXR limits the activation of stellate cells and hence may exert an antifibrotic effect by modulating the expression of SHP that in turn induces PPARγ (Pellicciari *et al.*, 2005). These data suggest that FXR ligands are potential targets for the treatment of (intrahepatic) cholestasis and liver cirrhosis.

In addition, ABCG5/G8, half transporters like BRCP (ABCG2), have been identified as biliary canalicular membrane sterol transporters, with a pivotal role in hepatic cholesterol excretion. Impairment of this transporter system has not only provided the mechanistic base for an inherited, but rare disease in human (Sitosterolemia) but also significantly increased the understanding of sterol trafficking in the organism. It is worthwhile to mention that the bovine sterol transporters share a responsive element in the promoter region for nuclear factor-kappaB (Viturro *et al.*, 2006). This link may result in a down-regulation of various efflux transporters as well as Phase I enzymes (Cytochrome P450 enzymes) during infections and inflammation. Bovine fatty liver disease might be associated with dysfunctions of these transporters, if there is any role in the aetiology, a possible therapeutic target would be LXR (Plosch *et al.*, 2002).

Finally the unique role of BCRP (ABCG2) in the function of stem cells should be mentioned. BCRP is highly expressed in SP cells (side population cells) of hematopoietic and non-hematopoietic sources, such as muscle, liver, brain and embryonic stem cells (van Herwaarden and Schinkel, 2006). It appears that BCRP not only protects stem cells from toxic compounds (including cytostatic drugs that are used in chemotherapy), but also conveys resistance to hypoxic conditions and may prevent early differentiation (Bunting, 2002; Scharenberg *et al.*, 2002; Krishnamurthy *et al.*, 2004). Although the mechanisms involved in the specific role of BCRP in stem cell survival remain to be elucidated, it may be speculated that targeted modulation of BCRP expression in these cells offer broad possibilities to advanced stem cell therapy (Tadjali *et al.*, 2006)

ABC transporters and veterinary issues

It is beyond the scope of this general discussion to review all data that link the expression and function of drug transporters with disease conditions. The few examples presented with respect to liver diseases, should serve only as an indication of the impact of ABC transporters in physiological and patho-physiological conditions. The few examples presented below should provide an indication for the impact of ABC-transporters in veterinary drug development or as genetic selection markers.

Drug residues in milk: One of the most recently classified ABC transporters is BCRP (ABCG2). Of major veterinary relevance is the expression of BCRP in the mammary gland of dairy cows and lactating small ruminants, where it transports structurally and functionally diverse compounds through the luminal surfaces (Jonker, 2005). The wide range of substrates transported by BCRP makes the protein a target for forthcoming therapeutic strategies to improve the efficacy of the treatment of for example bovine mastitis. In addition, BCRP is the target to control the excretion of undesirable substances into milk indented for human consumption (Merino *et al.*, 2005).

ABC transporters as genetic markers: BCRP is highly expressed also in mammary progenitor cells (Clayton *et al.*, 2004), indicating functional relevance in the protection or proliferation/differentiation and with possible consequences for mammary gland development. With the aim to use functional genetic polymorphisms in the selection of sires to increase the efficiency of milk production, genetic analysis suggested a role for BCRP as genetic marker. A SNP in the BCRP gene was found to correlate with milk yield, and fat and protein content of the milk (Cohen-Zinder *et al.*, 2005). These findings may result in the identification of genetic markers associated with milk production, with BCRP as a potential factor in the development of the mammary gland.

In conclusion, the recent findings indicate to overall importance of ABC transporters, not only for the prediction of the absorption, distribution and excretion of drugs and toxins, but also in the understanding of various physiological and patho-physiological conditions as well as inherited diseases. It remains a challenge for the forthcoming years to close the gap between the rapidly increasing knowledge in human medicine, and the application of this knowledge in veterinary sciences.

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Samenvatting in het Nederlands voor niet-ingewijden.

De farmacologie kent twee belangrijke onderdelen, de farmacodynamiek en de farmacokinetiek. De farmacodynamiek beschrijft het werkingsmechanisme van een geneesmiddel op de plaats waar het zijn effect uitoefent, bijvoorbeeld de remming van een enzym waardoor een product niet meer wordt gevormd dat ziekteverschijnselen veroorzaakt. Het is dus belangrijk dat het geneesmiddel de plek bereikt in het lichaam waar het werkzaam is. Dit betekent dat het geneesmiddel door (bijv.) de darm moet worden opgenomen en via de bloedcirculatie deze plaats moet kunnen bereiken, bijv. het hart. In de farmacokinetiek worden de processen beschreven die dit beïnvloeden. Het geneesmiddel zal echter onderweg in het lichaam verschillende celmembranen passeren en om deze passage te verbeteren of juist te voorkomen bestaan er speciale transporteiwitten.

Deze celmembraan-gebonden transporteiwitten worden onderscheiden in cellulaire opname- en cellulaire uitscheidingstransporters. De meeste cellulaire uitscheidingstransporters behoren tot de ATP-Binding Cassette (ABC) familie van membraangebonden eiwitten, die alle de energie gebruiken die vrijkomt na hydrolyse van ATP, om hun transport functie uit te oefenen. Deze grote familie bestaat uit 49 eiwitten met elk een eigen rol in het transport van stoffen over celmembranen. De meeste spelen een rol in de normale stofwisseling van cellen, echter een aantal kan naast lichaamseigen stoffen ook lichaamsvreemde stoffen transporteren zoals geneesmiddelen en giftige stoffen (toxinen).

Het eiwit uit de ABC familie dat als eerste is ontdekt is P-gp. De afkorting staat voor permeabiliteits-glycoproteine, een eiwit dat belangrijk is voor de permeabiliteit (doorlaatbaarheid) van celmembranen voor geneesmiddelen. De eerste bevindingen waren dat P-gp een ophoping van geneesmiddelen (veelal cytostatica) voorkomt in kankercellen, waardoor het therapeutisch effect tekort schiet. Sinds midden jaren negentig is ook de rol van P-gp in de farmacokinetiek bestudeerd en is gebleken dat P-gp de opname van veel medicijnen uit de darm remt en de uitscheiding via de lever bespoedigt, maar ook dat P-gp voorkomt dat medicijnen de hersencellen en de ongeboren vrucht kunnen bereiken. Na de ontdekking van P-gp zijn nog twee andere ABC-transporters gevonden die vergelijkbare functies hebben: BCRP en MRP2. BCRP en MRP2 transporteren niet alleen de toegediende geneesmiddelen zelf maar ook hun conjugaten, dit zijn geneesmiddelen of hun omzettingsproducten die gekoppeld zijn aan lichaamseigen verbindingen, zoals gluthathion, glucuronide en sulfaat, waardoor ze beter wateroplosbaar zijn. Hierdoor lossen ze goed op in de galvloeistof en urine, waarin ze uit gescheiden worden.

De meeste kennis over de genoemde transporteiwitten is vergaard in mensen en proefdieren (ratten en muizen), echter weinig is tot nu toe bekend over de rol in diersoorten die belangrijk zijn in de diergeneeskunde. Het doel van het onderzoek dat beschreven is in dit proefschrift, is de relevantie van deze transporters voor het varken met betrekking tot geneesmiddelen en toxinen te bestuderen.

In het eerste deel van het proefschrift wordt het onderzoek beschreven naar de mogelijke betrokkenheid van ABC-transporters in de kinetiek van drie stoffen die om uiteenlopende redenen belangrijk zijn voor het varken. We hebben voor deze proeven gebruik gemaakt van menselijke cellen, Caco-2 cellen, die uitgebreid zijn beschreven in andere studies, waaruit is gebleken dat ze zeer geschikt zijn om het transport te meten van stoffen door cellen.

Het antibioticum oxytetracycline is een oud, maar veel gebruikt middel in de varkenshouderij ter behandeling en voorkoming van luchtwegproblemen. Op grote schaal wordt oxytetracycline toegediend via het drinkwater of voer, maar de opname van oxytetracycline door de darm is zeer beperkt, slechts 3 tot 9 %. Een mogelijke verklaring hiervoor zou kunnen zijn dat de opname van oxytetracycline tegengewerkt wordt door ABC-transporters. De resultaten laten zien dat oxytetracycline een substraat is voor P-gp maar dat het effect op de opname minimaal is. Dit betekent dat het niet mogelijk is de opname van oxytetraycline door de darm te verbeteren door de functie van P-gp te blokkeren.

Danofloxacin-mesylaat is een antibioticum dat nog niet zo lang op de markt is en toegepast wordt voor de indicaties longontsteking, darmontsteking en melkklier ontsteking (mastitis). Danofloxacin-mesylaat wordt per injectie toegediend en moet dus in de darm, de long en de melk terecht komen. In hoofdstuk 2 laten we zien dat Danofloxacin-mesylaat wordt getransporteerd door meerdere transporters en hierdoor wordt uitgescheiden in bovengenoemde organen, hetgeen dus een voordelige eigenschap is. Deze kennis kan gebruikt worden voor de ontwikkeling van nieuwe medicijnen die hun werking uitoefenen in deze organen.

Zoals reeds genoemd worden niet alleen geneesmiddelen maar ook verscheidene toxinen door deze eiwitten getransporteerd. Ochratoxine A is een door schimmels geproduceerde gifstof waarvoor varkens zeer gevoelig zijn. Het veroorzaakt vooral nierafwijkingen, net als bij de mens. Ochratoxine A blijkt getransporteerd te worden door MRP2 en BCRP. Beide transporters zitten in de darm en uit recent onderzoek blijkt dat bepaalde stoffen in voeding, met name uit vruchten, deze transporters remmen met als gevolg een verhoogde opname van Ochratoxine A door de darm. In de nier leidt onvoldoende functie van deze transporters mogelijk tot een stapeling en vervolgens celschade. Dus veranderingen in de functie of in het aantal transporters in de nier kan gevolgen hebben voor deze cellulaire ophoping.

Deze voorbeelden laten zien dat de effecten van bepaalde geneesmiddelen en toxinen voor het varken afhankelijk zijn van de functie van transport-eiwitten. Specifieke kennis over de aanwezigheid en functie van deze eiwitten in het varken ontbrak echter.Het was wel bekend dat er verschillen zijn in de mate van aanwezigheid van de individuele transporter-types tussen diersoorten. Het belang van deze transporters in de diverse organen kan daardoor variëren per diersoort, hetgeen de extrapolatie van kinetische gegevens naar andere diersoorten bemoeilijkt. In de hoofdstukken 5 en 6 wordt de aanwezigheid van P-gp, BCRP en MRP2 in organen van het varken beschreven, waarbij een vergelijking wordt gemaakt met literatuurgegevens van andere diersoorten en de mens.

Twee verschillende benaderingen zijn gebruikt om de aanwezigheid van transporters aan te tonen. De eerste was immunohistochemische aankleuring van de drie eiwitten in organen van het varken die belangrijk zijn vanwege een barrière- en uitscheidingsfunctie. Door het gebruik van deze methode kunnen we laten zien waar de eiwitten zich bevinden. De tweede benadering was het meten van de expressie van deze transporters met behulp van de kwantitatieve PCR. Het voordeel van deze methode is de mogelijkheid om te kwantificeren. P-gp en BCRP worden in alle onderzochte organen tot expressie gebracht, waarbij BCRP een hogere expressie vertoont dan P-gp. Beide worden tot expressie gebracht in de baarmoeder, hersenweefsel, melkklier, hart, pancreas, milt en lymfocieten. P-gp en BCRP waren duidelijk aanwezig in de darm- en longepitheelcellen, en in de endotheliale bekleding van bloedvaten. MRP2 was aanwezig in levercellen, nierepitheel en in het darmepitheel van de dunne darm. Deze resultaten geven aan dat zowel P-gp als BCRP belangrijk zijn voor het beperken van de opname van geneesmiddelen en toxinen door de darmwand én voor de uitscheiding van deze stoffen in de darminhoud. Ook blijkt dat beide de uitscheiding van geneesmiddelen, bijvoorbeeld van danofloxacinmesylaat, in het longslijm kunnen stimuleren. MRP2 is, net als bij andere diersoorten, een belangrijke transporter in de lever en nieren zodat geneesmiddelen, toxinen en hun conjugaten het lichaam weer kunnen verlaten via de gal en urine.

In hoofdstuk 7 zijn de interacties van medicijnen met P-gp bestudeerd in lymfocieten van het varken. Structureel verschillende geneesmiddelen remmen de uitscheiding van Rhodamine 123 door P-gp. Ivermectine, cyclosporine, verapamil, ketoconazol en loperamide zijn zeer potente remmers en zodoende mogelijk ook substraten van P-gp. De gelijktijdige toepassing van deze geneesmiddelen of van deze geneesmiddelen met andere substraten voor P-gp geeft een hoog risico voor relatieve overdosering, door een verhoogde opname via de darm of door verhoogde concentraties in met name de hersenen. De hier toegepaste methode is eenvoudig op grote schaal toe te passen, niet alleen om interacties aan te tonen met P-gp, maar ook met andere transporteiwitten en kan daarom gebruikt worden als een snelle test om oude en nieuwe medicijnen te testen.

De verkregen resultaten van de expressie van de transporters en van de interacties met P-gp in het varken zijn vergeleken met de beschikbare gegevens uit de literatuur over de mens. Kennis van de overeenkomsten en verschillen tussen de mens en het varken maakt het namelijk mogelijk om beter onderbouwd beschikbare gegevens te extrapoleren van de mens naar het varken en andersom. En hoewel we veel overeenkomsten hebben gevonden op het nivo van de genetische codering (mRNA), de expressie en functie, zal vervolg onderzoek gericht moeten worden op de functionele karakteristieken van ABC-transporters in het varken om de belangrijke verschillen aan het licht te brengen.

De transporters die het onderwerp zijn geweest in dit promotie onderzoek, zijn de belangrijkste eiwitten in celmembranen van biologische barrières die geneesmiddelen en toxinen transporteren richting de holle ruimtes (lumina) in organen en voorkomen dat deze doordringen in kwetsbare weefsels zoals de hersenen. Om deze reden zijn ze in potentie ook belangrijk voor

de kinetische eigenschappen van andere geneesmiddelen en toxinen. Verder onderzoek op dit gebied kan daarom mogelijk verklaringen geven, niet alleen voor diersoortverschillen, maar ook voor inter-individuele variatie in de kinetiek van medicijnen en daarmee in de effectiviteit. Ook opent dit mogelijkheden voor het ontwikkelen en testen van nieuwe geneesmiddelen met als doel de functie van deze transporters te beinvloeden, zoals momenteel in de humane geneeskunde ter verbetering van de effectiviteit van bestaande geneesmiddelen of om juist de uitscheiding te bevorderen van toxinen en worden zo dus doelen voor de farmacodynamiek.

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Curriculum Vitae

Jan Schrickx was born on the 31st of December 1970 in Roermond, the Netherlands. He finished his secondary school (V.W.O. B.C. Schöndeln, Roermond) in 1990 and went to Utrecht to study Physics at the University of Utrecht. In 1991 he started his study in Veterinary Medicine at the same university. As a student, he did research for his students' thesis: the phytohaemagglutinin skin test in cattle, as an indicator for stress-induced immunomodulation?, and attended elective clinical rotations at the equine and bovine clinic of the University of California, Davis School of Veterinary Medicine, and at the Veterinary Medicine Teaching and Research Centre, Tulare, California, USA. Veterinary pharmacology attracted his attention by participating in a clinical field trial as part of the authorization procedure for a veterinary fluoroquinolone drug in pigs that was supervised by prof. dr. R van den Hoven. During this period in Utrecht, he was a member of the students association Veritas and also active for the veterinary students association DSK. After his graduation in 1999 as a Doctor of Veterinary Medicine, he had been working as a veterinary practitioner. First at a large dairy farm in Germany and in 2000 in a mixed animal practice in Volkel, the Netherlands, with major emphasis on dairy medicine. Thereafter, at the end of 2000, in a large animal practice in Scherpenzeel, the Netherlands, that was interrupted by the outbreak of foot and mouth disease in 2001. In that period he participated in the food and mouth disease eradication program after which he continued in Scherpenzeel. In September 2001 he started a PhD-program at the department of Veterinary Pharmacology, Pharmacy and Toxicology at the faculty of Veterinary Medicine, Utrecht University, under supervision of prof. dr. J. Fink-Gremmels and prof. dr. H. Vaarkamp. His work also included departmental duties in teaching general pharmacology and applied pharmacotherapy. He defended his PhD-thesis publicly on the 23rd of October 2006.

List of publications

J.A. Schrickx, Yuri Lektarau, J. Fink-Gremmels, Ochratoxin A secretion by ATP-dependent membrane transporters in Caco-2 cells. Archives of Toxicology 80: 243-249, 2006

J.A. Schrickx and J. Fink-Gremmels, P-glycoprotein mediated transport of oxytetracycline in the Caco-2 cell model. Journal of Veterinary Pharmacology and Therapeutics, 29, 2006, *in press*

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J.A. Schrickx and J. Fink-Gremmels, Expression of ABCB1, ABCC2 and ABCG2 in porcine tissues. *Submitted for publication in Toxicology Letters*

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