Transcriptional Regulation and Impact of ABC-transporters in Intestinal Cell Lines

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Abbreviations

AB	apical to basolateral
ABC	ATP-binding cassette
AhR	aryl hydrocarbon receptor
ANOVA	analysis of variance
ATP	adenosine-5'-triphosphate
BA	basolateral to apical
BCRP	breast cancer resistance protein
BLAST	basic local alignment search tool
bp	base pairs
Caco-2	human colon carcinoma cell line
CAR	constitutive androstane receptor
CD	Crohn's disease
cDNA	complementary DNA
COX-2	cyclooxygenase type 2
CYP450	cytochrome P450
DBD	DNA binding domain
DCR	dicer
DMEM	Dulbecco's minimal essential medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dsRNA	double strand ribonucleic acid
GAPDH	glyceraldehydes-3-phosphate
GR	glucocorticoid receptor
HBSS	Hank's balanced salt solution

HIV	human immunodeficiency virus
HRE	hormone response element
IBD	inflammatory bowel disease
Il	interleukin
iNOS	intrinsic nitric oxide synthase
kD	kilodalton
LY	lucifer yellow
LB	luria broth
LBD	ligand binding domain
5-LOX	5-lipoxygenase
LS180	human colon carcinoma cell line
MDR	multi-drug resistance
mRNA	messenger ribonucleic acid
MRP	multi-drug resistance associated protein
NCBI	National Center for Biotechnology Information
NFκB	nuclear factor κB
NRTI	nucleoside reverse transcriptase inhibitor
ORF	open reading frame
P(app)	apparent permeability coefficient
PCR	polymerase chain reaction
PGE2	prostaglandin E2
P-gp	P-glycoprotein
PhIP	2-Amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
pSUPER	vector system
PXR	pregnane X receptor
R123	rhodamine 123

RISC	RNA induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RT-PCR	reverse transcription polymerase chain reaction
RXR	retinoic X receptor (9-cis retinoic acid receptor)
SEM	standard error of the mean
shRNA	small haipin ribonucleic acid
siRNA	single strand ribonucleic acid
SNP	single nucleotide polymorphism
TM	transmembrane
TNF-α	tumor necrosis factor α
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
UC	ulcerative colitis
wt	wild-type
XRE	xenobiotic response element

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Summary

The intestine has an important role in facilitating and limiting absorption of drugs. Since the intestine forms a barrier to the external, epithelial cells have several properties to protect the body from microorganisms and toxins, and to control uptake of xenobiotics. This barrier system consists of tight-junction proteins which minimise paracellular uptake and on the other hand of membrane transport proteins and metabolic enzymes, which regulate transcellular uptake. Membrane transporters are very important in regulating drug absorption, distribution and extrusion. They are expressed in many tissues and regulate transport of endogenous and exogenous substances. ATP-binding cassette transporters (ABC-transporters) form a huge class of membrane transporters which are involved in drug transport. They exhibit large substrate specificity and effectively regulate drug absorption in the intestine. Induction or inhibition of these transporters may influence drug bioavailability of orally ingested drugs.

The aim of the thesis was to investigate the impact of ABC-transporters on intestinal drug absorption. The second purpose was to gain insight into transcriptional regulation of these transporters.

Two highly expressed ABC-transporters in the intestine, ABCB1 (P-gp, MDR1) and ABCG2 (BCRP), which have a broad and partly overlapping substrate specificity were of special interest in this thesis. The impact of P-gp and BCRP on transport of substrates, which are transported by both transporters, was investigated in the intestinal cell line Caco-2. A stable cellular *in vitro* system with single and concomitant knock-down of P-gp and BCRP was established using the method of mRNA silencing. Stable Caco-2 clones with selective, single knock-down of P-gp (siMDR1) and BCRP (siBCRP) and with concomitant knock-down of both transporters (co-silencing) were generated and characterised on the level of mRNA and protein expression. Additional functional characterisation was performed by transport and efflux studies using selective substrates of each transporter (chapter 3.1).

Compounds which are substrates of P-gp and BCRP were examined in this Caco-2 cell system by transport studies. The tyrosin-kinase inhibitor imatinib and the anti-retroviral drug abacavir are described to be substrates of both, P-gp and BCRP. Bidirectional transport of imatinib was observed in Caco-2 wild-type and BCRP-silencing clones, but no bidirectional transport was seen

in P-gp- and co-silencing clones. Since bidirectional transport means active transport processes, these results might suggest that P-gp possibly compensates transport in clones where only BCRP is silenced but BCRP does not when P-gp is silenced. We therefore concluded that P-gp might play a more important role in intestinal imatinib transport, but disturbing factors such as possible silencing off-target effects of our cell system have to be ruled out in further studies. Transport of abacavir showed a similar tendency as imatinib, but effects were only small and further evaluations have to be performed (chapter 3.2).

Regulation of P-gp in the intestinal cell lines Caco-2 and LS180 was investigated. Since budesonide is a frequently used glucocorticoid in inflammatory bowel disease (IBD), we investigated how budesonide influences P-gp expression in these intestinal cell lines. We observed differential effects in the two intestinal cell lines; budesonide showed an induction of P-gp in LS180 cells and a downregulation in Caco-2 cells. Expression levels of nuclear receptors revealed high expression of pregnane X receptor (PXR) only in LS180 cells and exclusive expression of glucocorticoid receptor (GR) in Caco-2 cells. Mifepristone, an anti-glucocorticoid, could not reverse the downregulation of P-gp by budesonide in Caco-2 cells. In PXR-transfected Caco-2 cells the budesonide-mediated downregulation of P-gp was abolished. Furthermore the expression of cytochrome P450 3A4 (CYP3A4), another target gene, was induced in PXR-transfected Caco-2 cells after budesonide treatment. Therefore, the induction of P-gp by budesonide in LS180 cells probably is mediated via PXR. However, the mechanism of the downregulation in Caco-2 cells still remains unclear, but GR does not seem to be involved (chapter 4.1).

In a collaboration, interactions of budesonide with the PXR-target gene CYP3A4 was investigated in comparison to other glucocorticoids. Induction studies in LS180 cell line and in a hPXR-transactivation assay showed, that budesonide significantly induced intestinal CYP3A4 while other glucocorticoids did not. *In vivo* data in mice showed that budesonide and dexamethasone induced intestinal CYP3a11 while only dexamethasone induced liver CYP3a11. These data indicated that budesonide has the potential to induce intestinal PXR target genes but only to a small extent and therefore, the risk for interactions seems to be low (chapter 4.2).

Caco-2 cells represent a good *in vitro* model for the small intestine but do not express functional active PXR. Therefore we aimed to establish a Caco-2 cell model with stable transfected PXR. We

successfully transfected Caco-2 cells with PXR. Induction of PXR target genes P-gp and CYP3A4 was achieved after induction with the PXR-ligand rifampicin. However, the effects were not as pronounced as in LS180 cells and therefore additional investigations are indicated (chapter 4.3).

In further studies, effects of curcuma longa L. extracts and single curcuminoids on transcriptional regulation of ABC-transporters, CYP enzymes and pro-inflammatory proteins were investigated. Curcumin is described as anti-inflammatory agent and is discussed as possible therapy for intestinal inflammation. Our data in tumor-necrosis-factor-α (TNF-α) induced LS180 cells indicate that curcuminoids reduce mRNA expression of different proteins involved in inflammation such as iNOS, TNF-α and COX-2. Additionally, the curcuma extract was shown to directly inhibit cyclooxygenase-2 (COX-2) activity (chapter 5.2). Curcuma extracts and curcuminoids showed no relevant effects on P-gp and CYP mRNA expression in LS180 cells indicating no interaction potential of curcuminoids and curcuma extracts on the level of transcriptional regulation (chapter 5.1). Effects of curcuma extracts and curcuminoids on inflammatory proteins and ABC-transporters or CYP expression have to be confirmed *in vivo*.

Aim of the Thesis

The aim of this thesis was to investigate the impact of ABC-transporters on drug absorption in the intestine. Of special interests therein were two important intestinal efflux transporters, P-glycoprotein and breast cancer resistance protein.

Furthermore, transcriptional regulation of intestinal P-gp and CYPs by topical effective anti-inflammatory drugs in the intestine was of interest. Of special concern was transcriptional regulation via the nuclear receptor PXR.

In order to fulfill the above mentioned aims, the following topics were investigated:

- Establishment of a stable Caco-2 cell culture model with selective knock-downs of P-gp and BCRP and additionally a concomitant knock-down of both transporters.
- Testing of drugs described to be substrates of both, P-gp and BCRP, and determining the impact of each transporter on transport of these substrates.
- Investigation of the influence of budesonide on transcriptional regulation of P-gp in Caco-2 and LS180 intestinal cell lines.
- Determination of the role of PXR in transcriptional regulation of P-gp and CYP3A4 by budesonide.
- Establishment of a PXR-inducible Caco-2 system by stable transfection of PXR.
- Investigation of curcuma extracts and curcuminoids on the regulation of ABC-transporters and CYPs.
- Investigation of a curcuma extract and curcuminoids on anti-inflammatory effects in an intestinal cell line.

1 Introduction

1.1 Drug Absorption in the Intestine

The intestine forms a barrier between the organism and the external environment. Major tasks of intestinal tissue comprise absorption of nutrients and protection against toxins and microorganisms. The intestine is highly colonised by bacteria; therefore protection against these bacteria and bacteria toxins is an important challenge of the intestinal epithelium. Intestinal epithelial cells form tight-junctions, which mainly consist of occludin and claudin. These proteins prevent paracellular uptake of e.g. toxins. Transcellular uptake is regulated by specific membrane pumps and channels (Baumgart and Dignass, 2002).

Tissue of the small intestine forms finger-like projections called villi with additional microvilli resulting in a huge surface extension. Therefore, most ingested nutrients and drugs are absorbed in the small intestine. In general passive diffusion along a concentration gradient is a common way for transcellular drug absorption in the intestine. However, several factors hinder drug absorption and can lead to poor bioavailability. Chemical and physicochemical properties of the drug such as the molecular weight, the amount of proton bond acceptors and donors or LogP-values (octanol-water partition coefficient) are important factors which determine drug absorption (Lipinski et al., 2001). Solubility in the intestinal fluid is a further requirement for drug absorption. Systemic bioavailability of drugs is also influenced by an additional set of different factors. Instability in acidic pH in the stomach or metabolism by the intestinal microflora or by hydrolytic and conjugative enzymes are possible limiting factors for drug absorption. In addition to the inactivation by conjugation (phase II metabolism), phase I intestinal metabolism is important for reducing bioavailability of oral drugs (Figure 1)(Benet et al., 1996). High expression level of the cytochrome P450 (CYP) enzyme family in the mature villus tip enterocytes was observed. Among phase I metabolising enzymes, CYP3A4 appears to be the most abundant CYP in human enterocytes and liver. (Shimada et al., 1994), (Watkins et al., 1987), (Kolars et al., 1992). Additionally, CYP3A4 is the most important CYP enzyme in drug metabolism since it is responsible for the metabolism of about 50% of available drugs (Benet et al., 1996). The fact that CYP3A4 is highly expressed at the villus tip enterocytes where it has

contact to the ingested drugs, suggests that intestinal metabolism exhibits an important impact on drug absorption.

In addition to intestinal metabolism, also transport proteins exhibit a significant influence on oral drug bioavailability. Carrier mediated uptake enables absorption of hydrophilic drugs. Bile-acid-, amino-acid- or oligopeptide-transporters belong to those transporters that facilitate absorption of e.g. L-dopa or cephalosporins (Hu and Borchardt, 1990), (Inui et al., 1992). In contrast to these uptake transporters, active efflux transporters are able to reduce drug bioavailability. ATP-binding cassette transporters represent a huge superfamily of active efflux transporters. Transporters of this superfamily were investigated in this thesis and are therefore described in detail (see chapter 1.2).

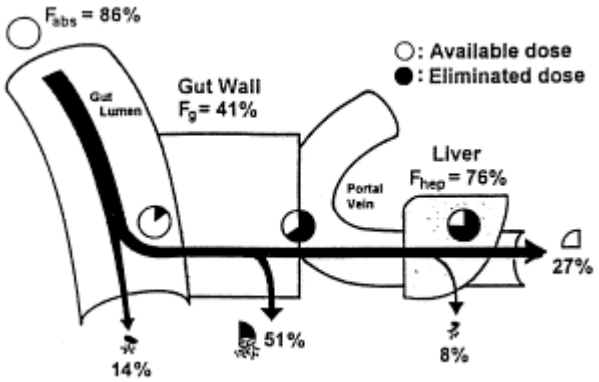


Figure 1 Schematic diagram for mechanisms involved in low bioavailability of cyclosporine A. 14% is not absorbed, exported by P-gp or degraded in the lumen. 51% is metabolised in the enterocytes and 8% in hepatocytes. These data point out the importance of enterocytes in limiting drug bioavailability (Benet et al., 1996).

1.2 ABC-Transporter

ATP-binding cassette transporters (ABC-transporters) represent a large superfamily of ATP-driven active efflux transporters. Seven subfamilies are described (ABCA-ABCG), therein members of ABCB, ABCC and ABCG subfamilies are involved in drug-transport. The structure of these transporters shows extensive sequence homology among the different subfamilies. ABC-transporters consist in general out of 12 transmembrane (TM) domains with two intracellular nucleotide binding sites. Half-transporters with only 6 TM domains and one nucleotide binding site are described in the ABCG subfamily. Some transporters of the ABCC subfamily which additionally comprise 5 TM domains at the N-terminus make an exception to this general structure.

ABC-transporters are in general located in the cell membrane, where they function as efflux pumps. Active extrusion of drugs requires ATP and can proceed against a concentration gradient. ABC-transporters were first discovered in tumour cells mediating as multi-drug-resistance protein. The first characterised member was P-glycoprotein (P-gp, MDR1, ABCB1) in Chinese hamster ovary cells (CHO) (Juliano and Ling, 1976). Identification of multi-drug resistance associated transporters (MRPs, ABCC) and breast cancer resistance protein (BCRP, ABCG2) followed afterwards. High expression of the mentioned transporters in cancer cells led to therapy resistance due to active export of anticancer drugs. Beside the expression in tumour cells, ABC-transporters are also expressed in healthy tissues. Predominantly, ABC-transporters are expressed in tissues which occupy a barrier or excretory function such as blood brain barrier (BBB), kidney, liver or intestine. Apical expression in the enterocytes suggests active extrusion into the gut lumen, while expression on the basolateral side of enterocytes suggests transport to the blood circulation (Figure 2) (Chan et al., 2004). Expression levels of ABC-transporters in the intestine were systematically analysed in biopsies from different segments of the gastrointestinal tract. All transporters showed alterations in their expression levels from the duodenum to the sigmoid colon. Most pronounced changes were observed for MRP2, with high levels in the small intestine and very low expression levels in the colon. MDR1 showed highest expression levels in the terminal ileum, while BCRP expression was highest in the duodenum and decreased to the rectum (Gutmann et al., 2005), (Zimmermann et al., 2005).

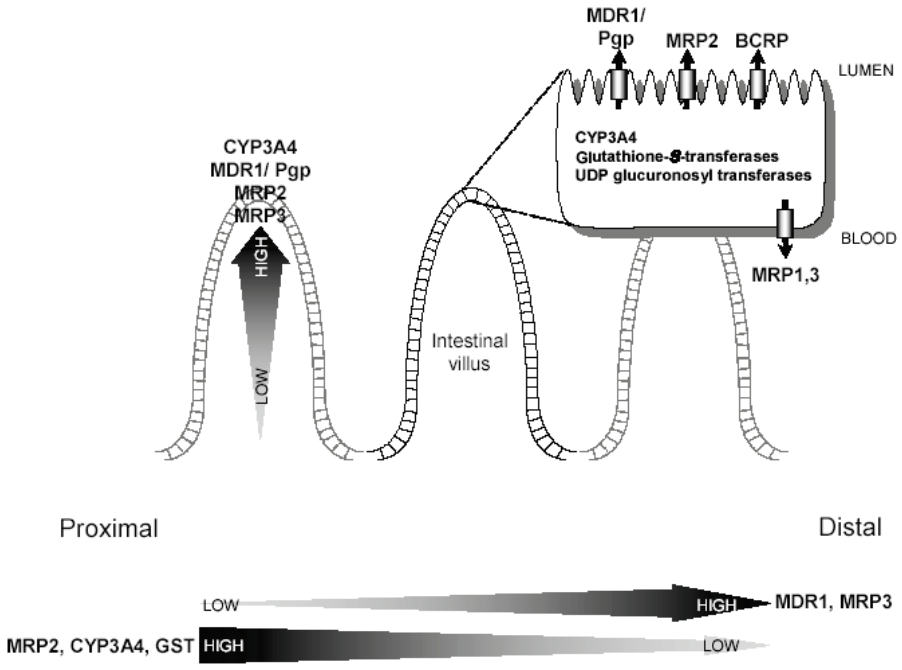


Figure 2 Expression of ABC-transporters in the small intestine. Expression is mainly in the villus tip enterocytes and varies from proximal to distal. Apical expression leads to increased efflux transport and reduced absorption while basolateral expression leads to increased absorption (Chan et al., 2004).

The ABC-transporters P-gp (ABCB1, MDR1) and BCRP (ABCG2) were of special interest in this thesis, since they play a major role in intestinal export function and have broad and partly overlapping substrate specificity.

1.2.1 ABCB1 (MDR1, P-gp)

P-glycoprotein is the first discovered ABC-transporter and therefore probably also the best characterised. P-gp was first described in drug resistant CHO cells as membrane glycoprotein with a molecular weight of 170 kD. It consists out of 12 TM domains and two nucleotide binding domains. It is arranged in cell membranes with the TM domains building an open ring of two halves (Figure 3). This arrangement suggests the mechanism of action. There are two models for drug-transport. The 'vacuum cleaner' model proposes that drugs interact with P-gp

within the hydrophobic part of the membrane bilayer, enter the transporter between the two halves and are transported to the extracellular space. In the 'flippase' model, it is suggested that P-gp can flip drugs from the inner to the outer leaflet of the bilayer or to the extracellular space (Hennessy and Spiers, 2007).

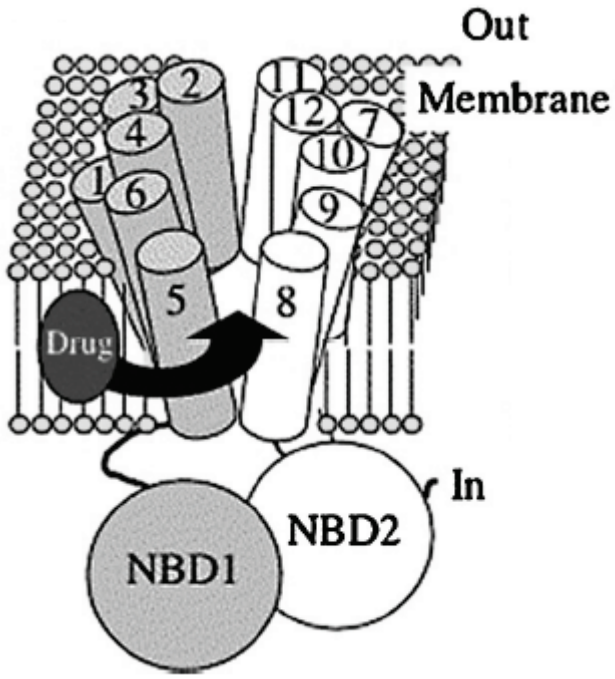


Figure 3 Arrangement of TM domains of P-gp and proposed entrance site for drugs between TM domains 5 and 8 or TM domains 2 and 11 (Hennessy and Spiers, 2007).

The expression of P-gp is found at high levels in tumour cells indicating its important role in multi-drug resistance in anticancer therapy. Besides tumour cells, P-gp is constitutively expressed in a wide variety of different normal tissues. The small intestine, colon, liver, kidney, placenta, blood-brain-barrier and adrenal gland express P-gp (Cordon-Cardo et al., 1990). P-gp is expressed mainly at the apical side where it has its barrier or excretory function. In intestinal tissue it is apically expressed in enterocytes of the villus tip and transports its substrates back to the intestinal lumen (Figure 2). This location and function indicates the important role of P-gp in extruding xenobiotics and toxins to protect the body. A huge number of drugs are substrates of P-gp. The chemical structure and pharmacological action of P-gp substrates differs considerably. Thus, P-gp recognises substrates from a broad range, but a tendency towards lipophilic cationic compounds was described (Zamora et al., 1988). Many important drugs of completely different

pharmacological classes such as antibiotics, anticancer agents, cardiac drugs, immunosuppressants, antihistamines and steroids are transported by P-gp. Drug-drug interactions possibly occur when two substrates of P-gp are applied concomitantly. While one drug modifies P-gp function, absorption or clearance of the other drug might change. Such interactions are of special importance when one of these drugs has a small therapeutic range. Relevant increases in plasma concentrations of e.g. digoxin were described when P-gp function was inhibited. (Hager et al., 1979).

On the other hand, induction of P-gp expression could lead to increased drug clearance or reduced absorption leading to insufficient therapy outcome. A broad set of drugs were described as inducers of P-gp. Pregnane-X-receptor (PXR) was described to mediate P-gp induction and is activated by a variety of xenobiotics (see chapter 1.3.2). Regulation of P-gp expression is very complex and far from being fully understood. Analysis on the promoter revealed a set of elements where in addition to PXR different factors could bind and regulate gene expression (Labialle et al., 2002).

In addition to drug-drug interactions, single nucleotide polymorphisms (SNPs) are stated to influence P-gp function and inducibility. Several SNPs were identified on the MDR1 gene, including the wobble mutation at C3435T which was described to be associated with decreased P-gp expression, inducibility and function (Hoffmeyer et al., 2000).

1.2.2 ABCG2 (BCRP)

Breast cancer resistance protein (BCRP) was first described in 1998 in MCF-7 breast cancer cells and in human placenta (Doyle et al., 1998), (Allikmets et al., 1998). BCRP consists only of 6 TM domains and one nucleotide binding domain and is therefore considered as half-transporter. Recent studies proposed that BCRP functions as a homodimer (Ozvegy et al., 2001). Like P-gp, BCRP shows polarised apical expression in tumour cells but also in normal tissue. BCRP expression is found in placenta, breast, ovary, small intestine, colon, blood brain barrier and liver (Maliepaard et al., 2001). The location of BCRP indicates its function. In placenta BCRP has a major role in the protection of the foetus against drugs, the expression in liver and intestine indicates its effects on reducing drug bioavailability and protection against toxins. Substrates of

BCRP are anticancer agents such as topoisomerase I inhibitors, mitoxantrone, prazosin and the food carcinogen PhIP (van Herwaarden et al., 2003), (Jonker et al., 2000), (Cisternino et al., 2004). BCRP substrates are partly overlapping with P-gp substrates. The amino acid 482 seems to be important in recognition of substrates since a SNP on this amino acid leads to resistance to known P-gp substrates such as rhodamine 123 or anthracyclines (Robey et al., 2003).

Clinical importance of BCRP was shown by Kruijtzer et al. by administering topotecan with a BCRP-inhibitor. Bioavailability of topotecan increased from 40% to 97% in patients while taking the inhibitor (Kruijtzer et al., 2002). This data clearly shows the effect of BCRP in limiting drug bioavailability.

Regulation of BCRP expression in the intestine is not fully elucidated but findings in Caco-2 cell lines showed that activation of the aryl hydrocarbon receptor (AhR) is involved (Ebert et al., 2005).

1.3 Regulation of Intestinal Transporters

Direct functional interaction with transporters in the intestine occurs via a set of different specific or unspecific inhibitors. First generation inhibitors of P-gp were developed for other indications. The calcium channel blocker verapamil or the immunosuppressant cyclosporine A belong to this generation. Unfortunately, these inhibitors are not selective to P-gp, since inhibition of e.g. BCRP and CYP3A4 was also described. Second generation drugs are structurally similar drugs to the first generation but modified to potentiate the inhibitory ability and to minimise other pharmacological effects. However, selectivity to P-gp was not achieved. Dex-verapamil (R-isomer of verapamil) and PSC833 (valspodar), a cyclosporine analogue are examples for second generation inhibitors. Inhibitors of the third generation have stronger potency and higher specificity (Hennessy and Spiers, 2007).

Besides direct inhibition, modulation of transporters function also can take place via intracellular signaling. P-gp function can be reduced or activated by protein kinase C (PKC) isoforms in different ways (Sachs et al., 1999). It was shown in hepatocytes that ABC-transporters may reside intracellular and are delivered to the canalicular membrane when increased transport function is demanded (Kipp and Arias, 2002). Ubiquitination which can determine degradation of proteins can also play a role in P-gp modulation. Zhang et al. showed that the ubiquitin-proteasome pathway regulates P-gp stability and function (Zhang et al., 2004). Regulation of ABC-transporter function was described to be influenced by endothelin via the endothelin-B receptor (Masereeuw et al., 2000).

1.3.1 Transcriptional Regulation

The mentioned short-term regulations can take place within minutes and have to be distinguished from long-term regulation which affects transcription of ABC-transporters and takes place within hours or days. Transcriptional regulation of P-gp is complex and covers many different factors which are not yet fully elucidated. Different proteins may bind to the MDR1 promoter and induce or reduce transcription. The MDR1-promoter, as well as the BCRP-promoter, lack a TATA-box but contain an initiator element. Several promoter elements have

been characterised on the MDR1 and BCRP promoters. (Labialle et al., 2002), (Bailey-Dell et al., 2001).

Transcriptional regulation via nuclear receptors is an important regulation of MDR1 and BCRP.

The nuclear receptors superfamily covers a large amount of structurally related, ligand activated transcriptional factors (1999). The subfamily III consists of hormonal receptors such as glucocorticoid- (GR), mineralocorticoid- (MR), progesterone- (PR), androstane- (AR) or oestrogen receptor (ER). These classic nuclear steroid hormone receptors have high affinity to steroids and regulate steroid expression by a negative feedback mechanism of the hypothalamic-pituary axis. The Subfamily II contains 9-cis retinoic acid receptor (RXR) which forms heterodimers with many other nuclear receptors from subfamily I. The latter subfamily comprises a large amount of different groups of nuclear receptors. Members of this subfamily are thyroid hormone receptor (TR), retinoic acid receptor (RAR), peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR) and vitamin D-receptor like receptors such as vitamin D receptor (VDR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR). Several members of the nuclear receptor superfamily such as GR, VDR, CAR, PXR and RXR have been shown to play a major role in transcriptional regulation of metabolic enzymes and efflux transporters (Ogg et al., 1999), (Thummel et al., 2001), (Honkakoski et al., 1998), (Burk et al., 2005), (Bertilsson et al., 1998), (Geick et al., 2001).

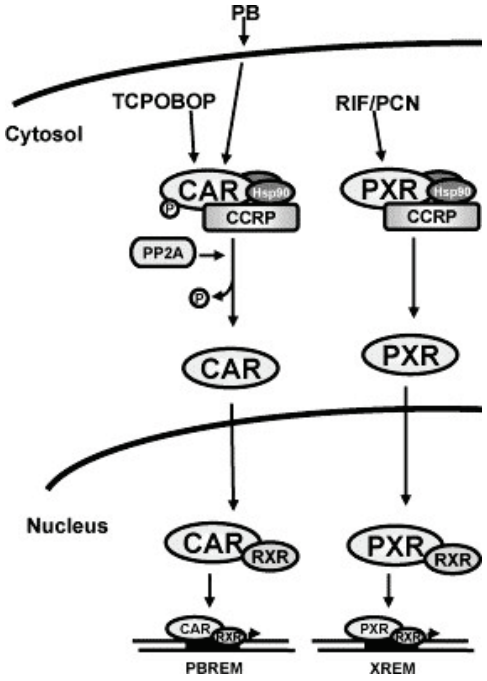


Figure 4 Nuclear receptors signal transduction. Ligand binding activates the nuclear receptors CAR or PXR, and frees them from co-repressors. In the following, the receptors translocate to the nucleus and build heterodimers with RXR. Transcriptional regulation is initiated upon binding to specific response elements of the promoter of the target gene. (Timsit and Negishi, 2007)

Among those receptors, PXR and CAR are important for MDR1 regulation. Regulation via PXR is of interest in this thesis since many drugs are ligands of PXR (see chapter 1.3.2). BCRP expression is not regulated via PXR but other nuclear receptors such as PPARγ (Szatmari et al., 2006), and aryl hydrocarbon receptor (AhR) (Ebert et al., 2005), (Ebert et al., 2007) or ER (Zhang et al., 2006) are described to be involved.

1.3.2 PXR

The structural organisation of PXR is divided in 4 general domains, a modulator region with activation function, a DNA binding domain (DBD) comprising two zinc-finger motifs, a hinge region which connects to the ligand binding domain (LBD) which is also associated with an activation function. The LBD of PXR is able to bind a variety of different ligands (Watkins et al., 2001). Such ligands are endogenous and synthetic steroids as well as a variety of different xenobiotics such as rifampicin, nifedipine, clotrimazole or paclitaxel (Kliewer et al., 1998), (Blumberg et al., 1998), (Lehmann et al., 1998), (Synold et al., 2001). The DBD is conserved

among species, sequence homology between human PXR and mouse or rat PXR is 96%, while the LBD is less conserved, sequence homology is about 76% (Kliewer et al., 2002). This observation might explain the fact that different ligands activate PXR in mouse and human.

Mechanistic insights in PXR mediated transcriptional regulation revealed ligand binding to the inactivated cytoplasmatic nuclear receptor. Then the following sequence of events takes place: conformational change, translocation to the nucleus, dimerisation with a co-factor (RXR) and final binding to response elements regulates target gene expression (Figure 4). These response elements are called xenobiotic response element (XRE) or hormone response element (HRE). They are organised as direct- (DR), everted- (ER) or inverted- (IR) repeats, with spacer base pairs in-between. These structures allow binding of the DBD of PXR which contains two zinc-finger motifs.

Target genes of PXR are metabolic enzymes and transporters. Several subfamilies of the cytochrome P450 (CYP) enzyme family have been shown to be regulated via PXR. Among those enzymes CYP3A4 is of special importance, since about 50% of drugs are metabolised by this isoform. ABC-transporters, MDR1 and some multi-drug-associated transporters (MRPs) are also regulated via PXR. Therefore, the interaction potential on the level of transcriptional regulation by PXR is important for drug bioavailability.

1.4 Inflammation of the Intestine

Ulcerative colitis (UC) and Crohn's disease (CD) are chronic inflammatory disorders of the intestinal tract. They are summarised as inflammatory bowel disease (IBD). In UC, the inflammation occurs in the mucosa and mucosal ulceration can develop. UC is mainly localised in the rectum and spreads to proximal parts of the intestine to a different extent. In CD, any part of the gastrointestinal tract can be affected, however the main site of inflammation is the terminal ileum and inflammation can occur segmental and discontinuously. In contrast to UC, not only the mucosa is affected but also all layers of the intestinal wall and granuloma are formed. The pathogenesis of both diseases is not yet fully elucidated. Different factors such as genetics, immune dysregulation, the microbial flora in the intestine and barrier dysfunction of intestinal epithelial cells may lead to the pathology of IBD. (Kucharzik et al., 2006), (Xavier and Podolsky, 2007).

ABC-transporters play an important role in maintaining the barrier function of the intestine, therefore their role in IBD is discussed. A downregulation of PXR and MDR1 gene expression in UC-patient compared to healthy controls was observed (Langmann et al., 2004). Furthermore, mdr1a knock-out mice (mdr1a-/-) were shown to spontaneously develop intestinal inflammation with a pathology similar to IBD (Panwala et al., 1998). SNP polymorphism of MDR1 and BCRP have been investigated and correlated to IBD. Up to now, it is not known whether there is an association between a polymorphism of MDR1 and BCRP and IBD. Different groups could not observe this association (Oostenbrug et al., 2006), (Fischer et al., 2007). In contrast to these findings, Fiedler et al. revealed an association between UC and the two MDR1 polymorphisms G2677T/A and C3435T (Fiedler et al., 2007). A meta-analysis gave an association between C3435T and UC, but no association with G2677T/A and none with CD (Annese et al., 2006).

Therapy options of IBD aim on reduction of inflammation and suppression of immune response. Steroids (e.g. budesonide) and 5-aminosalicylates and the immunosuppressants azathioprine and methotrexate are standard therapeutics. New options are biologicals such as the anti-TNF-α antibody infliximab. Treatment failure with glucocorticoids was described (Farrell and Kelleher, 2003). Since glucocorticoids are known substrates of P-gp and were shown to induce P-gp gene expression via PXR-pathway, the role of P-gp in therapy resistance is discussed. P-gp expression

was increased in peripheral blood monocytes in UC-patients after glucocorticoid treatment (Hirano et al., 2004). Furthermore, treatment failure in UC and CD was associated to an increase in P-gp expression in peripheral blood lymphocytes (Farrell et al., 2000).

Taken together, P-gp might be involved in disease susceptibility due to its barrier function in the intestine. Furthermore, it is possibly involved in glucocorticoid resistance due to its function as efflux transporter.

1.5 Curcuma Longa L.

Curcuma longa L. belongs to the family of Zingiberaceae (ginger family). It is a perennial plant and cultivated in southern parts of Asia. The powdered rhizome of *Curcuma longa* (turmeric) is of importance since it is widely used in food industry as spice and yellow pigment. It also has a long tradition in traditional Indian medicine, where it was used as agent to treat inflammatory diseases, hepatic disorders and skin wounds (Ammon and Wahl, 1991).

The main ingredients of *Curcuma longa*, which are believed to be responsible for its biological activities are the curcuminoids curcumin, demethoxycurcumin and bisdemethoxycurcumin. The curcuminoids make up about 5% of turmeric powder and the most abundant and best investigated is curcumin. *In vitro* data showed that curcumin interacts with a set of different molecular targets such as transcriptional factors, inflammatory cytokines, enzymes, kinases, growth factors and receptors. Anti-inflammatory, antioxidant and chemopreventive properties were shown for curcumin. Ongoing clinical trials aim to provide treatment of diverse cancer and inflammatory diseases (Goel et al., 2008). Treatment of inflammatory disorders in the intestine with curcumin is also of special interest. A study in UC-patients showed that curcumin is effective in maintaining remission (Hanai et al., 2006).

After oral intake, curcumin is hardly absorbed and the main part remains in the intestine. Interactions on absorption side could be the consequence, since it was described that curcuminoids interact with ABC-transporters. *In vitro* data revealed that the curcuminoids modulate P-gp function (Chearwae et al., 2004). A reduced expression of P-gp was also observed (Anuchapreeda et al., 2002), (Limtrakul et al., 2004). Inhibition or downregulation of intestinal P-gp can affect the intestinal barrier function and increased absorption of xenobiotics could be the consequence. Interaction studies with curcumin and BCRP showed that BCRP expression was induced by curcumin (Ebert et al., 2007).

Considering these data curcuminoids could be useful in treatment of inflammatory diseases of the intestine but the interaction potential with ABC-transporters has to be determined.

2 General Methods

2.1 Cell Culture

The LS180 and Caco-2 cell lines were purchased from ATCC (Manassas, USA). LS180 and Caco-2 cells were cultured in Dulbecco's MEM with Glutamax-I, supplemented with 10% (v/v) fetal bovine serum, 1% non essential amino acids, 1% sodium pyruvate, 50 µg/ml gentamycin (Invitrogen AG, Basel, Switzerland). Caco-2 clones additionally were treated with geneticin (Invitrogen) 1 mg/ml as selection antibiotic. Cells were maintained in a humidified 37°C incubator with a 5% carbon dioxide in air atmosphere.

2.2 Reverse Transcription, real time PCR (Taqman)

LS180 and Caco-2 cells were disintegrated by adding lysis buffer RLT (Qiagen, Hilden, Germany) and homogenised by using QIAshredder columns (Qiagen). Total RNA was extracted from cell lysates using the RNeasy Mini Kit (Qiagen). RNA was quantified with a Nanodrop Spectrophotometer (Witeg AG, Littau-Luzern, CH). The purity of the RNA preparations was high as demonstrated by the 260 nm / 280 nm ratio (range 1.8-2.1). After DNase I digestion (Gibco, Life Technologies, Basel Switzerland) 0.75 µg of total RNA was reversed transcribed by Superscript II (Gibco) according to the manufacturer's protocol using random hexamers as primers (Applied Biosystems, Rotkreuz, Switzerland).

TaqMan analysis was carried out on a 7900HT Sequence Detection System (Applied Biosystems). PCR conditions were 10 min 95°C followed by 40 cycles of 15 s 95°C and 1 min 60°C. Each TaqMan reaction contained 10 ng of cDNA in a total volume of 10 µL. qPCR™ Mastermix Plus from Eurogentec (Seraing, Belgium) was used. Primers and probes were used at concentrations of 900 nM and 225 nM, respectively. They were synthesised by Invitrogen (Basel, Switzerland) and by Eurogentec (Seraing, Belgium), respectively. Primers and probes were designed according to the guidelines of Applied Biosystems with help of the Primer Express 2.0 software. Corresponding sequences of primers and probes for TaqMan analysis are shown in

Table 1. All samples were run in triplicates and not reverse-transcribed RNA served as a negative control.

For absolute quantification we used external standard curves. Standards were gene-specific cDNA fragments that cover the TaqMan primer/probe area and they were generated by PCR. Sequences of the corresponding primers are shown in Table 2. The PCR products were purified by running a 1.5% agarose gel and a subsequent gel extraction (gel extraction kit, Qiagen). The standards were quantified using the PicoGreen reagent (Molecular Probes, Eugene, OR, USA) and were checked by sequencing (Microsynth GmbH, Balgach, Switzerland). For some genes there were used cDNA samples to generate a standard curve.

Table 1 Primers and Probes for TaqMan Analysis

Gene	Probe
GAPDH	5`-CGCCTGGTCACCAGGGCTGC-3`
MDR1	5`-AAGCTGTCAAGGAAGCCAATGCCTATGACTT-3`
BCRP	5`-CCATTGCATCTTGGCTGTCATGGCTT-3`
GR alpha	5`-TTTCAACCACTTCATGCATAGAAT-3`
GR beta	5`-CATAACATTTTCATGCATAGAATCCAAGAGTTTTGTCA-3`
PXR	5`-AGCCCTTGCATCCTTCACATGTCATGA-3`
CYP3A4	5`-TTCTCCTGGCTGTCAGCCTGGTGC-3`
CYP1A2	5`-CACAGCCATCTCCTGGAGCCTCATGTA-3`
COX-2	5`-TTCCTACCACCAGCAACCCTGCCA-3`
5-LOX	5`-CTTCGAGCGTGGCGCGGTG-3`
TNF-alpha	5`-TAGCCCATGTTGTAGCAAACCCTCAAGCT-3`
Il-8	5`-CTTGGCAAAACTGCACCTTCACACAGA-3`
iNOS	5'-TGGCAAGCACGACTTCCGGGTG-3'

Gene	Forward Primer (FW) and Reverse Primer (REV)
GAPDH	FW: 5`-GGTGAAGGTCGGAGTCAACG-3` REV: 5`-ACCATGTAGTTGAGGTCAATGAAGG-3`
MDR1	FW: 5`-CTGTATTGTTTGCCACCACGA-3` REV: 5`-AGGGTGTCAAATTTATGAGGCAGT-3`
BCRP	FW: 5`-CAGGTCTGTTGGTCAATCTCACA-3` REV: 5`-TCCATATCGTGGAATGCTGAAG-3`
GR alpha	FW: 5`-GGCAGCGGTTTTATCAACTGA-3` REV: 5`-AATGTTTGGAAGCAATAGTTAAGGAGA-3`
GR beta	FW: 5`-AACTGGCAGCGGTTTTATCAA-3` REV: 5`-TGTGAGATGTGCTTTCTGGTTTTAA-3`
PXR	FW: 5`-GGCCACTGGCTATCACTTCAA-3` REV: 5`-GTTTCATGGCCCTCCTGAAA-3`
CYP3A4	FW: 5`-TCTCATCCCAGACTTGGCCA-3` REV: 5`-CATGTGAATGGGTTCCATATAGATAGA-3`
CYP1A2	FW: 5`-CAATGACGTCTTTGGAGCAGGAT-3` REV: 5`-CAATCACAGTGTCCAGCTCCTTC-3`
COX-2	FW: 5`-GAATCATTCACCAGGCAAATT-3` REV: 5`-TTTCTGTACTGCGGGTGGAAC-3`
5-LOX	FW: 5`-TGGACAAGCCCTTCTACAACG-3` REV: 5`-CTCGTCCACAGTCACGTCGT-3`
TNF-alpha	FW: 5`-TCTTCTCGAACCCCGAGTGA-3` REV: 5`-CCTCTGATGGCACCACCAG-3`
Il-8	FW: 5`-CTCTTGGCAGCCTTCCTGATT-3` REV: 5`-TATGCACTGACATCTAAGTTCTTTAGCA-3`
iNOS	FW: 5'-TGCAGACACGTGCGTTACTCC-3' REV: 5'-GGTAGCCAGCATAGCGGATG-3'

Table 2 Primers for cDNA Standards

Gene	Forward Primer	Reverse Primer
GAPDH	5`-ACATCGCTCAGAACACCTATGG-3`	5`-GCATGGACTGTGGTCATGAGTC-3`
MDR1	5`-ACAGTCCAGCTGATGCAGAGG-3`	5`-CCTTATCCAGAGCCACCTGAAC-3`
BCRP	5'-TTTCAGCCGTGGAACTCTTT-3'	5'-TGAGTCCTGGGCAGAAGTTT-3'
GR alpha	5`-TACCCTGCATGTACGACCAA-3`	5`-TTTTGGTATCTGATTGGTGATGA-3`
GR beta	5`-TACCCTGCATGTACGACCAA-3`	5`-TTGTCGATGAGCATCAGTTG-3`
PXR	5`-GCAGTCCAAGAGGCCCAGAA-3`	5`-CGTCGGACATGATCATCTCCTTC-3`
CYP3A4	5'-TAGTGATGGCTCTCATCCCAGA-3'	5'-TGAAGGTTGGAGACAGCAATGA-3'
CYP1A2	5'-ACTTTGACAAGAACAGTGTCCGG-3'	5'-GCCAAACAGCATCATCTTCTCA-3'
COX-2	5`-CATAGGGCTTCAGCATAAAGCG-3`	5`-ACCCTCTATCACTGGCATCCC-3`

2.3 Western Blot

Proteins were extracted with protein extraction buffer (20 mM Tris-HCl, 1% Igepal CA-630, 0.5 mM sodium orthovanadate) including 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride (Sigma-Aldrich, St. Louis, MO, USA) and protease inhibitor cocktail tablet, Complete Mini (Roche Diagnostics, Germany). The quantification of the protein content was performed with the BCA protein assay kit (Pierce Chemical, Rockford, IL, USA). Protein concentration was determined by measuring the absorbance at 562 nm with Spectra MAX 250 Microplate Spectrophotometer (Molecular Devices Corporation, California, USA).

For immunoblotting, 50 µg of total protein extract was mixed with Laemmli sample buffer (Bio Rad Laboratories, Reinach, Switzerland) and transferred to the polyacrylamide gel. Gel electrophoresis was performed with a Mini Protean 3 Electrophoresis Cell (Bio Rad) applying 80 V for 15 min for the stacking gel (4% acrylamide) and 120 V for 1 hour for the separating gel (6.5-10% acrylamide). After electrophoresis, proteins were blotted to the nitrocellulose membrane (250 mA for 2.5 hours) using a Mini Trans-Blot Cell (Bio Rad). Protein transfer was verified by Ponceau S staining. The membrane was blocked overnight at 4°C with PBS

containing 5% milk powder and 0.05% Tween 20. After washing three times for 15 minutes (0.05% Tween in PBS), the membrane was incubated for 2 hours at room temperature with the respective primary antibody in PBS containing 0.05% Tween and 1% milk powder. As loading control beta-actin mouse monoclonal antibody (abcam, Cambridge, UK) was used. After the first incubation the membrane was washed 3 times for 15 min and then incubated with the secondary, horseradish peroxidase-conjugated, rabbit anti-mouse or goat anti-rabbit IgG (Amersham, Buckinghamshire, UK) diluted 1:1000. Secondary antibody incubation was performed for 1 hour at room temperature. Membranes were washed, and protein detection was performed with the enhanced chemiluminescence system (ECL-Detection-Kit, Amersham). The molecular weight was identified by using Precision Plus Protein™ Standard Dual Color (Bio Rad).

Table 3 Antibodies

Protein	Antibody	Concentration (Dilution)
MDR1	C219 (Alexis Corporation, Lausen, CH)	0.1 mg/ml (1:100)
BCRP	BXP-21 (Alexis)	0.25 mg/ml (1:100 – 1:1000)
PXR	PXR Rabbit Polyclonal Antibody (abcam, Cambridge, UK)	0.53 mg/ml (1:500)
Beta-actin	mouse monoclonal antibody (abcam)	1 mg/ml (1:1000 – 1:10000)

2.4 Transport

Caco-2 cells were seeded on type I collagen 5 µg/cm^2 (Becton Dickinson, Basel, CH) precoated Transwell® filters (polycarbonate 12 well, pore size 0.4 µm or polyester [clear], 12 well, pore size 0.4 µm) (Corning, Baar, Switzerland) in a density of 660‘000 cells/cm^2. Cells were cultured for 3 days in DMEM high glucose (see chapter 2.1). After 2 or 3 days medium was changed. Cells were incubated with Intestinal Epithelial Differentiation Medium (BD) and 0.1% MITO+ Serum Extender (BD). On the 4th or 5th day, medium was exchanged again and on the 5th or 6th day the transport-assay was performed.

Before starting the assay, both sides of the Transwell® were washed 3 times with pre-warmed HBSS (Gibco) supplemented with 1mM pyruvate and 10 mM Hepes adjusted to a pH of 7.4 (HBSS-P). A pre-incubation with or without the respective inhibitor dissolved in HBSS-P on the apical side (0.5 ml) and HBSS-P at the basolateral side (1.5 ml) was performed for 15 minutes. For apical-to-basolateral (AB) transport, at time t=0 substrates and tightness-marker with or without inhibitor were given to the apical donor chamber and on the basolateral donor compartment HBSS-P was added. Transport was performed at 37°C and 120 rpm over 2 hours. Samples were taken after 10, 20, 40, 60, 90 and 120 minutes out of the basolateral donor compartment. Volume was replaced with HBSS-P. For basolateral-to-apical (BA) transport, the basolateral side acted as donor chamber and the apical side was the acceptor chamber. Thus, substrate and tightness-marker were added to the basolateral side and samples were taken out of the apical side. However, if an inhibitor was used, it was added to the apical acceptor compartment. Samples were taken at indicated time points out of the acceptor compartment (apical) and volume was replaced.

After 2 hours, samples out of the donor chamber were also taken for drawing the balance of transport. Transport-buffer was removed and the Transwell® was placed on ice. Both sides of the Transwell® were washed 3 times with ice-cold HBSS-P. Transwell®-filters were cut for measurement of the remaining substance in the cells. Radioactive-labeled (^{14}C or ^{3}H) or fluorescent substrates were used. Insta Gel plus scintillation liquid was added to the radioactive samples and analysis was performed on a scintillation counter (Packard TriCarb2000, Canberra Packard S.A.). Detection of fluorescent samples was carried out on a fluorescent reader (HTS 7000 Plus Bioassay Reader, Perkin Elmer Ltd., Buckinghamshire, UK). To confirm tightness of the monolayer ^{14}C-sucrose (0.6 µCi/ml) (Perkin Elmer, Schwerzenbach, Switzerland) or Lucifer Yellow (20 µM) (Sigma Aldrich, Buchs, CH) was used. LY was measured at 428 nm excitation and 536 nm emission.

The apparent permeability coefficient (P_{app}) was calculated for tightness-markers in order to determine the tightness of the monolayers. P_{app} is calculated with the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0}$$

The term $\frac{dQ}{dt}$ is the steady-state flux, A stands for the surface of the filter and C_0 is the initial concentration of the donor chamber (Hubatsch et al., 2007).

2.5 Uptake

Cells were seeded 50'000 cells/cm^2 on 24-well plates (Falcon) and incubated for 2-3 weeks and medium was changed every 3-4 days (chapter 2.1). Before starting the assay, cells were washed 3 times with pre-warmed HBSS supplemented with 1 mM pyruvate and 10 mM Hepes (HBSS-P) and incubated for 15 min at 37°C and 120 rpm with HBSS-P with or without inhibitor. After this pre-incubation cells were incubated with the respective substrate with or without inhibitor for the indicated time. In this step influx and efflux of the substrate goes on, while in the cells where an inhibitor is added, the efflux is inhibited. After this period of time the plate was placed on ice and samples of the supernatant were taken. Cells were washed 3 times with ice cold HBSS-P with or without inhibitor. In order to achieve cell-lysis, 5% Triton X-100 was added and cells were incubated again for 1 hour. The lysate was homogenised and samples were taken for measurement of the remaining substance in the cells. Radioactive-labeled (^{14}C or ^{3}H) compounds were given to Insta Gel plus scintillation liquid and analysis was performed on a scintillation counter (Packard TriCarb2000, Canberra Packard S.A.). Fluorescent compounds were analysed on a fluorescent reader (HTS 7000 Plus Bioassay Reader, Perkin Elmer Ltd.).

2.6 Efflux

Cells were seeded and cultured as described for the uptake assay. All monolayers were washed 3 times with pre-warmed HBSS-P (37°C) and were pre-incubated for 15 min at 37°C and 120 rpm with corresponding inhibitors dissolved in HBSS-P. The solutions were removed and all cells were incubated with HBSS-P containing the test-compounds and corresponding inhibitors for 30

min at 37°C and 120 rpm. After loading the plate was put on ice and the cells were washed 3 times with ice-cold HBSS-P with or without the test inhibitors. Subsequently HBSS-P (37°C) alone and with inhibitors was added to the cells, and the efflux took place the indicated time at 37°C and 120 rpm. After this period of time, the reaction was stopped by putting the plate on ice and samples from the supernatant were taken for measurement of efflux. Afterwards, cells were rinsed 3 times with ice cold HBSS-P with or without inhibitors. Then, they were lysed at 37°C for approximately 1 hour using 5% Triton-X. Cells, which were treated with radioactive labeled compounds, were transferred to scintillation vials. After adding Insta Gel plus, the samples were analysed in a scintillation counter (Packard TriCarb2000). The cells loaded with fluorescent drugs were analysed on a Perkin-Elmer HTS 7000 Bio Assay Reader using the indicated settings.

2.7 Statistics

If not otherwise stated, groups were compared to control group by analysis of variance (ANOVA). If this analysis revealed significant differences and more than one treatment group was included in the analysis, pairwise comparisons of treatment groups with the control group was performed subsequently using Dunnett's two-sided multi-comparison test. All tests were performed using the SPSS for Windows software (version 15.0). The level of significance was $P<0.05$.

3 Knock-down (Silencing) of ABC-Transporters

3.1 Establishment of the Silencing Method

3.1.1 Abstract

Background and Purpose: RNA knock-down or silencing is a new method to inhibit protein expression by cutting mRNA sequence specifically. P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) are two important ATP-binding cassette transporters (ABC-transporters) in the intestine. They are expressed at relatively high levels at the apical membrane of enterocytes and figure as efflux transporters of various drugs and toxins. Highly specific chemical inhibitors of P-gp and BCRP are lacking. Therefore, we aimed to establish an intestinal cell culture model with selective knock-down of P-gp and BCRP and with concomitant knock-down of both (co-silencing).

Experimental Approach: Specific sequences for P-gp and BCRP silencing were designed using an open access algorithm, synthesised and integrated into the pSUPER vector. Caco-2 cells were transfected with these vectors containing a neomycin resistance gene for selection. Stable clones were achieved by culture under antibiotic pressure. Validation of the specific knock-down of the silencing clones was performed on mRNA level by real-time RT-PCR and on protein level by Western blot analysis. Functional inhibition of P-gp and BCRP was measured using efflux and transport assays with the specific P-gp substrates ^{3}H-digoxin and rhodamine 123 (R123), and the specific BCRP substrate ^{14}C-PhIP.

Key Results: Stable knock-down of P-gp on mRNA level was achieved by reducing mRNA expression 75% and 95% in P-gp- and co-silencing clones compared to control. Knock-down of BCRP yielded a reduction of 90% and 80% mRNA expression in BCRP- and co-silencing clones, respectively. On protein level, no P-gp could be detected in the P-gp- and co-silencing clones. BCRP protein was not detectable as dimer in BCRP- and co-silencing clones but the monomer was still as detectable as in the controls. Transport assays from apical-to-basolateral (AB) through Caco-2 monolayers with ^{3}H-digoxin revealed significantly higher transport rates when P-gp was silenced or inhibited with verapamil. In addition, remaining R123 in the efflux

assays showed functional inactive P-gp in the P-gp-silencing clone. Transport assays AB of ^{14}C-PhIP, a substrate of BCRP, also showed significant higher transport rates when BCRP was silenced or inhibited with prazosin. Efflux assays with PhIP also showed functional inactive BCRP in the BCRP-silencing clone.

Conclusions and Implications: A stable intestinal cell culture model with a selective knock-down of P-gp and BCRP was established. Using this system with single and concomitant knock-down of P-gp and BCRP, the impact of each transporter on substrates, which are transported by both ABC-transporters, could be determined.

3.1.2 Introduction

Silencing or RNA interference (RNAi) is a sequence specific knock-down of genes, which is based on the target specific recognition of the messenger RNA (mRNA) and its following cleavage. RNAi was suggested to be important for the protection of the genome against mobile genetic information such as viruses or transposons. Silencing first was observed in pigmented Petunia plants in 1990 and was termed post-transcriptional-gene-silencing (PTGS). In a transfection experiment, intending to over-express the chalcone synthase for achieving more purple petunias, the contrary effect was observed. Transgenic petunias showed less mRNA expression of chalcone synthase (Napoli *et al.*, 1990). In 1992, it was shown, that exogenous genes transfected into Neosporum crassa could inactivate sequence specifically the corresponding endogenous gene. This observation was named 'quelling' (Romano and Macino, 1992). First insights in the mechanism of silencing was given by Fire et al. who could demonstrate that double-stranded RNA (dsRNA) silenced the corresponding gene in Caenorhabditis elegans (C. elegans) (Fire *et al.*, 1998). Later on, siRNA (small interfering RNA) consisting of ~25 nucleotides dsRNA were identified as main component of gene silencing (Hamilton and Baulcombe, 1999). Zamore et al. identified even smaller siRNA of 21- to 23- nucleotides, which cleaves the target mRNA in 21- to 23-nt intervals (Zamore *et al.*, 2000). This processing of dsRNA to siRNA in the cytoplasm is catalysed by a nuclease of the RNAse III class and was called Dicer (DCR) (Bernstein *et al.*, 2001). Elbashir et al. revealed that 21- and 22- nucleotide sense and antisense strand with 2 nucleotides overhang at the 3'- terminal are the main actors of RNAi. Using chemically synthesised siRNA, they showed that siRNA can be uncoupled from dsRNA and functional gene silencing in mammalian cells can be achieved with synthetic siRNA (Elbashir *et al.*, 2001).

These siRNAs are then further incorporated into a nuclease-complex which was named RNA-induced-silencing-complex (RISC) (Hammond *et al.*, 2000). Martinez et al found that the siRNA double-strand is unwounded and a single-strand remains in the RISC. This single-strand should match to the target mRNA so that the nuclease is able to cleave the target mRNA (Martinez *et al.*, 2002). The strand which is less strong bound to the complementary strand at his 5'-end is preferentially inserted into the RISC. Therefore, the antisense strand which fits to the target mRNA should have a weaker 5'- end (Schwarz *et al.*, 2003). The cleavage position was suggested

to be in the middle of this ~21nt siRNA, Elbashir et al. proclaimed that the cleavage position is located 7-10nt downstream of the 5'-terminus (Elbashir *et al.*, 2001) (Figure 5).

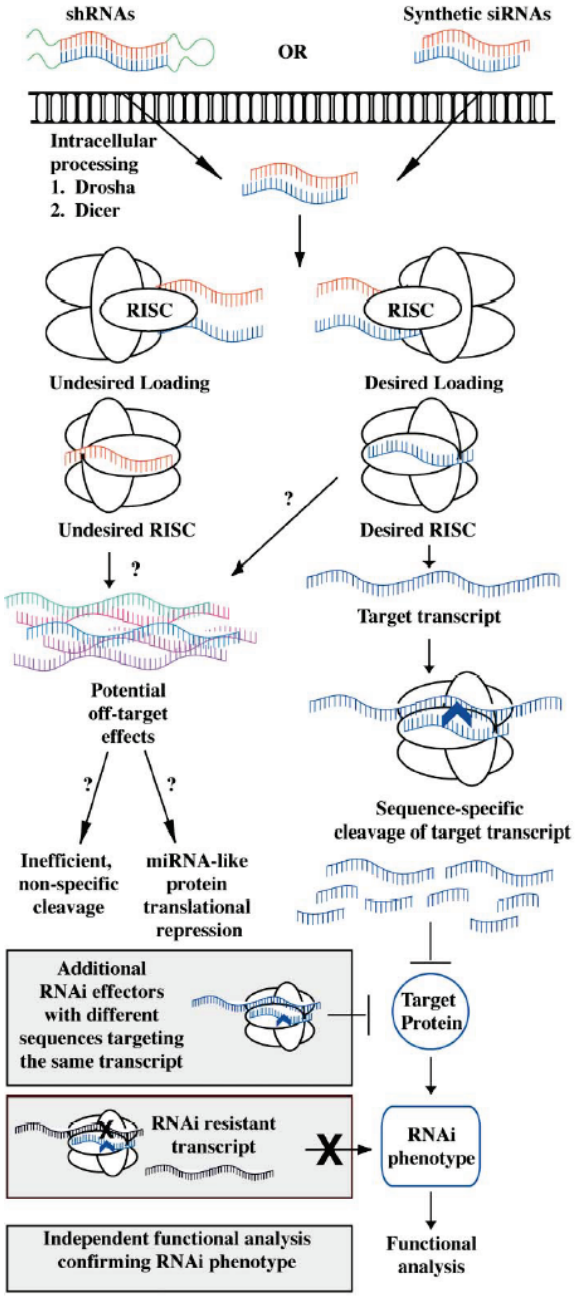


Figure 5 Critical steps in the RNAi mechanism. shRNA is first cut to siRNA and then incorporated into the RISC. Incorporation of the antisense strand leads to target cleavage, while incorporation of the sense strand leads possibly to inefficient or non-specific effects (Huppi et al., 2005).

With this information about the conditions for a knock-down of genes, several *in vitro* assays for targeted silencing were established. One of these methods is the plasmid-based stable transfection of silencing sequences. The target specific DNA sequence which is incorporated into a plasmid forms after transcription a short-hairpin-RNA (shRNA) and is as effective as synthetic siRNA. Brummelkamp et al. designed the pSUPER-vector which was used in our studies (Brummelkamp *et al.*, 2002).

We aimed to establish an intestinal cell system with a stable and selective knock-down of the ABC-transporters P-glycoprotein (P-gp, MDR1, ABCB1) and breast cancer resistance protein (BCRP, ABCG2), as well as of both transporters simultaneously. The lack of defined high specific chemical inhibitors for the respective transporters was the rationale to establish this system for intestinal transport studies. These two transporters form an important intestinal barrier for various xenobiotics. A knock-down of either P-gp or BCRP has the potential to influence the bioavailability of several drugs.

Table 4: Silencing terms

RNAi	RNA interference: Regulatory mechanism of gene expression. Inhibition of gene expression via mRNA degradation by a complementary dsRNA (or siRNA, shRNA). The natural function of RNAi is supposed to be protection of the genome against e.g. viruses.
dsRNA	Double-stranded RNA: Long RNA duplexes which trigger RNAi within the region of homology to mRNA sequences. Intracellular they are cut in 21–23-nt RNA fragments which recognise the target mRNA.
siRNA	small interfering RNA: A short double-stranded RNA molecule of ~21nt size that effects RNAi
shRNA	short hairpin RNA: DNA of the target gene inserted into a vector which is intracellular transcribed to a hairpin RNA and subsequently processed to siRNA.
DCR	Dicer: A nuclease of the RNase III family which cleaves dsRNA to 20-25 nucleotides in length with 3' overhangs of 2 to 3 nucleotides.
RISC	RNA induced silencing complex: A complex of different proteins which have a nuclease activity. These proteins assemble with the siRNA and are responsible for the target specific cleavage of the mRNA.

3.1.3 Methods

Materials

^{3}H-Digoxin (9 Ci/mmol) and ^{14}C-sucrose (588 mCi/mmol) was purchased from Perkin Elmer AG (Schwerzenbach, CH). ^{14}C-PhIP (10 mCi/mmol) was from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Lucifer Yellow (LY) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in phosphate buffered saline (PBS) (Invitrogen, Basel, CH). Prazosin (Sigma-Aldrich) was dissolved in DMSO. Verapamil (Sigma-Aldrich) was dissolved in water. Rhodamine 123 (Molecular Probes, Eugene, OR, USA) was dissolved in ethanol.

Design of shRNA

ABCB1 (MDR1) (Acc.No. NM_000927) and ABCG2 (BCRP) (Acc. No. NM_004827) shRNA sequences were designed using the siRNA design algorithm of Whitehead Institute for Biomedical Research (http://jura.wi.mit.edu/bioc/siRNAext/home.php). A sequence pattern based on investigations of Reynolds et al. was chosen (Reynolds *et al.*, 2004). This siRNA pattern consists of the sequence N4AN6TN2HN5WN2, while N= any nucleotide, H= A, T or C, W= A or T (following the Nomenclature for Incompletely Specified Bases in Nucleic Acid Sequences, Nomenclature Committee of the International Union of Biochemistry, NC-IUB). Further parameters for the siRNA search were a GC percentage from 30 to 70%, no sequence with 4 or more T, A or G in a row, only less than 7 consecutive GC in a row and a siRNA-ending with TT. Among the proposed siRNA sequences, siRNA were chosen which lay in the open reading frame (ORF) and which exhibit a negative thermodynamic value (ΔG = ΔG 5'-sense-strand – ΔG 5'-antisense-strand) (Schwarz *et al.*, 2003), (Khvorova *et al.*, 2003). These sequences were then aligned using the NCBI Blast tool. The thermodynamic profile of these designed sequences was checked using the iRNAi program (http://mekentosj.com/irnai/). This program suggests sequences for the forward and reverse strand which contain the selected siRNA sequence, nucleotides for the loop of the shRNA and nucleotides at the end which fit into the pSUPER-vector digested with the restriction enzymes BglII and HindIII. For both genes, MDR1 and BCRP, one sequence was chosen. Additionally, other sequences earlier described in literature were used as positive control (Celius *et al.*, 2004), (Li *et al.*, 2005). As negative control, a functional

and targeting sequence with an inaccurate thermodynamic profile was chosen for both genes. Under these conditions, the RISC should incorporate the sense-strand of the siRNA and no silencing effect should be provoked. Secondly, a non-targeting but functional sequence was taken. This sequence was described earlier by Taniguchi et al. (Taniguchi *et al.*, 2006). Sequences for the shRNA are listed in Table 5.

Table 5: Sequences for shRNA

MDR1 human, Acc. No: NM_000927

siMDR1h1

Target sequence	5`-AAGGCCTAATGCCGAACACATTG-3`
Reference	Celius T, Biochem Biophys Res Commun. 2004:5;324:365-71
Sequence for pSUPER	FW: 5`-GATCCCC **GGCCTAATGCCGAACACAT** TTCAAGAGA **ATGTGTTCGGCATTAGGCC** TTTTTGGAAA-3`
	RV: 5`-AGCTTTTCCAAAAA **GGCCTAATGCCGAACACAT** TCTCTTGAA **ATGTGTTCGGCATTAGGCC** GGG-3`

siMDR1h2

Target sequence	5`-TTGGAGGATTATGAAGCTAAATT-3`
Reference	-
Sequence for pSUPER	FW: 5`-GATCCCC **GGAGGATTATGAAGCTAAA** TTCAAGAGA **TTTAGCTTCATAATCCTCC** TTTTTGGAAA-3
	RV: 5`-AGCTTTTCCAAAAA **GGAGGATTATGAAGCTAAA** TCTCTTGAA **TTTAGCTTCATAATCCTCC** GGG-3

siMDR1h3 neg

Target sequence	5`-TGATAAAGAACTCTTAGCGTATG-3`
Reference	-
Sequence for pSUPER	FW: 5`-GATCCCC **ATAAAGAACTCTTAGCGTA** TTCAAGAGA **TACGCTAAGAGTTCTTTAT** TTTTTGGAAA-3
	RV: 5`-AGCTTTTCCAAAAA **ATAAAGAACTCTTAGCGTA** TCTCTTGAA **TACGCTAAGAGTTCTTTAT** GGG-3

BCRP human, Acc. No: NM_004827:

siBCRPh1

Target sequence	5`-AAGGTTGGAACTCAGTTTATCCG-3`
Reference	Li WT, Neoplasma. 2005;52:219-24
Sequence for pSUPER	FW: 5`-GATCCCC **GGTTGGAACTCAGTTTATC** TTCAAGAGA **GATAAACTGAGTTCCAACC** TTTTTGGAAA-3`
	RV: 5`-AGCTTTTCCAAAAA **GGTTGGAACTCAGTTTATC** TCTCTTGAA **GATAAACTGAGTTCCAACC** GGG-3`

siBCRPh2

Target sequence	5`-GTGGAGGCAAATCTTCGTTATTA-3`
Reference	-
Sequence for pSUPER	FW: 5`-GATCCCC **GGAGGCAAATCTTCGTTAT** TTCAAGAGA **ATAACGAAGATTTGCCTCC** TTTTTGGAAA-3`
	RV: 5`-AGCTTTTCCAAAAA **GGAGGCAAATCTTCGTTAT** TCTCTTGAA **ATAACGAAGATTTGCCTCC** GGG-3`

siBCRPh3 neg

Target sequence	5`-TGTAATTCAGGTTACGTGGTACA-3`
Reference	-
Sequence for	FW: 5`-GATCCCC **TAATTCAGGTTACGTGGTA** TTCAAGAGA **TACCACGTAACCTGAATTA** TTTTTGGAAA-3`
	RV: 5`-AGCTTTTCCAAAAA **TAATTCAGGTTACGTGGTA** TCTCTTGAA **TACCACGTAACCTGAATTA** GGG-3`

Negative control, scrambled sequence:

siscrambled

Target sequence	5'-GCTATCGCTACGTGTAAGT-3'
Reference	Taniguchi H, Development. 2006;133:1923-31 Supplementary Material
Sequence for pSUPER	FW: 5'-GATCCCC **GCTATCGCTACGTGTAAGT** TTCAAGAGA **ACTTACACGTAGCGATAGC** TTTTTGGAAA-3'
	RV: 5'-AGCTTTTCCAAAAA **GCTATCGCTACGTGTAAGT** TCTCTTGAA **ACTTACACGTAGCGATAGC** GGG-3'

Plasmid Preparation

pSUPER-vector was digested with the restriction enzymes BglII and HindIII (Fermentas Inc., Ontario, Canada). On a 1% agarose gel the resulting two sequences were separated and the opened plasmid was extracted using QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. The previously described designed sequences were synthesised by Invitrogen (Invitrogen AG, Basel, CH). The forward and reverse strand were annealed and afterwards ligated into the pSUPER-vector (OligoEngine, Seattle, USA) at the BglII and HindIII sites with T4 DNA ligase (Fermentas).

These plasmids were transformed into Sure®2 Supercompetent cells (Stratagene, La Jolla, CA, USA) following the manufacturers protocol. Bacteria were then plated on a LB-Ampicillin-Agar (ampicillin 100 µg/ml) overnight at 37°C. The following day, clones were picked and again let grown on a control LB-Ampicillin-Agar plate and in 2ml LB-Ampicillin-Medium overnight at ~240 rpm at 37°C. Plasmid isolation was performed following the protocol Nucleospin® Plasmid (Macherey-Nagel, Oensingen, CH).

The obtained plasmids were checked with a control digestion using HindIII and XbaI restriction enzymes (Fermentas Inc., Ontario, Canada). When correctly ligated we obtained two fragments with the size of ~4400 bp and 313 bp, which was analysed on an agarose gel. A sequencing analysis was performed by microsynth (Microsynth AG, Balgach, CH) using T3 seq primer.

Clones containing correct plasmid sequences were grown again overnight at 37°C in 250 ml LB-Ampicillin-Medium at ~240 rpm. Plasmid isolation was performed with the Nucleobond® AX500 Kit (Macherey-Nagel, Oensingen, CH) following the protocol.

Transfection and Generation of Stable Caco-2-Silencing Clones

Caco-2 cells were transfected using Lipofectamine™ 2000 (Invitrogen). For transient transfection, cells were seeded on 12-well plates and transfection was started when cells reached confluence of ~60%. Transfection was performed following the manufacturers protocol using 2 µg Plasmid DNA and 4 µl Lipofectamine™ 2000 each in a volume of 100 µl Opti-MEM I (Invitrogen) and 1 ml DMEM without gentamycin per well. RNA extraction was carried out after different time points after transfection.

For stable transfection, 1 day before transfection, Caco-2 cells were seeded on 6-well-plates in Dulbecco's modified Eagle's medium with Glutamax-I, supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate, 50 µg/ml gentamycin. Transfection was started when cells reached a confluence of ~60%. Transfection was performed following the manufacturers protocol using 4 µg Plasmid DNA and 10 µl Lipofectamine™ 2000 each in a volume of 250 µl Opti-MEM I and 2 ml DMEM without gentamycin per well. In the case of co-silencing, both plasmids, siMDR1h2 and siBCRPh2, were mixed in the same proportion and transfected into the cells. For obtaining stable clones, cells were diluted 1:40 into 10 mm Petri dishes after 72 hours and the selection antibiotic geneticin (Invitrogen) was added in a concentration of 1.5 mg/ml. Every 3-4 days DMEM with geneticin was replaced. After 2 weeks clones were picked using Scienceware® Cloning discs (Sigma-Aldrich) and further cultured in 24-well plates with a geneticin concentration of 1 mg/ml.

Each clone was passaged over 6 passages and mRNA expression of the gene of interest was performed. The best silencing clone was chosen for further experiments. Evaluation of the silencing clones was performed on mRNA level using real time RT-PCR (Taqman), on protein level by western blot analysis and on activity level using different functional assays such as uptake-, efflux- and transport-assay. These methods as well as cell culture conditions are described in chapter 2.

3.1.4 Results

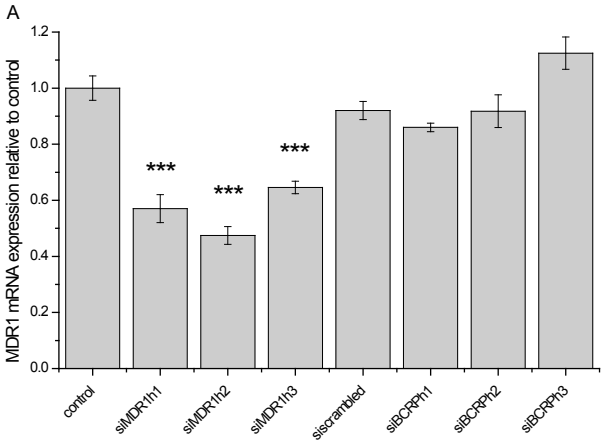
Transient Transfection

mRNA Expression of MDR1 and BCRP

MDR1 and BCRP silencing plasmids were transfected into Caco-2 cells and mRNA expression was measured after transfection. Best silencing effects with MDR1-silencing clones were achieved after 24 or 48 hours. The plasmids siMDR1h1, siMDR1h2 and siMDR1h3 showed 40-50% reduction of MDR1 mRNA expression whereas the negative control (siscrambled) did not significantly alter MDR1 mRNA expression. As a further control experiment, effects of BCRP silencing plasmids on MDR1 mRNA expression were analysed. The introduction of siBCRP plasmids had no reducing effect on the level of MDR1 mRNA expression (Figure 6A).

On the other hand, 96 hours after transfection with siBCRP plasmids (siBCRPh1,h2,h3) BCPR mRNA expression was reduced to a level of 60-70% compared to control. Transfection of the negative control (siscrambled) resulted in a 10% reduced BCRP mRNA expression level, however this effect was not significantly different compared to control. The MDR1 silencing plasmid siMDR1h1 exhibited a 40% reduction on BCRP mRNA expression, whereas siMDR1h2 showed no significant reduction in BCRP mRNA expression (Figure 6B).

The negative control siMDR1h3 and siBCRPh3 showed, despite their inaccurate thermodynamic profile, effects on the expression levels of the respective transporters and where therefore not used for further experiments (Figure 6A and B).



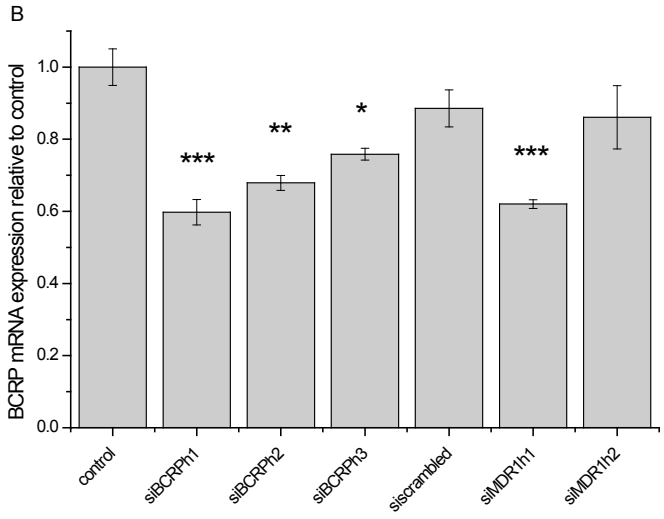


Figure 6 mRNA expression of MDR1 (A) and BCRP (B) after transfection with silencing plasmids. mRNA expression was measured after 96 hours (except A: siMDR1h1-3 after 48 hours) and determined by quantitative real-time PCR. Results are normalised to control cells and expressed as mean ± s.e.m. (n=3). $^{*}p < 0.05$, $^{**}p < 0.01$ $^{***}p < 0.001$ vs. control cells.

Stable Transfection

mRNA Expression of MDR1 and BCRP

Several clones were cultivated over 6 passages and mRNA was extracted in every passage. The best clones were chosen according to the best silencing effect of the desired gene and to the absence of silencing effects on the other gene. The chosen clones contained the sequences siMDR1h2, siBCRPh2 and siscrambled. Data of mRNA expression of these clones are shown in Figure 7.

Silencing of MDR1 mRNA expression was achieved up to 75% in the single siMDR1-transfection and up to 95% in the co-silencing clone (cosil), where both siMDR1 and siBCRP were transfected at the same time. The BCRP-silencing clone, siBCRP, and the negative control, siscrambled, exhibited no significant effects on MDR1 mRNA expression.

BCRP silencing effects on mRNA level were achieved with the single BCRP-transfection, siBCRP, up to 90% and in the co-silencing transfection up to 80%. Unfortunately, the siMDR1-clone and the negative control also reduced BCRP mRNA expression about 25% percent.

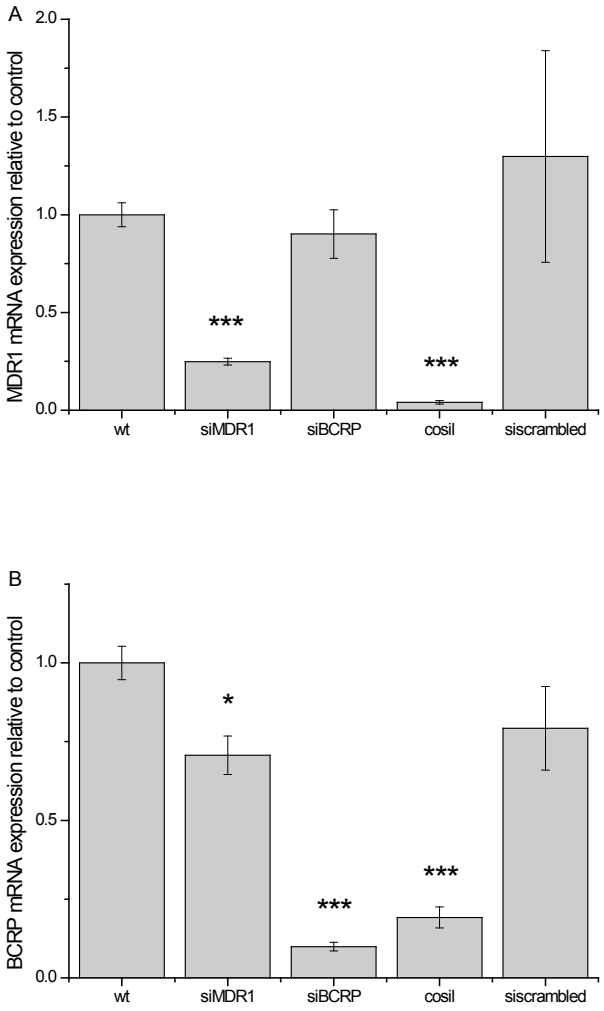


Figure 7 mRNA expression of MDR1 (A) and BCRP (B) in stable silencing clones. mRNA expression was determined by quantitative real-time PCR and results are normalised to wild-type (wt) cells and expressed as mean ± s.e.m. (n=3-5). $^{*}p < 0.05$, $^{**}p < 0.01$ $^{***}p < 0.001$ vs. wild-type cells. Statistics in (A) were performed after exclusion of siscrambled.

Protein Expression of MDR1 and BCRP

In a next step Western blot analysis was performed to confirm the knock-down on protein level. In the Western blot assay, MDR1 protein expression can be detected as a band at a molecular weight of 170 kD. As expected in the MDR1-silencing clones (siMDR1 and cosil), no protein expression was detectable, while in the wild-type Caco-2 cells, in the negative control (siscrambled) and in the BCRP-silencing clone (siBCRP) a clear MDR1 protein expression was observed (Figure 8A)

It is known from literature (Kage *et al.*, 2002) that BCRP forms homodimers to be functionally active. Typically, BCRP protein expression is localised at 72 kD but since we did not heat our samples with reducing agents, dimers of BCRP should also be detectable at ~150 kD. In Figure 8B, the expression of BCRP monomers and dimers is clearly detectable. At 150 kD, BCRP dimers are only detected in wild-type cells, in the negative control (siscrambled) and in the MDR1-silencing clone. BCRP-dimers are not visible in the BCRP silencing clone (siBCRP) nor in the co-silencing clone (cosil). Nevertheless, BCRP monomer expression is seen in all clones at more or less the same intensity (Figure 8B)

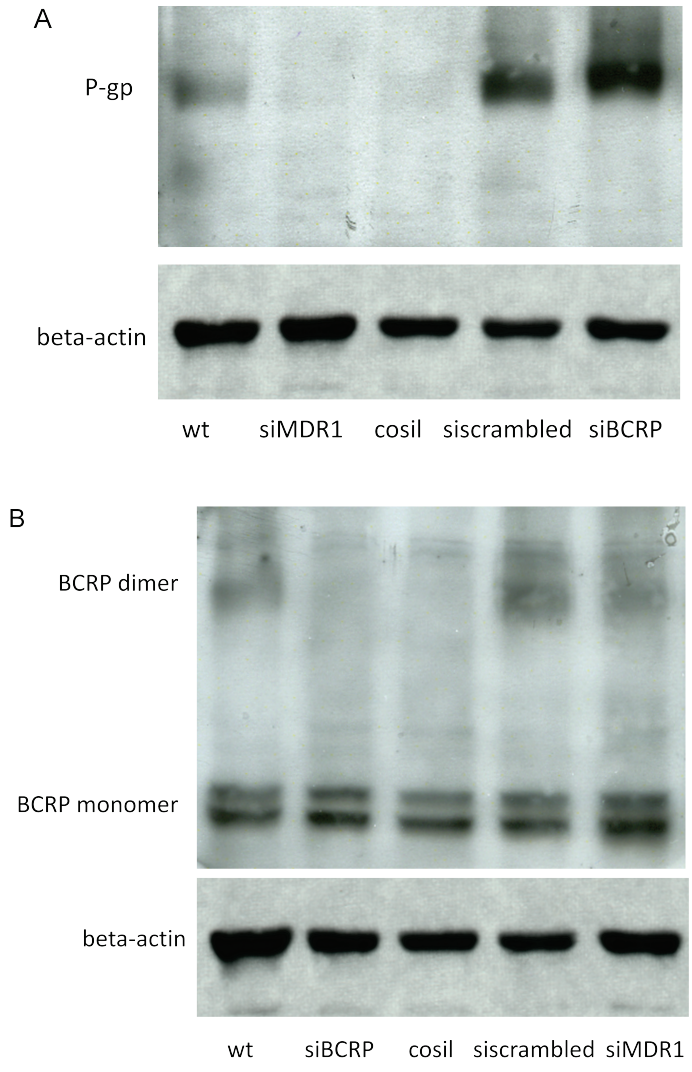


Figure 8 Protein expression of MDR1 (A) and BCRP (B). Beta-actin served as loading control. Protein was extracted 14 days after seeding and analysis was performed using Western blot technique.

Functional Activity of MDR1 and BCRP

Functional activity of MDR1 and BCRP was assessed by transport and efflux experiments using specific substrates. Transport was performed in the direction apical to basolateral with or without an inhibitor for the respective protein. Since MDR1 and BCRP are working as efflux transporters on the apical side, silencing or inhibition of the transporters should result in an increase in apical to basolateral flux. This was clearly shown in the experiments shown in Figure 9. The MDR1 specific substrate ^{3}H-digoxin showed significant higher transport rates from apical to basolateral when MDR1 was inhibited by verapamil. In the case of MDR1-silencing, the transport rate to the basolateral compartment is similarly increased as shown in the clones siMDR1 and cosil (Figure 9A). ^{14}C-sucrose was used as tightness marker. All clones had a comparable tightness. After 2 hours, only 2.5 - 3.25% sucrose was transported from apical to basolateral. The apparent permeability coefficient (P*app*) for sucrose was determined and was around 1.7 - 2.1*10^{-9} cm/s.

^{14}C-PhIP, which is according to literature (van Herwaarden *et al.*, 2003) (Pavek *et al.*, 2005) a specific substrate of BCRP, was used in the transport studies for BCRP. When BCRP was inhibited with prazosin in wild-type cells, an increase in transport from apical to basolateral was observed compared to not-inhibited wild-type Caco-2 cells. A comparable increase in transport was also seen in the silencing-clones siBCRP and cosil (Figure 9B). In these transport experiments, Lucifer Yellow (LY) was used as tightness marker. The amount of LY transport after 2 hours was between 2 - 4.5%. Calculations of P*app* were about 1.3 – 2.9*10^{-9} cm/s and confirmed, that transport studies were performed across cells forming a tight monolayer.

The negative control (siscrambled) was also used in these transport experiments and showed similar effect on ^{3}H-digoxin and on ^{14}C-PhIP transport compared to Caco-2 wild-type cells.

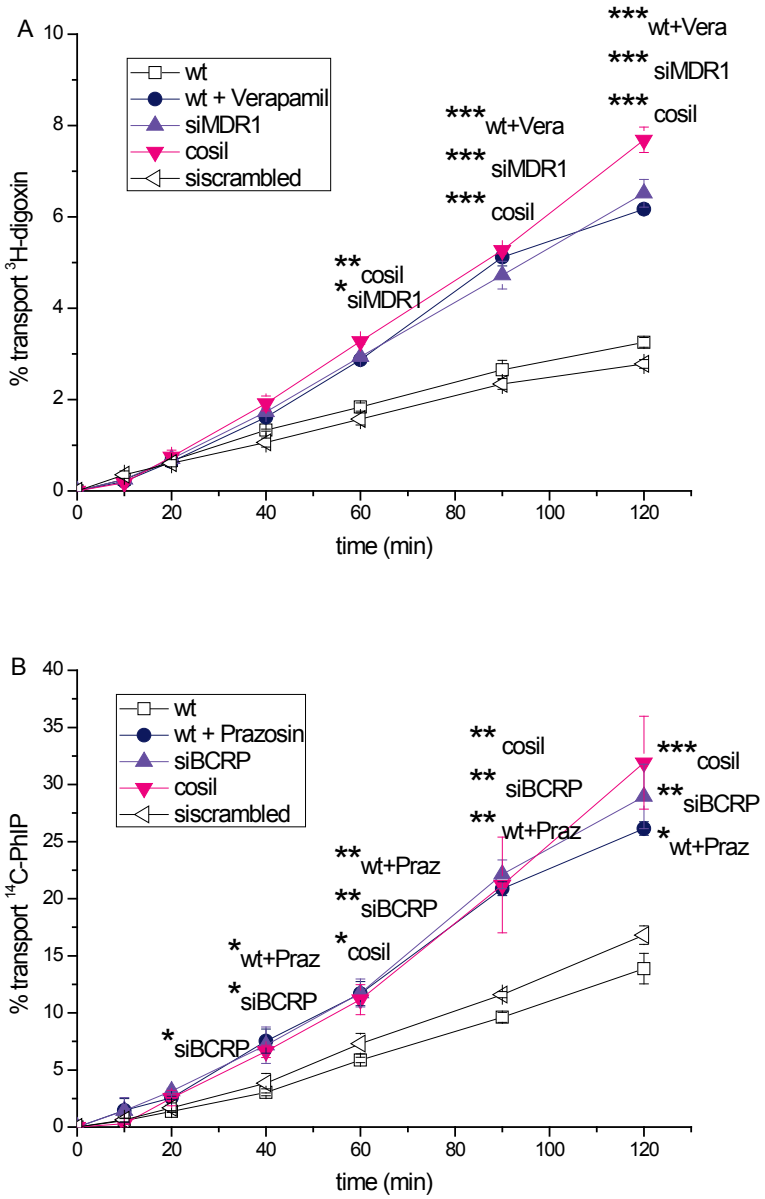
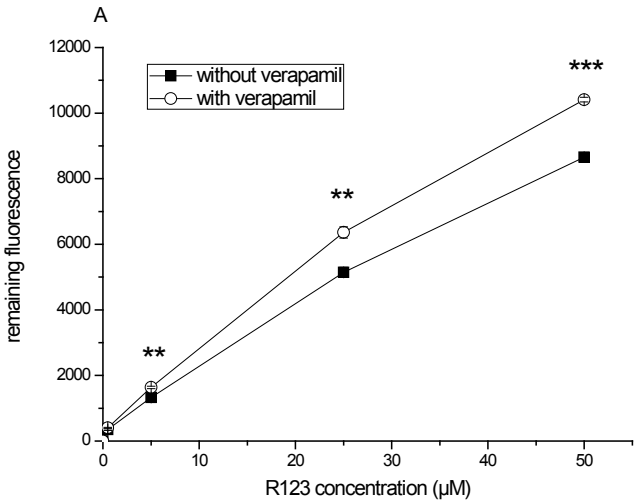
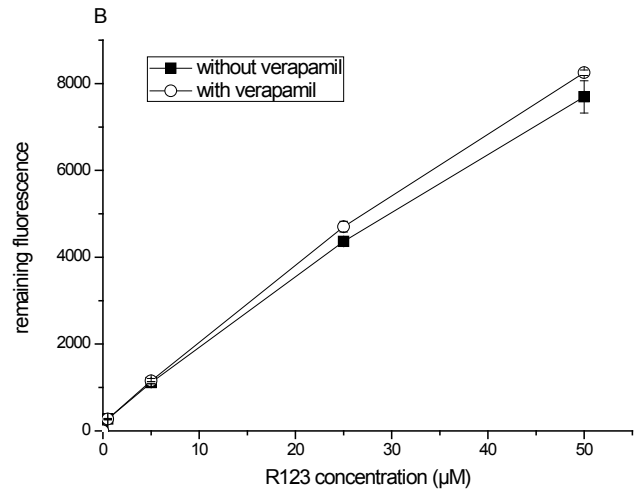


Figure 9 Apical to basolateral transport of ^{3}H-digoxin (66.6 nM, 0.6 µCi/ml) (A) and 14C-PhIP (4 µM, 0.04 µCi/ml) (B) through Caco-2 monolayers. Verapamil 100 µM and prazosin 50 µM were used as inhibitors of P-gp and BCRP, respectively. Data represent means of n=3 ± s.e.m. $p < 0.05$, $^{**}p < 0.01$ $^{***}p < 0.001$ vs. wild-type cells.

For efflux experiments, cells were pre-loaded with a specific substrate while efflux of MDR1 or BCRP was inhibited by verapamil and prazosin, respectively. After preloading, a defined time of efflux followed where the substrate was transported out of the cells. The remaining substrate in the cells was measured after 1 hour efflux period and compared to cells which were inhibited during the efflux period.

In MDR1-silencing clones rhodamine 123 (R123) and in BCRP-silencing clones ^{14}C-PhIP were used as specific substrates. Figure 10 and Figure 11 show the results of these assays. In wild-type cells and in siscrambled-clones R123 remaining substance in the cells differed between inhibited and not-inhibited cells (Figure 10A and C). In siMDR1-clones, no difference in these two conditions can be seen (Figure 10B). Since in siMDR1-clones MDR1 function is already inhibited due to the knock-down, the inhibitor verapamil exhibits no additional effect on the function of MDR1. In wild-type cells and in the silencing control, MDR1 is still functionally active and can be inhibited by verapamil (Figure 10A and C).





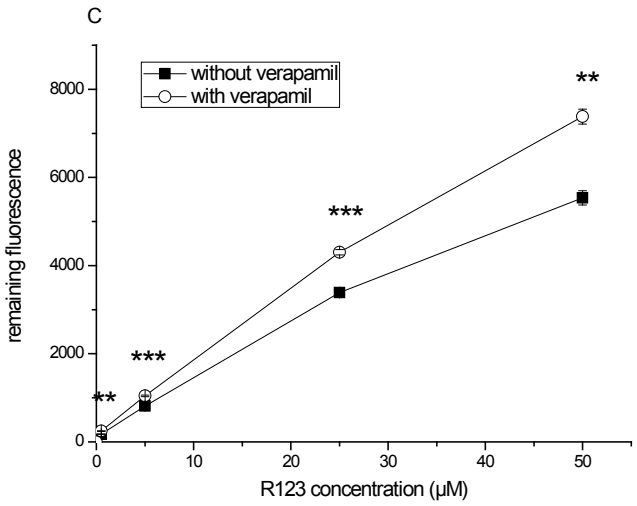


Figure 10 MDR1 activity measured using R123 accumulation assay. Cells were loaded with R123 in different concentrations and residual accumulation of R123 was measured in the cells after 60 min efflux. Fluorescence was measured at 485 nm excitation and 535 nm emission. Remaining substance was compared between not inhibited or verapamil (100 µM) -inhibited wild-type (A), siMDR1 (B) and siscrambled (C) Caco-2 cells. A difference is indicative for P-gp function. Results are expressed as mean±s.e.m. (n=3). $^{*}p < 0.05$, $^{**}p < 0.01$ $^{***}p < 0.001$

Residual accumulation of PhIP in the wild-type Caco-2 cells showed a significant difference after inhibition with prazosin compared to not-inhibited cells, indicating functional active BCRP expression (Figure 11A). Unfortunately, this difference was not significant in the negative control siscrambled (Figure 11C). In siBCRP-clones, no difference was observed after inhibition of the efflux compared to not-inhibited cells, which indicates the lack of BCRP activity in these silencing clones (Figure 11B).

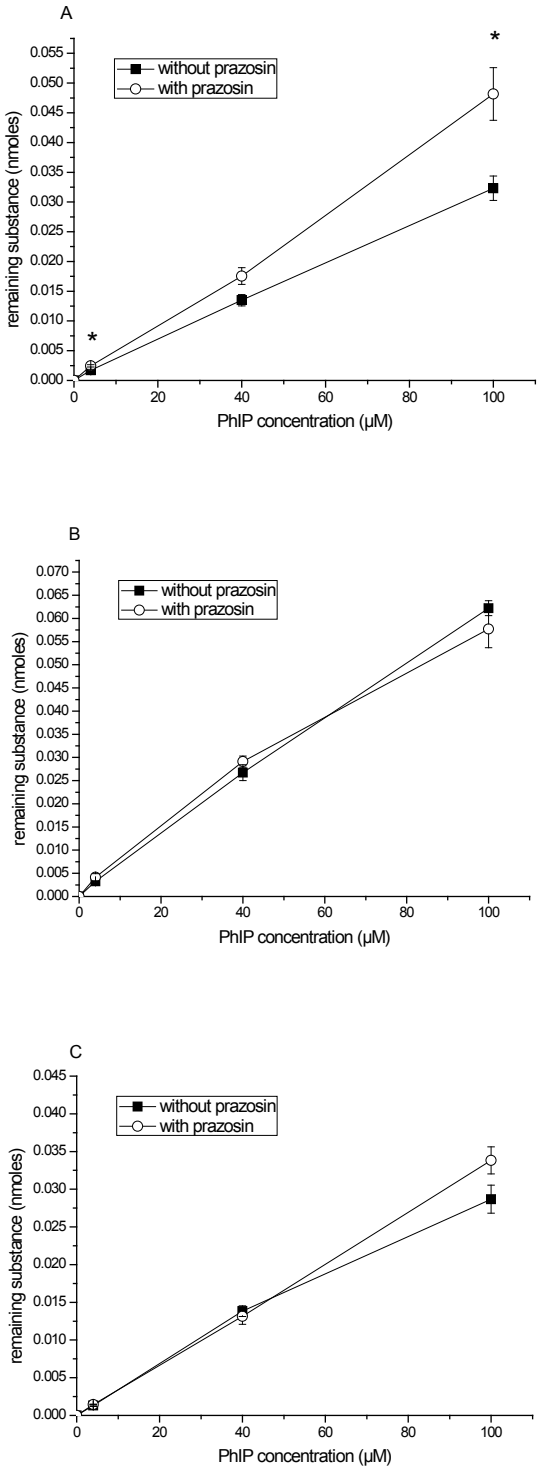


Figure 11 BCRP activity measured using PhIP accumulation assay. Cells were loaded with ^{14}C-PhIP in different concentrations and residual accumulation was measured in cells after 60 min efflux. Remaining substance was compared between not inhibited or prazosin (50 μM) -inhibited wild-type (A), siBCRP (B) and siscrambled (C) Caco-2 cells. A difference is indicative to BCRP function. Results are expressed as mean±s.e.m. (n=3). $^{*}p < 0.05$

3.1.5 Discussion

Silencing is a method which inhibits protein formation by target specific mRNA cleavage. Since the availability of completely specific inhibitors of either BCRP or MDR1 is very limited, silencing is an appropriate method for selective inhibition of the respective transporter function. In the present study we established a vector-based method to permanently knock-down MDR1 and BCRP in Caco-2 cells. We used pSUPER vector which was developed for stable silencing (Brummelkamp *et al.*, 2002). Specific sequences for silencing were designed using established algorithms and earlier described sequences were used as positive control, additionally. As negative controls, targeting and functional sequences with an inaccurate thermodynamic profile and a non-targeting but functional sequence were used.

Transient transfection revealed good silencing effects of the positive controls and the newly designed sequences. With the exception of siMDR1h1, none of our tested sequences exhibited any silencing effects on the other investigated gene after transient transfection. The observed off-target effect of siMDR1h1 on BCRP gene expression was expected since Celius et al. (Celius *et al.*, 2004) already observed this effect.

Unfortunately, the targeting and functional sequences with the inaccurate thermodynamic profile (siMDR1h3 and siBCRPh3) which should serve as negative controls also showed silencing effects. These sequences were specific for the target gene but had an inaccurate thermodynamic profile. Therefore, the RISC should incorporate predominantly the sense strand and no matching to the mRNA should occur. Concerning our results, we conclude that the RISC did not selectively enter only the sense strand. The antisense strand probably was also included despite the inaccurate thermodynamic profile. These data show that it is delicate to design a negative control using targeting and functional sequences. Therefore, an additional negative control which was non-targeting but functional was used. This sequence was earlier described in the literature as scrambled silencing control (Taniguchi *et al.*, 2006). As we expected, this sequence had no effects on MDR1 and BCRP mRNA expression.

Stable silencing Caco-2 clones were selected after 6 passages and the clones with the continuous best silencing effects were chosen for further studies. Clones kept their silencing effects for at least 30 passages and functional assays could be performed with this stable cell lines.

Silencing of MDR1 was analysed on mRNA, protein and functional level. Silencing of MDR1 in Caco-2 cells was already earlier published by (Celius *et al.*, 2004), (Watanabe *et al.*, 2005). They achieved a downregulation of MDR1 mRNA expression of 96 and 90%, respectively. We approached these results, since our knock-down of MDR1 mRNA was between 75 - 95%. On protein level, no MDR1 could be detected by Western blot analysis. The functional activity assays confirmed the knock-down of MDR1. ^{3}H-digoxin transport studies in our silencing clones showed results similar to verapamil-inhibited wild-type Caco-2 cells, which means a functional inhibition of MDR1 activity. R123 efflux assay also endorsed the lack of MDR1-function in the siMDR1 clones.

Stable knock-down of BCRP in Caco-2 cells was not yet reported. Stable BCRP silencing was earlier performed in BeWo cells by (Evseenko *et al.*, 2007). They reported a reduction of BCRP mRNA expression of about 65%. In our model, we achieved 80 - 90% reduction of BCRP mRNA expression, which even exceeded this result. Western blot analysis for the detection of BCRP protein expression revealed an interesting finding. Since we did not work under reducing conditions, dimers of BCRP should be detectable at ~150 kD in Western blot analysis as described previously for Caco-2 cells (Xia *et al.*, 2005). Our protein data show a reduction of BCRP dimer expression in the silencing clones compared to controls but the monomer at 72 kD varied ambiguously. This fact has to be discussed; a lack of sensitivity in our Western blot analysis might be one reason why no difference at 72 kD can be detected. However, further analysis of BCRP activity clearly demonstrated a reduction of BCRP function in our clones. We confirmed the BCRP-silencing effect measuring BCRP activity in PhIP-transport and –efflux assays. These assays all proved a reduced BCRP activity in the BCRP silenced cells. Unfortunately, a slight decrease in BCRP mRNA expression was also measured in siMDR1 and siscrambled clones, but on protein level BCRP dimer expression still was clearly detectable. However, this fact has to be taken into account when analysing BCRP function in siMDR1 and siscrambled clones.

Knock-down of both, MDR1 and BCRP, together in a single clone is described for the first time. Since both transporters have overlapping substrate specificity, a possible compensation by one transporter must be taken into account while the other is inhibited. Relevant interaction of drug absorption by MDR1 and BCRP in the intestine can be predicted, while working in Caco-2 cells which is a common model for intestinal drug absorption (Artursson *et al.*, 2001). The impact of MDR1 and BCRP on the transport of any substrate can be determined in this model and might be an interesting tool to predict drug interactions for the *in vivo* situation.

3.2 Transport Studies in Caco-2 Silencing Clones

3.2.1 Abstract

Background and Purpose: P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) play a major role in determining the oral absorption of various drugs. Abacavir and Imatinib are both transported by P-gp and BCRP. We aimed to identify the influence of P-gp and BCRP on drug absorption when one or both transporters are lacking.

Experimental Approach: Caco-2 wild-type (wt) cells and clones with a stable knock-down of P-gp, BCRP or P-gp and BCRP (co-silencing) were used. Bidirectional transport and uptake assays were performed to measure the influence of each transporter on transport of ^{3}H-abacavir and ^{14}C-imatinib.

Key Results: A difference in bidirectional transport means active transport processes while no difference stands for passive diffusion. Bidirectional transport with ^{14}C-imatinib revealed a significant difference in transport from apical to basolateral (AB) and from basolateral to apical (BA) in wild-type cells and BCRP-silencing clones which indicates active transport processes. However in P-gp- and in co-silencing clones no difference in bidirectional transport was observed which indicates absence of active efflux transport. Residual accumulation after transport was significantly higher only in P-gp- and co-silencing clones compared to wild-type. Uptake studies with ^{14}C-imatinib only reached significant higher accumulation in the co-silencing clone compared to wt. Imatinib as inhibitor of BCRP-function could nullify the difference in transport AB and BA of the specific BCRP substrate PhIP in wt cells. Concentration equilibrium transport assay with ^{3}H-abacavir resulted in a slight but significant difference in AB and BA transport in wt and BCRP-silencing clones, but not in P-gp- and co-silencing clones.

Conclusions and Implications: Our results with imatinib and abacavir might suggest that P-gp plays a more important role than BCRP in intestinal transport of co-substrates. Nevertheless, further studies are indicated to verify this observation. Furthermore, possible interferences of our cell culture model such as silencing off-target effects have to be ruled out.

3.2.2 Introduction

Gastro-intestinal drug absorption is of major importance since oral drug formulations are widely used. Drug solubility and permeability are physicochemical properties that influence drug absorption. Lipinski et al. proposed four important properties including logP (octanol:water partition coefficient), H-bond-donor and –acceptor and molecular weight, which determine drug solubility and permeability (Lipinski *et al.*, 2001). In the last years, intestinal metabolism and active efflux transport in the intestine have been recognised as major determinants of drug bioavailability (Benet *et al.*, 1996). Active efflux into the intestinal lumen or metabolism in the intestinal cells can drastically lower bioavailability of drugs. Active efflux is mainly mediated by the ATP-binding-cassette-transporters family. Among these transporters, P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) play an important role. Their expression is high at excretory sites amongst other tissues also in the intestine. They are expressed at the apical brush border membrane of the intestine and form a barrier for various drugs and toxins (Cordon-Cardo *et al.*, 1990), (Thiebaut *et al.*, 1987), (Maliepaard *et al.*, 2001). Both transporters have broad and partly overlapping substrate specificity. Interaction of drugs at the level of efflux-transport can be the consequence.

Imatinib and abacavir belong to newly discovered substrates of both, P-gp and BCRP. Imatinib is an anti-proliferative drug and is primarily used in Philadelphia chromosome positive chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL) or gastrointestinal stromal tumours. It belongs to the family of tyrosine-kinase inhibitors and inhibits selectively the BCR-ABL fusion protein, the tyrosinkinase-receptor c-KIT and the platelet-derived growth factor receptors (Buchdunger *et al.*, 2000), (Druker *et al.*, 2001), (van Oosterom *et al.*, 2001). Imatinib is orally taken and its bioavailability is about 98% (Peng *et al.*, 2004). Bioavailability of imatinib is high but varying plasma AUC (area under the curve) are described, possibly due to metabolism and transport processes (Peng *et al.*, 2005). Recently, it was described that imatinib is a substrate of human P-gp and BCRP (Hamada *et al.*, 2003), (Burger *et al.*, 2004).

Abacavir is an anti-retroviral drug and belongs to the class of nucleoside reverse transcriptase inhibitors (NRTI). It is used in the therapy of HIV infections in combination with other NRTIs, non-NRTIs and protease inhibitors (Josephson *et al.*, 2007). The oral bioavailability of abacavir is

about 83% (Chittick *et al.*, 1999). Two recently published studies showed that abacavir is a substrate of human P-gp and murine Bcrp1 (Shaik *et al.*, 2007), (Pan *et al.*, 2007).

Interactions of these two substrates in an intestinal model are not yet published. A suitable *in vitro* model for intestinal transport processes are Caco-2 cells (Hidalgo *et al.*, 1989). It was shown that the expression of ABC-transporters in Caco-2 cells have in general a good correlation to the expression level in the human intestine (Taipalensuu *et al.*, 2001).

The aim of this study was to investigate how co-substrates of P-gp and BCRP are transported in the situation where one or both of these transporters are inhibited.

3.2.3 Methods

Materials

^{14}C-Imatinib mesylate (52.74 mCi/mmol) was kindly gifted by Novartis Pharma AG (Basel, CH). ^{14}C-PhIP (10 mCi/mmol) was from Toronto Research Chemicals Inc. (Toronto, Ontario, Canada). Abacavir and ^{3}H-abacavir (0.5 Ci/mmol) was purchased from Moravek Biochemicals, (Brea, CA, USA). ^{14}C-sucrose (588 mCi/mmol) was from Perkin Elmer (Schwerzenbach, CH) Lucifer Yellow (LY) (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in phosphate buffered saline (PBS) (Invitrogen, Basel, CH). Prazosin (Sigma-Aldrich) was dissolved in DMSO. Elacridar was kindly gifted from Roche (Basel, CH) and dissolved in DMSO.

mRNA Expression of MDR1 and BCRP: see chapter 2.2

Sequences for primers and probes are listed in **Table 1**.

Protein Expression of MDR1 and BCRP: see chapter 2.3

Bidirectional Transport of Imatinib and Abacavir

Transport assay was performed as described in chapter 2.4. The assay with^{14}C-imatinib was performed with Caco-2 cells grown on polycarbonate filters, while for ^{3}H-abacavir and for ^{14}C-PhIP polyester filters were used. For bidirectional transport assays, samples were taken after, 20,

40, 60, 90 and 120 min. Lucifer Yellow (20 µM) was used as tightness-marker in the ^{14}C-imatinib and ^{14}C-PhIP assays and ^{14}C-sucrose (0.6 µCi/ml) was used in the assay with ^{3}H-abacavir.

Concentration equilibrium transport assay was performed for abacavir transport. On both sides the same concentration of the substrate was added and the tightness-marker was added only at the apical side. At every time point samples were taken on the apical and basolateral side. Removed volume was not replaced after sampling in order not to change the developing concentration gradient. Therefore only small sample sizes of the same proportion were taken on both sides.

3.2.4 Results

Validation of Cell System

Validation of Caco-2 silencing is described in detail in chapter 1.1.

Imatinib

Imatinib is a substrate of P-gp and BCRP. Using ^{14}C-imatinib in Caco-2 cells we investigated whether these two transporters can compensate imatinib-efflux when one or both of these transporters are downregulated.

An uptake assay was performed for one hour using elacridar (10 µM) as inhibitor of both transporters. An increase of ^{14}C-imatinib as remaining substance in the cells indicates less efflux processes and consequently lower activity of P-gp and BCRP. In Figure 12, remaining ^{14}C-imatinib in not inhibited cells normalised to elacridar-inhibited cells are shown. In the P-gp-silencing clone (siMDR1) and in the co-silencing clone (cosil) more ^{14}C-imatinib accumulates in the cells compared to wild-type Caco-2, but significance is only reached in the cosil-clone. In the BCRP-silencing clone (siBCRP) no alteration is observed compared to wild-type cells. These results show that accumulation of ^{14}C-imatinib tends to increase when P-gp activity is decreased but not when only BCRP is downregulated.

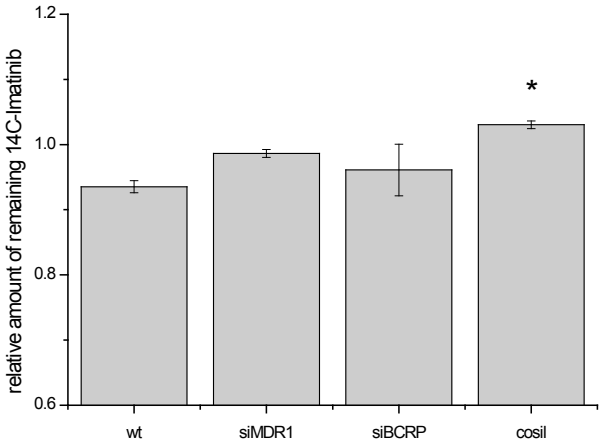


Figure 12 Accumulation of ^{14}C-imatinib (10 µM, 0.02 uCi/ml) in Caco-2 cells after 1 hour uptake. Data represent ^{14}C-imatinib normalised to elacridar (10 µM)-treated cells. An increase in ^{14}C-imatinib is indicative for decreased efflux function. Results are expressed as mean±s.e.m. (n=3). *p<0.05

Transport assays were performed to study bidirectional transport of ^{14}C-imatinib through a monolayer. Transport in both directions from apical to basolateral (AB) and from basolateral to apical (BA) was measured. More transport BA means active efflux on the apical membrane. In Figure 13 we can see that Caco-2 wild-type cells have an asymmetry in transport direction. More ^{14}C-imatinib is transported to the apical compartment which stands for active efflux function on the apical side. This asymmetry is reversed in P-gp- and co-silencing clones (siMDR1 and cosil). In BCRP-silencing clone (siBCRP) the asymmetry of transport is still present. Therefore, efflux activity at the apical side is still present in BCRP-silencing clones, but in P-gp- and co-silencing clones, no active efflux can be seen.

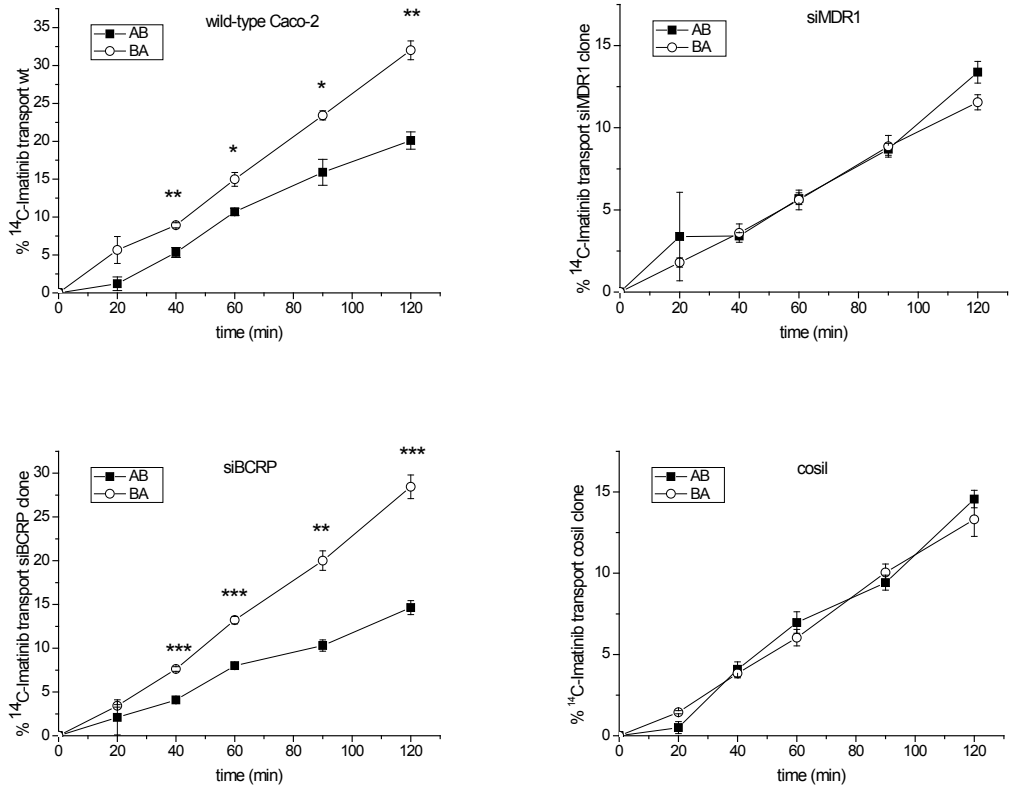


Figure 13 Bidirectional transport of ^{14}C-imatinib (11,4 µM, 0.6 µCi/ml) in Caco-2 wild-type cells and silencing clones. Percentage of transport apical to basolateral (AB) and basolateral to apical (BA) is plotted against time. Data represent mean±s.e.m. (n=3). *p<0.05, **p<0.01, ***p<0.001

Measurement of remaining ^{14}C-imatinib in wild-type, MDR1-, BCRP- and co-silencing clones revealed a comparable result (Figure 14). Higher levels of ^{14}C-imatinib are indicative for reduced efflux function. In clones where P-gp activity was abolished by the knock-down, significantly more imatinib accumulates in the cells. In the clone where only BCRP is silenced, the accumulation in the cells is not significantly increased.

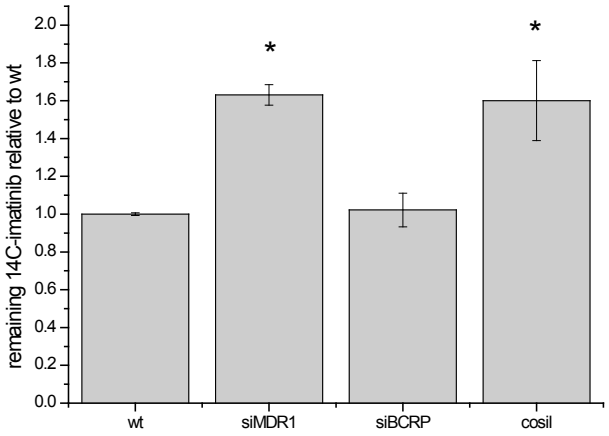


Figure 14 Remaining ^{14}C-imatinib (11.4 µM, 0.6 µCi/ml) in wild-type Caco-2 and silencing clones after 2 hours transport. Data represent means±s.e.m. (n=3). *p<0.05

In a control experiment, we tested ^{14}C-PhIP-transport across wild-type Caco-2 cells and Caco-2 wild-type cells inhibited with imatinib and in BCRP-silencing clone (Figure 15). Transport of PhIP across Caco-2 wild-type cells shows a wide asymmetry while in cells, which are inhibited with imatinib, this asymmetry decreases which indicates inhibition of BCRP efflux activity at the apical side. In the BCRP-silencing clone this asymmetry is also reversed indicating loss of efflux transport.

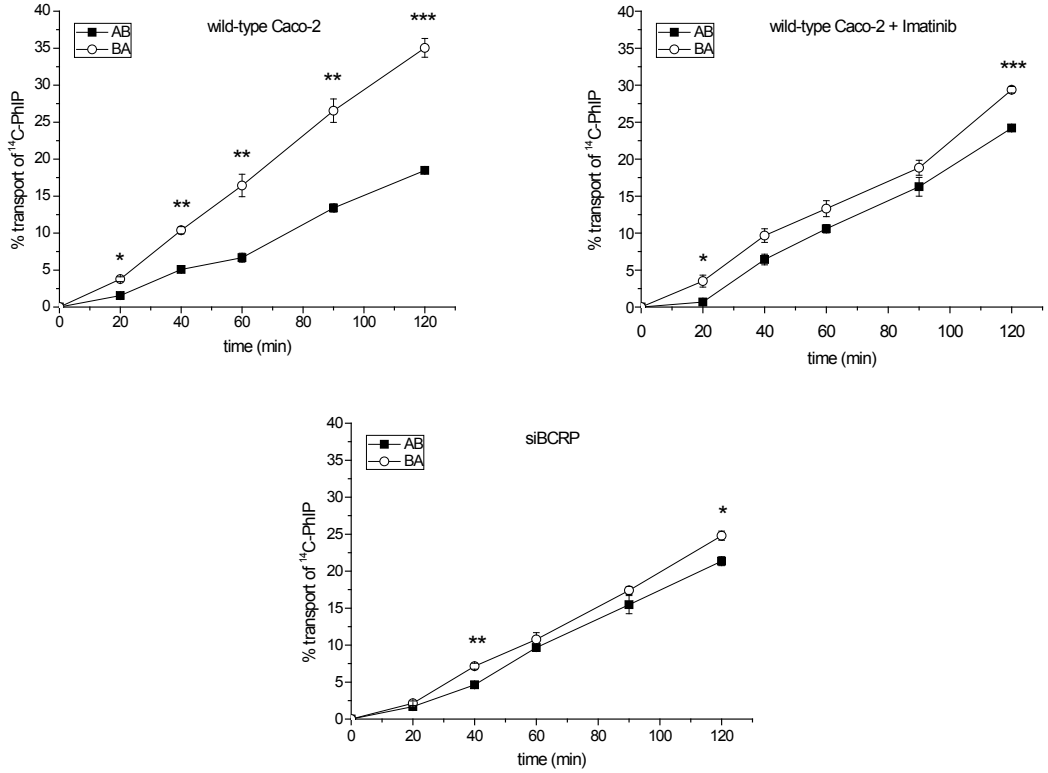


Figure 15 Bidirectional Transport of ^{14}C-PhIP (4 µM, 0.04 µCi/ml) across Caco-2 wild-type cells without inhibitor, with prazosin (50 µM) and imatinib (100 µM) as inhibitors and in BCRP-silencing clone (siBCRP). Percentage of transport apical to basolateral (AB) and basolateral to apical (BA) is plotted against time. Data represent mean±s.e.m (n=3). *p<0.05, **p<0.01, ***p<0.001

Abacavir

Abacavir was described as substrate of P-gp and Bcrp. We evaluated its transport by P-gp and BCRP in the intestinal cell model Caco-2. Abacavir showed good permeability over the cell monolayer and discrimination between the different clones was not possible. Therefore we worked with a concentration-equilibrium where on both sides of the membrane the same concentration of drug was applied. Using this system, passive diffusion in the direction of the concentration gradient is reduced. In Figure 16, concentration of ^{3}H-abacavir in the respective compartment is shown at the indicated time-points. A difference in drug concentration between the compartments means active transport. Differences of concentration in each compartment are minimal but there still can be seen some effects. A significant difference is observed in Caco-2

wild-type cells, where both tranporters P-gp and BCRP are active. No significant difference is seen in the co-silencing clone (cosil). A reduced difference is also seen in the P-gp-silencing clone (siMDR1), but in the BCRP-silencing clone (siBCRP) concentration in both compartments is clearly differing.

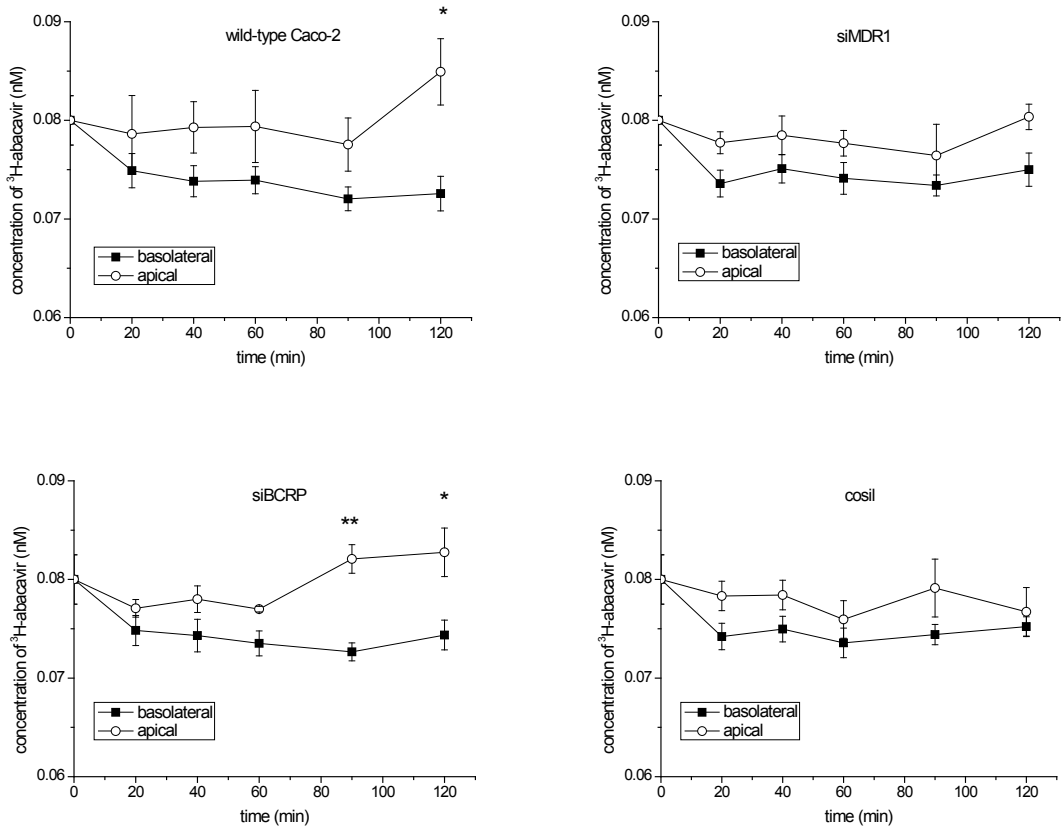


Figure 16 Concentration-equilibrium transport assay. Concentration of ^{3}H-abacavir (80 nM, 0.04 µCi/ml) in both compartments are shown for the different clones (wt, siMDR1, siBCRP, cosil). Data represent mean±s.e.m. (n=3). $^*p<0.05$, $^{**}p<0.01$

3.2.5 Discussion

In this study we investigated imatinib and abacavir transport through monolayers of Caco-2 cells. Caco-2 cells were shown to represent an ideal *in vitro* model for intestinal transport processes (Hidalgo *et al.*, 1989). We established a model of single and concomitant knock-down of P-gp and BCRP. Using this model, we tested interactions of these substrates with the transporters when one or both are silenced.

Imatinib is an orally applied anti-cancer drug, which can evoke therapy resistance after long-term treatment. A possible explanation for therapy resistance is the overexpression of active efflux-transporters. The role of ABC-transporters in therapy resistance is discussed. Several studies showed that imatinib is a substrate of P-gp (Hamada *et al.*, 2003), (Dai *et al.*, 2003), (Mahon *et al.*, 2003). Studies in Caco-2 cells showed that imatinib induced P-gp and BCRP expression which lead to reduced intracellular drug accumulation (Burger *et al.*, 2005). We could confirm that P-gp plays a certain role in transport of imatinib in the intestinal model Caco-2. When P-gp is silenced, only around half of the amount of imatinib is transported to the apical compartment (Figure 13) and almost twice as much remains in the cells compared to wild-type Caco-2 cells (Figure 14).

Furthermore, we silenced BCRP in Caco-2 cells. Interactions between imatinib and BCRP are intensively discussed in literature. Several authors demonstrated that imatinib is a substrate of BCRP while others concluded that it is only an inhibitor of BCRP. Jordanides et al. (Jordanides *et al.*, 2006) and Houghton et al. (Houghton *et al.*, 2004) both showed that imatinib is an inhibitor of BCRP function, but is not transported by BCRP. In contrast to these results several authors revealed that imatinib is not only an inhibitor, but also a substrate of BCRP (Burger *et al.*, 2004), (Breedveld *et al.*, 2005). Shukla et al. demonstrated that imatinib interacts at the transport-substrate binding sites of BCRP and P-gp (Shukla *et al.*, 2007). Furthermore, Nakanishi et al. showed that imatinib-induced cytotoxicity in BCRP-transfected cell lines is lower than in not-transfected cell lines. (Nakanishi *et al.*, 2006).

Our results confirm the fact that imatinib is an inhibitor of BCRP function, since it inhibited the transport of the specific BCRP-substrate PhIP (Figure 15). Transport studies of ^{14}C-imatinib in our cell model show interesting findings. In Caco-2 cells where only BCRP is silenced, there is no

difference in ^{14}C-imatinib transport compared to wild-type cells. Remaining imatinib in cells after two hours transport shows a slight but not significant increase in BCRP-silenced clones. An explanation for this observation might be P-gp-function. In the BCRP-silencing clone P-gp still is highly expressed and possibly compensates the loss of BCRP-function. However, on the other hand, in P-gp-silencing clones, we cannot see a compensation of BCRP-transport when P-gp activity is lacking. But in the co-silencing clone, where both, P-gp and BCRP, are silenced, remaining ^{14}C-imatinib after two hours transport is higher compared to the single P-gp-silencing clone. This result might indicate that P-gp and BCRP both transport imatinib but P-gp possibly plays a more important role. These conclusions are in accordance with results of Bihorel et al. (Bihorel *et al.*, 2007a), (Bihorel *et al.*, 2007b). In a mouse model, knock-out of mdr1a/1b (-/-) increased the brain-to-blood ratio but knock-out of bcrp1(-/-) showed the same ratio compared to wild-type mice. However, inhibition of P-gp and BCRP in wild-type mice with elacridar increased the brain-to-blood ratio compared to single inhibition of P-gp with valspodar or zosuquidar.

The second investigated substrate in our cell system was the nucleoside reverse transcriptase inhibitor (NRTI) abacavir. Among anti-HIV drugs interactions at transporters-side are well known. Many anti-HIV drugs have inhibitory potencies on P-gp and BCRP (Weiss *et al.*, 2007), (Storch *et al.*, 2007). In these studies, abacavir was described to be a very weak inhibitor of P-gp and BCRP. In addition, recent investigations revealed that abacavir is a substrate of human P-gp and mouse Bcrp1 (Shaik *et al.*, 2007), (Pan *et al.*, 2007). In our Caco-2 system transport of abacavir by P-gp and BCRP was determined. Transport of abacavir from apical to basolateral was extensive and interactions between the different silencing clones were hardly detectable (data not shown). Therefore, a concentration-equilibrium transport assay was performed in order to minimise the effect of passive diffusion. Considering results of abacavir transport, effects of P-gp and BCRP on abacavir absorption are small but detectable. When both transporters are silenced, almost the same concentrations on both sides of the membrane were detected, while in wild-type cells more abacavir was measured in the apical compartment probably due to active efflux. Silencing of P-gp reduced this apical-to-basolateral transport more clearly than silencing of BCRP. Reasons for this observation again might be compensation of transport by P-gp when BCRP is silenced. On the other hand, since single P-gp-silencing is not as effective as co-silencing

of both transporters, BCRP still is active but probably is not able to compensate the loss of P-gp function.

Summarising our findings with imatinib and abacavir, we could see that single silencing of P-gp had more potent effects on transport of these two substrates than single silencing of BCRP. Possible explanations could be a stronger affinity of these substrates to P-gp than to BCRP. Interestingly, both substrates showed a similar tendency. A further explanation to this fact also could be due to our system. On mRNA level we observed a slight downregulation of BCRP in P-gp-silencing clones (chapter 3.1.4). However, this downregulation is very low but further experiments have to be performed to verify a full functionally active BCRP in P-gp-silencing clones.

4 Regulation of ABC-Transporters

4.1 Effects of Budesonide on P-glycoprotein Expression in Intestinal Cell Lines

Running Title: Budesonide and P-glycoprotein Regulation

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4.1.1 Abstract

Background and Purpose*:* P-glycoprotein (P-gp) is an important efflux transporter that supports the barrier function of the gut against invading antigens and against administered drugs. Since glucocorticoids, such as budesonide, are frequently used during inflammatory bowel disease we investigated how budesonide influences P-gp expression in different intestinal cell lines.

Experimental Approach: LS180 and Caco-2 cells were incubated with budesonide and changes in P-gp expression were determined on mRNA, protein and functional level. The mRNA expression levels of glucocorticoid receptor (GR) and pregnane X receptor (PXR) were determined in the investigated cell lines. PXR receptor was transiently transfected into Caco-2 cells.

Key Results: Budesonide showed an induction of P-gp in LS180 cells and a downregulation in Caco-2 cells. Expression levels of nuclear receptors revealed high expression of PXR only in LS180 cells and exclusive expression of GR in Caco-2 cells. Mifepristone, an antiglucocorticoid, could not reverse the downregulation of P-gp by budesonide in Caco-2 cells. In PXR-transfected Caco-2 cells the budesonide-mediated downregulation of P-gp was abolished. Furthermore the expression of cytochrome P450 3A4 (CYP3A4), another PXR target gene, was induced in PXR-transfected Caco-2 cells after budesonide treatment.

Conclusions and Implications: Budesonide has the potential to influence MDR1 expression *in vitro*. In LS180 cells, the induction of MDR1 by budesonide probably is mediated via PXR. The mechanism of the downregulation in Caco-2 cells still remains unclear, but GR does not seem to be involved. Further studies are required to evaluate how budesonide alters P-gp expression *in vivo*.

4.1.2 Introduction

Glucocorticoids are an important therapeutic option in treating inflammatory disorders like asthma, rheumatoid arthritis or inflammatory bowel disease (IBD). Budesonide is a newer synthetic glucocorticoid that is increasingly used in IBD patients (Kane *et al.*, 2002). If applied in a controlled release formulation, budesonide is after oral administration topically released in the distal small intestine and colon, the predominant sites of inflammation (Klotz and Schwab, 2005). There, it is well absorbed, but it is extensively pre-systemically metabolized (Schwab and Klotz, 2001). Consequently, upon oral administration budesonide exerts strong anti-inflammatory effects in intestinal tissue with minimal systemic side effects (Hofer, 2003).

The molecular mechanism of glucocorticoid action involves the intracellular binding to the glucocorticoid receptor (GR), followed by a translocation of activated GR to the nucleus. There, it stimulates or inhibits gene expression by binding to glucocorticoid response elements on DNA (Wright *et al.*, 1993). Moreover, ligand-bound GR can repress a number of pro-inflammatory genes by physically associating with transcription factors via direct protein-protein interactions. Examples are the repression of nuclear factor-κB (NF-κB) or activator protein-1 (AP-1) (Smoak and Cidlowski, 2004).

Glucocorticoids such as dexamethasone are further known to activate the pregnane X receptor (PXR) (Kliewer *et al.*, 1998). PXR belongs to the group of orphan nuclear receptors that function as heterodimers with the retinoic X receptor (RXR) (Chawla *et al.*, 2001). They are important regulators of xenobiotic metabolism and upon activation they can induce the expression of metabolizing enzymes (e.g. cytochrome P450 3A4) and drug transporters such as P-glycoprotein (P-gp) (Bertilsson *et al.*, 1998), (Geick *et al.*, 2001).

P-gp is the gene product of the multi-drug resistance gene 1 (MDR1, gene symbol: ABCB1). It is an adenosine-triphosphate-dependent drug efflux pump with wide substrate specificity (Juliano and Ling, 1976). It has barrier function in tissues such as kidney, blood brain barrier and intestine (Cordon-Cardo *et al.*, 1990). In the intestine, P-gp is localized in the apical membrane of epithelial cells and is continuously expressed along the intestinal tract (Thiebaut *et al.*, 1987), (Zimmermann *et al.*, 2005). Therefore, P-gp can significantly influence the bioavailability of many drugs including glucocorticoids, as it has been shown previously that budesonide,

dexamethasone, and prednisone are substrates of this transporter (Dilger *et al.*, 2004), (Fromm, 2003), (Ueda *et al.*, 1992). Besides the induction through PXR, the expression level of MDR1 is dependent on a complex transcriptional regulation with a redundancy of signalling pathways (Labialle *et al.*, 2002).

Several publications demonstrated that dexamethasone induces MDR1 expression in the intestine of rats (Lin *et al.*, 1999), (Perloff *et al.*, 2004), (Yumoto *et al.*, 2001). So far, there is no explanation how glucocorticoids, in particular budesonide, influence MDR1 expression in human intestinal cells. Here, we investigated how the glucocorticoid budesonide influences the expression of MDR1 in different human intestinal cell lines. We chose Caco-2 cell line, as it shows transporter expressions comparable to those in the human jejunum (Taipalensuu *et al.*, 2001). Furthermore, these cells are an established model for small intestinal transport. (Hidalgo *et al.*, 1989). Secondly, we used LS180 cells, which are a suitable model for intestinal gene induction studies (Bhat *et al.*, 1995), (Thummel *et al.*, 2001), (Zhou *et al.*, 2004). Their expression level of efflux transporters is similar to human colonic tissue (Pfrunder *et al.*, 2003). The findings of this study could be relevant in evaluating the involvement of P-gp in glucocorticoid action and glucocorticoid side effects.

4.1.3 Methods

Chemicals

Budesonide (Sigma-Aldrich, St. Louis, MO, USA), rifampicin (Fluka Chemie, Buchs SG, Switzerland), and actinomycin D (Sigma-Aldrich) were dissolved in dimethylsulfoxide (DMSO). Mifepristone (Sigma-Aldrich) and rhodamine 123 (Molecular Probes, Eugene, OR, USA) were dissolved in ethanol.

Cell Culture

The LS180 (used between passage 40 and 50) and Caco-2 cell line (used between passage 54 and 70) were purchased from ATCC (Manassas, USA). LS180 and Caco-2 cells were cultured in

Dulbecco's MEM with Glutamax-I, supplemented with 10% (v v^{-1}) fetal bovine serum, 1% non essential amino acids, 1% sodium pyruvate, 50 µg mL^{-1} gentamycin (Invitrogen AG, Basel, Switzerland). Cells were seeded into 12 well plastic culture dishes (3.8 cm^2 per well, BD Falcon AG, Allschwil, Switzerland) and were maintained in a humidified 37°C incubator with a 5% carbon dioxide in air atmosphere. After Caco-2 cells had reached confluence or nearly confluence for LS180, they were treated with the substances as indicated or with vehicle alone. Medium was changed every 24 hours. Toxicity was tested in advance for all applied substances using sulforhodamine B staining (Sigma-Aldrich) (Skehan *et al.*, 1990).

Real-time RT-PCR: see chapter 2.2

Sequences for primers and probes are described in Table 1. Sequences for GR alpha and GR beta were adopted from a previous paper (Pedersen *et al.*, 2003).

Western Blot Analysis

LS180 and Caco-2 cells were incubated for 72 hours with 25 µM budesonide or with vehicle (0.25% DMSO) as a negative control. LS180 cells additionally were incubated with 10 µM rifampicin.

For protein extraction and western blot see chapter 2.3.

Rhodamine 123 Accumulation Assay

LS180 and Caco-2 cells were incubated for 72 hours either with medium containing 20 µM budesonide or vehicle only (0.2% DMSO). LS180 cells were additionally incubated with 10 µM rifampicin. Medium was changed every day. Following drug treatment, efflux assay with rhodamine 123 (R123) was performed as described in chapter 2.6.

R123 fluorescence was analysed with a HTS 7000 Plus Bio Assay Reader (Perkin Elmer Ltd., Buckinghamshire, UK) with 485 nm excitation and 535 nm emission filters. The ratio of intracellular R123 fluorescence in the absence and presence of verapamil is indicative of the activity of P-gp. This approach has previously been reported for the measurement of P-gp activity in LS180 cells (Collett *et al.*, 2004).

mRNA Decay Measurement

The measurement of mRNA stability was performed in Caco-2 cells using actinomycin D as an inhibitor of transcription. Cells were preincubated for 1 hour with budesonide 25 µM or vehicle. Actinomycin D (5 µg mL^{-1}) was added and total RNA was extracted after different time points. The expression level of MDR1 mRNA after 0, 4, 8, 24 and 48 hours was determined by real-time reverse transcription (RT-)PCR.

Transfections

The Full ORF Expression vectors of PXR (IOH34726-pT-Rex-DEST30) was purchased from the Deutsches Ressourcenzentrum für Genomforschung GmbH. The empty vector was obtained by cutting the PXR plasmid with the restriction enzymes EcoRV and Mlu I followed by purification on a 0.5% agarose gel. The cut ends were blunt-ended with T4 DNA polymerase and self-circulated with T4 DNA ligase. Transfection was performed with Lipofectamine 2000 (Invitrogen) during 48 hours following the manufacturer's protocol using 1.6 µg DNA and 4 µL Lipofectamine 2000. As negative control the empty vector was used. After transfection Caco-2 cell were incubated with budesonide 25 µM and rifampicin 10 µM or vehicle only. Transfection was verified by measuring PXR mRNA expression using TaqMan analysis.

Statistics

Treatment groups were compared to control group by analysis of variance (ANOVA). If this analysis revealed significant differences and more than one treatment group was included in the

analysis, pairwise comparisons of treatment groups with the control group was performed subsequently using Dunnett's two-sided multi-comparison test or multiple unpaired two-sided t-test with Bonferoni's correction, as appropriate, to account for the multiplicity of testing. All tests were performed using the SPSS for Windows software (version 14.0). The level of significance was $P<0.05$.

4.1.4 Results

MDR1 mRNA Expression

The basal MDR1 mRNA expression level was higher in Caco-2 cells compared to LS180 cells. The effect of budesonide on MDR1 mRNA expression was investigated in these two different intestinal cell lines (LS180 and Caco-2). Cells were incubated for 48 hours with increasing budesonide concentrations and mRNA expression was analysed using real-time RT-PCR. In LS180 cells, we observed a dose-dependent induction of MDR1 mRNA expression by budesonide (Figure 17A). Rifampicin as a positive control also showed an induction. In Caco-2 cells, MDR1 mRNA expression decreased in a dose-dependent manner after budesonide treatment (Figure 17B).

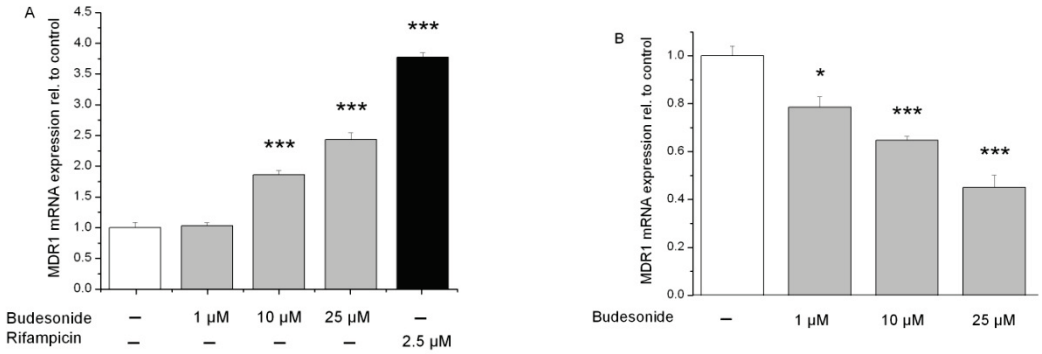

Figure 17: Effect of budesonide on MDR1 mRNA expression in LS180 (A) and Caco2 (B) cell lines. Cells were incubated for 48 hours and mRNA expression was determined by quantitative real-time PCR. Rifampicin was used as a positive control for MDR1 induction in LS180 cells. Results are normalized to control cells and expressed as mean ± s.e.m. (n=4). $^{*}p < 0.05$, $^{***}p < 0.001$ vs. control cells.

MDR1 Protein Expression

The observed changes in MDR1 mRNA expression in LS180 and Caco-2 cells were confirmed on protein level by Western blot analysis (Figure 18). In three independent assays cells were incubated for 72 hours with 25 µM budesonide and 10 µM rifampicin. Compared to control cells, MDR1 protein levels increased in LS180 cells and decreased in Caco-2 cells after budesonide treatment.

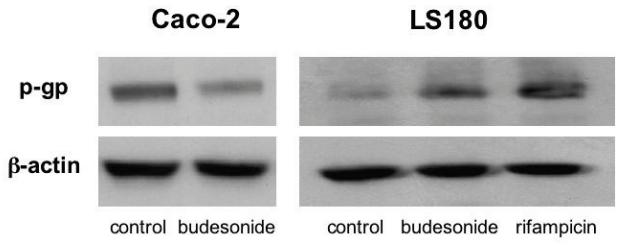

Figure 18 Effect of budesonide on P-gp protein expression. LS180 and Caco-2 cells were incubated for 72 hours with 25 µM budesonide or vehicle. Rifampicin was used as a positive control for MDR1 induction in LS180 cells. P-gp protein and beta-actin were determined by Western blot analysis. Beta-actin was used as loading control.

Measurement of P-gp Activity

R123 is a fluorescent P-gp substrate that can be used for the determination of P-gp activity (Figure 19). After passive diffusion into the cells it is actively transported out of the cell by P-gp. Measurement of cellular R123 accumulation revealed that the observed changes of MDR1 expression are reflected by changes in P-gp function. In LS180 cells, where MDR1 is induced by budesonide, relative R123 uptake was significantly lower compared to control cells. Similar effects were observed for rifampicin, a positive control for MDR1 induction in LS180 cells. In Caco-2 cells, the decreased MDR1 expression led to a significant accumulation of intracellular R123.

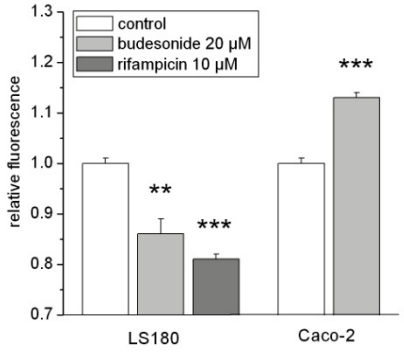

Figure 19 P-gp activity was assessed using Rhodamine 123 accumulation in LS180 and Caco-2 cells. Cells were pre-treated with 20 µM budesonide or vehicle for 72 hours. Rifampicin was used as a positive control for P-gp induction. Data represent Rhodamine 123 fluorescence normalized to verapamil treated cells. A decrease in intracellular fluorescence is indicative of an increase in P-gp activity and *vice versa*. Results are expressed as mean ± s.e.m. (n=3-4). $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control cells.

Expression of the Nuclear Receptors PXR and GR in Intestinal Cell Lines

To elucidate the differential effects of budesonide on the expression of MDR1 in the cell lines studied, we determined whether this could be attributed to differential expression levels of nuclear receptors (Figure 20). Glucocorticoids exert their anti-inflammatory effects through the GR-α. However, the PXR can also be activated by glucocorticoids like dexamethasone. Real-time PCR analysis (n=4 for both cell lines) revealed low expression levels of PXR in Caco-2 cells and high expression in LS180 cells. GR alpha, on the other hand, was exclusively expressed in Caco-2 cells. As the GR beta apparently exhibits inhibitory effects on the GR alpha, we analysed also the expression rate of this receptor. However, no GR-β mRNA was detectable in both cell lines. There was no effect of budesonide treatment on these receptors. In addition, basal MDR1 mRNA expression was not correlated to PXR expression level in both investigated cell lines.

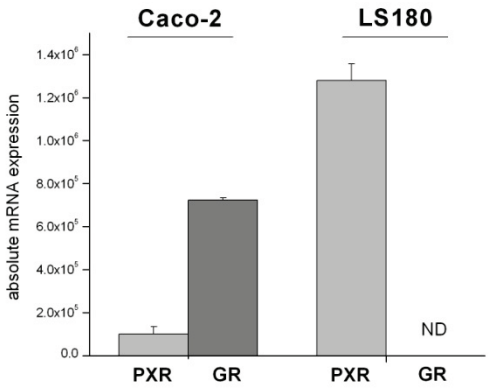

Figure 20 Expression of PXR and GR alpha mRNA in the investigated intestinal cell lines Caco-2 and LS180. Expression was determined by quantitative real-time PCR. Results are expressed as mean ± s.e.m. (n=4). ND = not detectable.

Effect of Mifepristone on MDR1 mRNA Expression in Caco-2 Cells

To determine whether the GR plays a role in the budesonide-induced downregulation of MDR1 in Caco-2 cells, mifepristone (RU486) a known anti-glucocorticoid was used. Mifepristone could not reverse the effect of budesonide when co-applied; when the drug was given alone, it induced a similar downregulation of MDR1 (Figure 21).

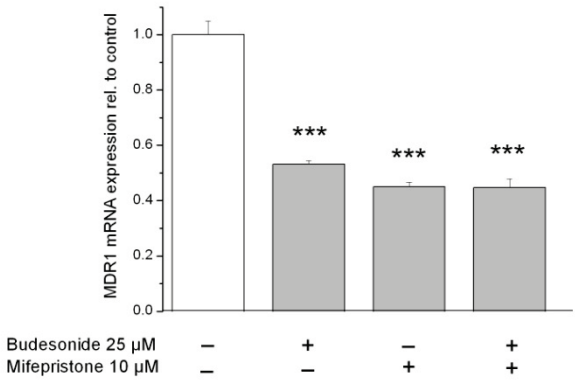

Figure 21 Effect of the anti-glucocorticoid mifepristone on MDR1 mRNA expression. Cells were incubated for 48 hours and mRNA expression was determined by quantitative real-time PCR. Results are normalized to control cells and expressed as mean ± s.e.m. (n=4). ***$p < 0.001$ vs. control cells.

MDR1 mRNA Stability

We have shown that budesonide decreases the expression of MDR1 in Caco-2 cells on the mRNA level. However, beside a transcriptional regulation, this result could also reflect a decrease in mRNA stability. Therefore, we determined the decay of MDR1 mRNA after the addition of the transcription inhibitor actinomycin D in Caco-2 cells (Figure 22). The stability of MDR1 mRNA was not different between control cells and cells treated with budesonide. For both treatments the half-life of MDR1 mRNA in Caco-2 cells appeared to be about 20 h.

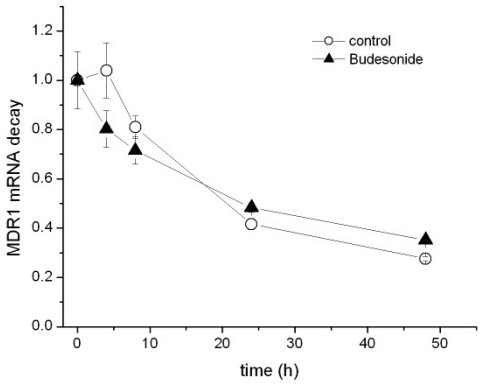

Figure 22 Effect of budesonide on MDR1 mRNA stability in Caco-2 cells. Cells were treated with 25 µM budesonide or vehicle while transcription was inhibited with actinomycin D (5 µg ml^{-1}). mRNA expression was determined by quantitative real-time PCR after 0, 4, 8, 24 and 48 h incubation. Each data value is expressed relative to the expression at 0 hours. Data represent mean ± s.e.m. (n=3).

Transfection of PXR into Caco-2 Cells

To investigate whether PXR is involved in the regulation of MDR1 by budesonide, PXR was transiently transfected into Caco-2 cells. Caco-2 cells express only low levels of endogenous PXR and known PXR activators such as rifampicin are not able to induce PXR target genes in Caco-2 cells (data not shown and (Pfrunder *et al.*, 2003)). In a control experiment, cells transfected with the empty vector showed the expected downregulation of MDR1 mRNA after budesonide treatment and no changes after rifampicin treatment compared to control cells. On the other

hand, PXR transfection could reverse the downregulation of MDR1 mRNA after budesonide treatment. Rifampicin, the positive control for PXR activation, induced MDR1 mRNA in PXR-transfected Caco-2 cells (Figure 23A).

CYP3A4, another target gene of PXR, is only slightly expressed in Caco-2 cells. Nevertheless, in parallel to MDR1, we could see a significant induction of CYP3A4 in PXR-transfected Caco-2 cells after treatment with budesonide and rifampicin (Figure 23B).

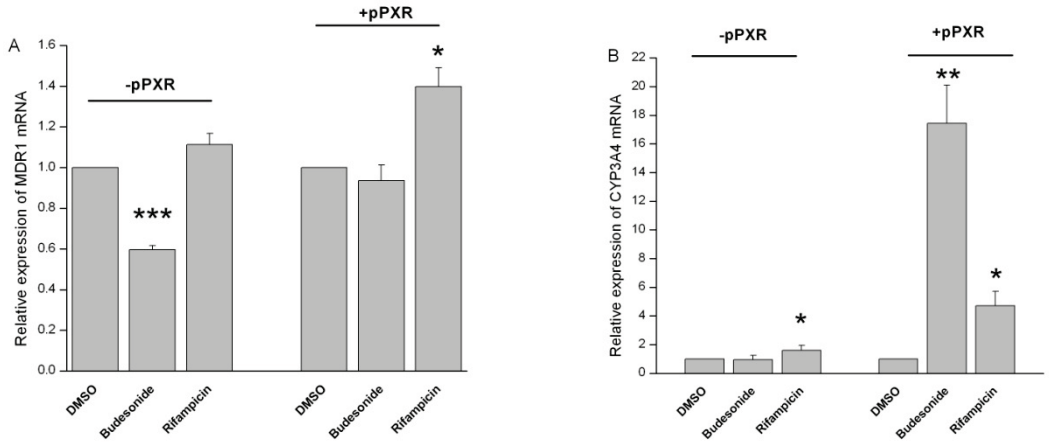

Figure 23 Expression of MDR1 (A) and CYP3A4 (B) in Caco-2 cells transfected with the empty vector (-pPXR) and PXR vector (+pPXR) after treatment with budesonide, rifampicin or vehicle only. In two independent experiments, cells were transfected during 48 hours, followed by 48 hours drug-treatment. mRNA expression was determined by quantitative real-time PCR. Results are normalized to the respective control cells and expressed as mean ± s.e.m. (n=5-6). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control cells.

4.1.5 Discussion

At present, there are no data about the intestinal regulation of MDR1 by budesonide. Given that this glucocorticoid is often used in the treatment of IBD, we investigated its effects on MDR1 expression in two different, frequently used intestinal cell lines. Our results indicate that the regulation of this efflux transporter by budesonide is complex. MDR1 expression was induced in LS180 cells; in contrast, it was downregulated in Caco-2 cells.

An altered intestinal expression of P-gp can have important clinical implications. On the one hand, an induction of MDR1 can lead to an increased efflux of P-gp substrates out of the enterocytes (Westphal *et al.*, 2000). Ineffective therapy or even therapy resistance could be the

outcome. On the other hand, a decreased expression can impair the barrier function of the intestinal epithelial cells. In this respect, it was shown that mice deficient for mdr1a developed an inflammation of the large intestine similar to IBD (Panwala *et al.*, 1998). The inflammation was dependent on the presence of intestinal bacteria, suggesting a function of P-gp to protect the body from toxins produced by intestinal bacteria. This hypothesis is in concordance with data from patients with ulcerative colitis, where the expression of MDR1 and PXR was significantly reduced in mucosal biopsy specimens from non-affected regions of the colon and terminal ileum (Langmann *et al.*, 2004). Here, we have shown that in Caco-2 cells MDR1 expression was repressed by budesonide in a dose-dependent manner. An enhanced mRNA decay is not responsible for the observed MDR1 downregulation (as shown in the experiments with actinomycin D), although increased MDR1 mRNA decay with dexamethasone has been reported in primary rat hepatocytes (Schuetz *et al.*, 1995).

In contrast to LS180 cells, the GR was highly expressed in Caco-2 cells. This led us to assume that the GR could mediate the observed downregulation of MDR1 in this cell line. The downregulation by budesonide occurred, however, in the range of micromolar concentrations only, despite the fact that the GR receptor has a high affinity for glucocorticoids. Furthermore, the addition of mifepristone (RU486), an established antagonist at the GR, did not abolish this effect. In fact, mifepristone repressed MDR1 expression in the same way as budesonide. Therefore, the involvement of the GR can most likely be ruled out, but the mechanism behind the repression of MDR1 in Caco-2 cells remains still unclear. However, a not yet identified unspecific effect appears to be most likely.

In contrast, when we used LS180 cells as an intestinal model, incubations with budesonide showed an increase in MDR1 expression. We assumed that budesonide might be able to activate PXR, leading to an induction of MDR1 and other PXR target genes.

An unspecific induction of MDR1 through toxic drug effects could be ruled out by performing cytotoxicity assays prior to the incubation procedures. In our experiments, the induction occurred with budesonide concentrations starting from 10 µM. This is in concordance with the fact that PXR is a receptor with only low affinity for glucocorticoids, since the affinity of corticosterone for PXR was reported to be in the range of 10 - 30 µM (Sheppard, 2002). The applied budesonide

concentrations up to 25 µM are high, but they could represent relevant local concentrations in the gut lumen after oral administration or when the drug is applied rectally as an enema. The induction of MDR1 in the intestine through glucocorticoids has been shown previously for dexamethasone in the rat (Lin *et al.*, 1999), (Perloff *et al.*, 2004), (Yumoto *et al.*, 2001). The mechanism of this upregulation by glucocorticoids seems to involve PXR. Pascussi et al. demonstrated that dexamethasone activates the nuclear receptor PXR (Pascussi *et al.*, 2001). Furthermore it has been shown that PXR mediates MDR1 induction (Geick *et al.*, 2001).

From our experiments, there are several lines of evidence to support the suggestion that budesonide induces MDR1 expression through activation of PXR. Firstly, PXR expression was high in LS180 cells, where we observed an induction of MDR1. Secondly, rifampicin, a known activator of PXR (Geick *et al.*, 2001), induced MDR1 in parallel with budesonide in LS180 cells. Finally, the PXR target genes MDR1 and CYP3A4 were induced in PXR-transfected Caco-2 cells compared to cells transfected with the empty vector.

In PXR-transfected Caco-2 cells, the induction of MDR1 apparently resulted in a reversal of the previously observed downregulation. For CYP3A4, on the other hand, a clear induction could be seen. Surprisingly, this induction of CYP3A4 was even more prominent with budesonide compared to rifampicin.

PXR is known to be expressed in the human intestine. Therefore, budesonide treatment might lead to increased intestinal levels of P-gp and CYP3A4. Thus, potential drug-drug interactions have to be taken into account, when substrates of P-gp or CYP3A4 are co-administered. On the other hand, the intestinal expression level of PXR itself is a factor that can vary in diseases, such as ulcerative colitis, where PXR has been shown to be downregulated (Langmann *et al.*, 2004). Consequently, results from *in vivo* studies in healthy subjects cannot directly be applied to the situation in patients with IBD.

In conclusion, we have shown in this study that budesonide has the potential to influence the expression of intestinal MDR1 *in vitro*. The investigated intestinal cell lines showed opposing regulatory effects of this transporter. Further studies have to be carried out to evaluate the impact of glucocorticoid treatment on intestinal P-gp expression *in vivo*.

4.2 Collaborative Project:

PXR-mediated Induction of Human CYP3A4 and Mouse Cyp3a11 by the Glucocorticoid Budesonide

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4.2.1 Abstract

Budesonide, a glucocorticoid with a high first-pass metabolism, is used for the oral treatment of inflammatory bowel disease. Cytochrome P450 3A4 (CYP3A4) is an enzyme involved in the metabolism of numerous drugs, including budesonide. Since inhibition or induction of CYP3A4 is often the cause of drug-drug interactions we analysed how budesonide affects the activity and expression of this enzyme. CYP3A4 activity was assessed by the metabolism of a luminogenic substrate (luciferin-benzylether) using recombinant human CYP3A4 protein. We observed no inhibition of the metabolism in the presence of budesonide at concentrations up to 25 µM. Induction experiments in human LS180 colon carcinoma cells showed an increased expression of CYP3A4 mRNA after budesonide treatment. Transactivation assays revealed that budesonide activates the CYP3A4 promoter via the pregnane X receptor (PXR). In mice, oral budesonide administration (25 mg/kg) for 4 days induced the murine homolog Cyp3a11 in the intestine 3-fold, whereas liver expression was notably less influenced. In knockout mice devoid of PXR, budesonide-mediated inductions were reduced compared to wild-type mice. In conclusion, we could demonstrate that budesonide is not an efficient inhibitor but rather an inducer of CYP3A via a PXR-mediated mechanism. In vivo, however, oral budesonide administration to mice showed only modest gene induction, which occurred mainly in the intestine. Therefore, the risk for budesonide-mediated drug interactions seems to be low but cannot be ruled out entirely.

4.2.2 Introduction

Glucocorticoids are substances with strong anti-inflammatory properties mediated through specific activation of the glucocorticoid receptor (GR). However, beside their high affinity for the GR, it has been shown for some glucocorticoids that they are also low-affinity ligands of the pregnane X receptor (PXR, NR1I2) (Lehmann et al., 1998; Sheppard, 2002). PXR is a promiscuous transcription factor expressed in liver and intestine that induces the expression of genes involved in xenobiotic metabolism and transport, thereby stimulating the detoxification and elimination of endogenous substances, drugs, and other xenobiotics (Geick et al., 2001; Pascussi et al., 2003). One of the PXR target genes is cytochrome P450 3A4 (CYP3A4), which encodes one of the most important Phase I drug-metabolizing enzymes in humans. Since more than 50% of all prescribed drugs are CYP3A4 substrates, induction (or inhibition) of this enzyme are often the cause of clinically relevant drug-drug interactions (Lin and Lu, 1998). Therefore, it is a relevant characteristic of a drug to be able to induce CYP3A4 expression or, on the other hand, inhibit CYP3A4 activity.

Studies in rodents revealed that treatment with the glucocorticoid dexamethasone induces the expression of the efflux transporter P-glycoprotein and the metabolizing enzyme cytochrome P450 3A (Lin et al., 1999; Jin et al., 2006). They further showed that these inductions are PXR-mediated and eventually lead to changes in the pharmacokinetics of co-administered drugs due to an enhanced drug clearance. Consequently, co-application of a PXR-activating ligand might reduce or even abolish the efficacy of a given drug therapy.

Budesonide is a synthetic glucocorticoid that is amongst others used orally in patients with inflammatory bowel disease. It acts locally in the gut lumen where it is readily absorbed, followed by extensive pre-systemic metabolism, primarily by CYP3A4 (Jonsson et al., 1995) (Lu et al., 2008). As a consequence, budesonide has high topical anti-inflammatory activity, but considerably lower systemic activity than conventional glucocorticoids (Thalen et al., 1989). So far there is only limited information about the impact of budesonide treatment on the activity and expression of drug-metabolizing enzymes. Though, induction of CYP3A5 by budesonide through a GR-mediated pathway in the human lung adenocarcinoma cell line A549 has been reported (Hukkanen et al., 2003). In the colon carcinoma cell line Caco-2, we recently found that budesonide led to an induction of CYP3A4 when the cells were transfected with human PXR

(Maier et al., 2007). However, there is no direct evidence yet that budesonide is an activator of PXR and, in turn, can induce drug-metabolizing enzymes in vivo. The rationale of the present study was therefore to clarify whether this glucocorticoid can induce CYP3A in vitro and in vivo, and to assess if PXR activation is involved. Since budesonide is an excellent CYP3A4 substrate, we further determined whether it could inhibit the activity of this enzyme. All together, this knowledge might help to assess the risk for possible CYP3A4 interactions mediated by budesonide.

4.2.3 Methods

Materials

Budesonide, dexamethasone, hydrocortisone, prednisolone, prednisone, and pregnenolone-16α-carbonitrile (PCN) were from Sigma Aldrich (St Louis, MO, USA). Rifampicin was from Fluka Chemie (Buchs SG, Switzerland). The pGL3-CYP3A4-XREM (-372/+7.8 kb) luciferase reporter construct was a kind gift from Dr. Richard Kim (Vanderbilt University, Nashville, USA), the pCDG-hPXR expression vector was generously provided by Dr. R.M. Evans (The Salk Institute for Biological Studies, La Jolla, CA). The pCMV-mPXR was obtained from Dr. B. Forman (Beckman Research Institute of the City of Hope National Medical Center, Duarte, California). The LS180 cell line was purchased from ATCC (Manassas, USA).

Determination of CYP3A4 Inhibition

The inhibitory potency of budesonide on CYP3A4 metabolism was measured using the P450-Glo assay for CYP3A4 from Promega (Madison, WI, USA). Assays were carried out according to the instructions of the manufacturer. Briefly, reactions were performed in 96 well plates in a total reaction volume of 50 µL. Each reaction contained 1 pmol of CYP3A4 (cell membranes prepared from baculovirus-infected insect cells). As a CYP3A4 substrate luciferin-benzylether at 30 µM, which is metabolized to luciferin, was applied. Budesonide and ketoconazole (control inhibitor of CYP3A4) were used between 0.025 and 25 µM. Control reactions with no inhibitor (0.1% acetonitrile) and with membranes devoid of CYP3A4 (for background measurements) were

performed in parallel. After a preincubation of 10 minutes reactions were started by adding a NADPH regeneration solution. Metabolic reactions were carried out at room temperature for 60 minutes. After adding a luciferin detection reagent the reactions were stopped and CYP3A4 activity could be assessed by measuring the emerging luminescence.

Measurement of CYP3A4 mRNA Induction in Human Intestinal Cells

The procedure for measuring CYP3A4 induction in LS180 cells was described before (Maier et al., 2007). Briefly, cells were treated with budesonide, dexamethasone, rifampicin or vehicle (0.25% DMSO) for 48 hours. Total RNA was extracted and 0.5 µg was reverse transcribed. Real-time PCR analysis (TaqMan) was carried out on a 7900HT Sequence Detection System (Applied Biosystems) using qPCR Mastermix Plus from Eurogentec (Seraing, Belgium). Sequences of primers and probes have been reported by us (Maier et al., 2007). For absolute quantification external standard curves were used. Expression of CYP3A4 was normalized to the expression of GAPDH. Drug treatments did not significantly influence GAPDH levels.

CYP3A4 Reporter Gene Assay

LS180 cells were seeded in 96 well plates, incubated overnight, and transfected with 75 ng/well of nuclear receptor expression vector (pCDG-hPXR or pCMV-mPXR), 210 ng/well of the CYP3A4 luciferase reporter construct (pGL3-CYP3A4-XREM), and 15 ng/well of the renilla luciferase expression control vector (pRL-TK; Promega, Madison, WI, USA), using Exgen500 in vitro transfection reagent (Fermentas, St. Leon-Rot, Germany) in 150 mM NaCl. After overnight transfection, the drugs (dissolved in DMSO) were added to the plates and after 48 hours, cells were lysed with Passive Lysis Buffer (Promega, Madison, WI, USA). The reporter activities of firefly luciferase and renilla luciferase in the cell lysates were determined using the Dual-Luciferase Reporter (DLRTM, Promega, Madison, WI, USA) Assay System. Luminescence was recorded on a Mithras LB940 microplate reader (Berthold Technologies, Bad Wildbad, Germany). Cells without hPXR or mPXR transfection did not yield significant luminescent signals.

Measurement of Cyp3a11 and Oatp2 mRNA Induction in Mice

Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Pxr-knockout mice were kindly provided by Dr. R.M. Evans (The Salk Institute for Biological Studies, La Jolla, CA) and were crossed back to a homogeneous (>99%) FVB genetic background. Mice used in experiments were male, between 8 and 15 weeks of age and kept in a temperature-controlled environment with a 12-h light/dark cycle. They received a semisynthetic diet (Reference diet 20% casein, Hope Farms, Woerden, The Netherlands) and acidified water ad libitum.

For induction studies, wild-type and Pxr-knockout mice were given an oral dose of dexamethasone or budesonide (both 25 mg/kg), or vehicle (corn oil) for 4 consecutive days. Each group consisted of 4 animals. On day 5, mice were sacrificed, intestines and livers were isolated and immediately placed in RNAlater (Qiagen). Total RNA was extracted using the RNeasy mini kit (Qiagen) and 1 µg was reverse transcribed using Superscript II (Invitrogen, Carlsbad, CA) and random hexamers (Applied Biosystems) according to the supplier's protocols. Real-time PCR was performed on a 7500 real-time cycler system (Applied Biosystems) using SyBr Green PCR master mix and QuantiTect Primer Assays (Qiagen) for the genes of interest (Cat.No.: QT00170590 (Cyp3a11), QT01065232 (Oatp2), QT00095242 (ß-actin)). Then 45 PCR cycles (denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min) were performed. All samples were run in duplicates and non-reverse-transcribed RNA served as a negative control. Analysis of the data was done by relative quantification using the 2-ΔΔCt method as described (Livak and Schmittgen, 2001). Briefly, for all samples Ct values of the target genes were normalized to the Ct values of ß-actin and subsequently normalized to the values of vehicle treatment. This was independently done for both wild-type and Pxr-knockout mice. ß-actin was chosen as a reference gene since the expression was not influenced by drug treatment.

Statistical analysis

Data from control and test groups were analysed for statistical significance using Student`s t-test (unpaired, two-tailed). $p<0.05$ was considered statistically significant.

4.2.4 Results and Discussion

In this study we addressed the question whether the glucocorticoid budesonide can inhibit the activity or induce the expression of the drug-metabolising enzyme CYP3A4. Both inhibitors and inducers can lead to alterations in the pharmacokinetics of co-administered CYP3A4 substrates resulting in potential drug-drug interactions (Lin and Lu, 1998). Regarding this problem only little information is available about budesonide.

We used recombinant CYP3A4 protein and a luminogenic CYP3A4 substrate to assess the inhibitory potency of this glucocorticoid *in vitro*. When testing budesonide concentrations up to 25 µM the activity of CYP3A4 was not inhibited (Figure 24). At higher concentrations we started to observe poor solubility of the drug. It thus seems unlikely that much higher free drug concentrations occur *in vivo*. Ketoconazole, a known potent CYP3A inhibitor *in vivo*, displayed an ideal inhibition curve in this test system (Figure 24). The IC_{50} we found for ketoconazole (about 0.5 µM) was on the high end, but still well within the range reported for various CYP3A4 substrates. So far the lack of clinical interactions with budesonide has been attributed to the low systemic concentrations (Edsbacker and Andersson, 2004). Our experiments show that even at high drug concentrations there is no significant inhibition of metabolism of a model substrate by CYP3A4. Although we cannot fully exclude that budesonide-mediated inhibition might occur for some other CYP3A4 substrates, our data do suggest that budesonide will not substantially inhibit the metabolism of other drugs *in vivo*.

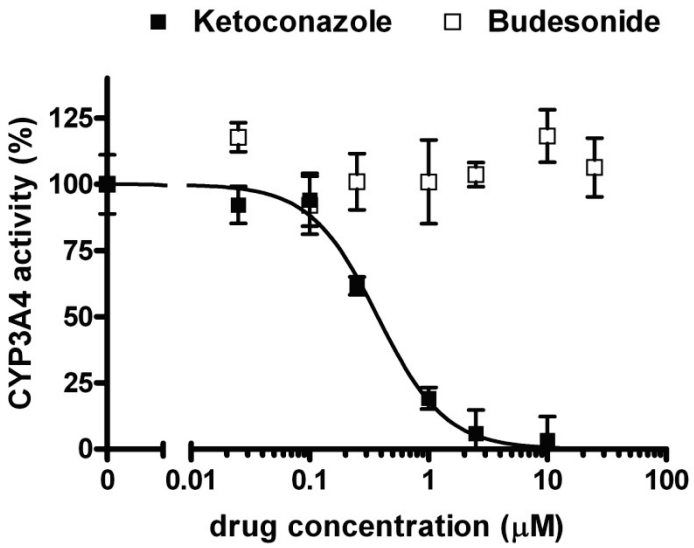

Figure 24 Determination of the effect of budesonide on CYP3A4 acitvity. CYP3A4-containing cell membranes were incubated with budesonide or ketoconazole (control inhibitor of CYP3A4). Luciferin-benzylether was applied as a CYP3A4 substrate and the metabolism to luciferin was analysed after 60 minutes by measuring the resulting luminescence. Data points represent mean values +/- SEM (n = 4). For ketoconazole a sigmoidal inhibition curve could be fitted.

For assessing whether budesonide is an inducer of CYP3A4 we applied LS180 human intestinal cells, a cell line which is effectively used to screen substances for their ability of PXR-mediated gene induction (Gupta *et al.*, 2008). Here, budesonide demonstrated a dose-dependent induction of the endogenous CYP3A4 mRNA (Figure 25). Dexamethasone, on the other hand, did not change the expression of this gene.

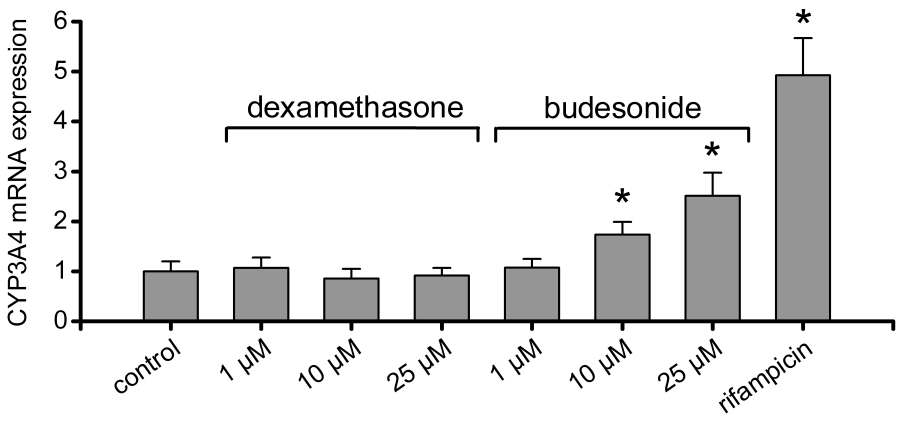

Figure 25 Effect of dexamethasone and budesonide on the expression of CYP3A4 mRNA in intestinal cells. LS180 cells were incubated with increasing concentrations (1, 10, 25 µM) of dexamethasone and budesonide for 48 h. mRNA expression of CYP3A4 was measured by real-time PCR and normalised to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). DMSO (control) and rifampicin (10 µM) were used as negative and positive controls, respectively. Results are mean values (± SD) pooled from 3 independent experiments (each n = 3). * $p < 0.05$ vs control (using Students unpaired, two-tailed t test).

PXR transactivation assays in LS180 cells transfected with a human PXR expression vector and a CYP3A4 promoter-containing reporter construct revealed that budesonide is a good activator of human PXR (Figure 26a). We observed a more than 5-fold increase of the reporter gene activity whereas all other glucocorticoids, including dexamethasone, did not show a significant activation in this intestinal cell line. When transfecting mouse PXR, both dexamethasone and budesonide activated the CYP3A4 promoter around 6-fold (Figure 26b). Budesonide thus activates both human and mouse PXR. The observed species difference for dexamethasone is consistent with previous reports that dexamethasone is a much better activator of mouse Pxr than human PXR (Moore *et al.*, 2000).

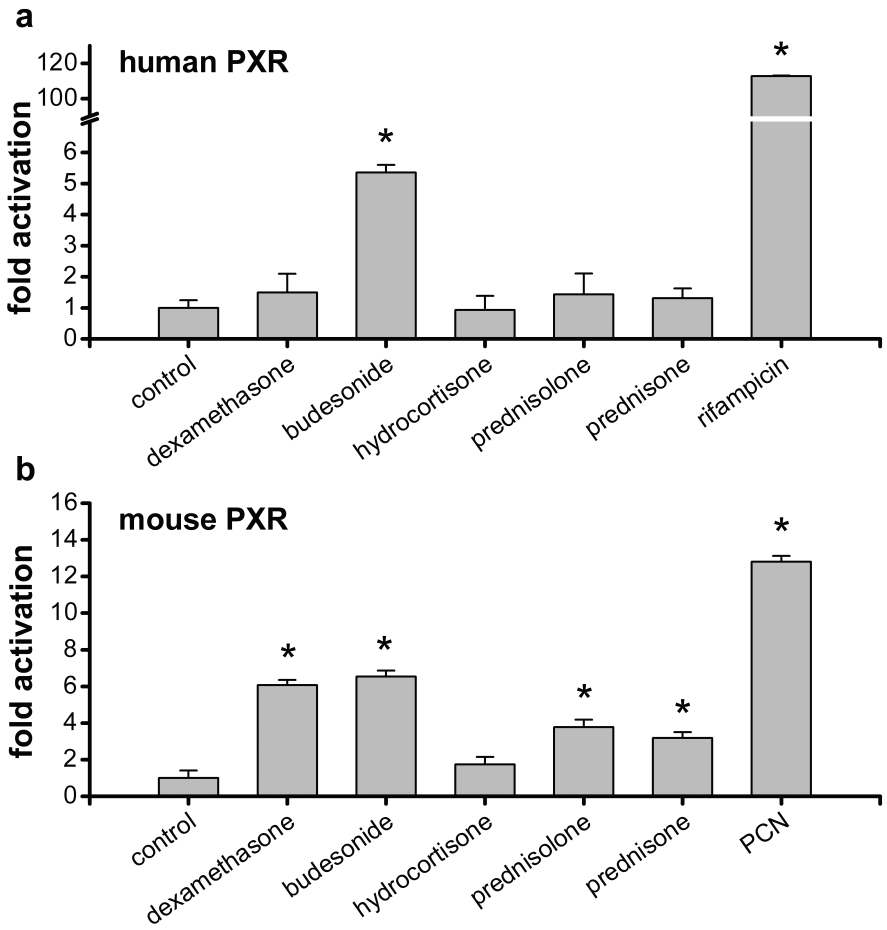

Figure 26 Transactivation of the CYP3A4 promoter by human and mouse PXR in the presence of glucocorticoids. LS180 cells were cotransfected with either human PXR (A) or mouse PXR (B) and a human CYP3A4 promoter-driven firefly-luciferase construct together with a renilla-luciferase expression control vector. Cells were incubated with different glucocorticoids (100 µM), rifampicin (10 µM), pregnenolone-16α-carbonitrile (PCN, 10 µM), or DMSO (control) for 48 h, followed by measurement of the luciferase activity. The firefly-luciferase signal was normalised to the renilla-luciferase signal. Results are expressed as mean values ± SD (n = 3). * $p < 0.05$ vs control.

Given these results, budesonide might be a likely candidate for drug-drug interactions, due to its ability to activate PXR and induce CYP3A4 expression. However, a characteristic quality of budesonide is its extensive first pass metabolism leading to rapid drug degradation and an estimated systemic availability in humans of only 10% after oral treatment (Lundin *et al.*, 2003). Therefore we investigated whether these pharmacokinetic properties resulted in a reduced or even abolished ability to induce PXR-target genes *in vivo*.

This question was addressed by assessing how a 4-day oral drug treatment influenced the expression of the mouse PXR target gene Cyp3a11 in wild-type and *Pxr*-knockout mice. In line

with the activation of mouse PXR *in vitro*, both budesonide and dexamethasone induced Cyp3a11 expression in the duodenum of wild-type mice (around 3-fold, Figure 27a). Induction was also observed in the jejunum, albeit less pronounced (around 2-fold). In *Pxr*-knockout mice, however, there was still a treatment-related induction of Cyp3a11 observable. Especially for dexamethasone, the induction levels were at least as high in the absence of PXR. In contrast, with budesonide induction levels did decrease significantly in *Pxr*-knockout mice in both duodenum and jejunum ($p < 0.05$), although in duodenum they were not completely reduced to the expression levels of vehicle treatment (Figure 27a). These data suggest that budesonide-mediated upregulation of intestinal Cyp3a11 is substantially mediated by PXR, but that dexamethasone-mediated induction is less dependent on PXR.

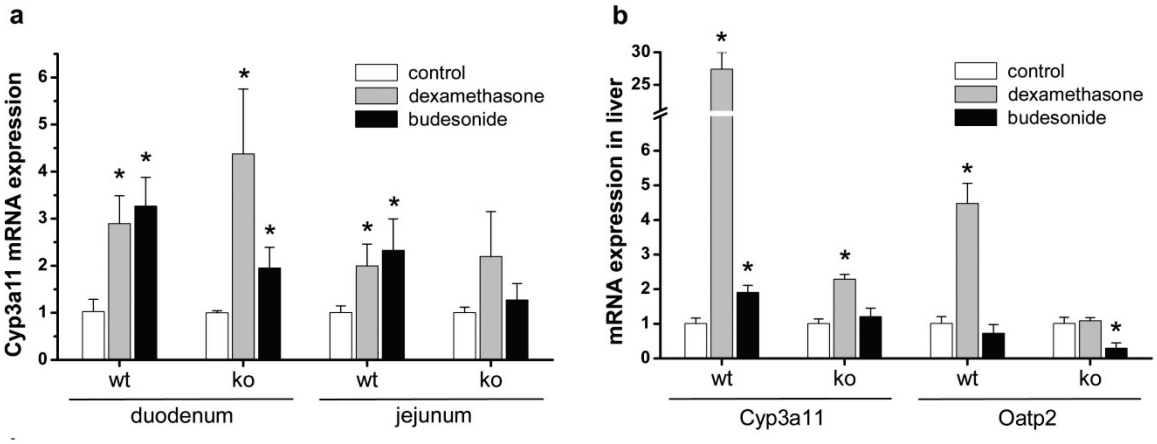

Figure 27 Intestinal and hepatic mRNA expression in mice after dexamethasone and budesonide treatment. The drugs (25 mg/kg/day) or corn oil (control) were given orally to wild-type (wt) and *Pxr*-knockout (ko) mice for 4 days. Expression levels were determined with real-time PCR and normalised to the expression of ß-actin. A: mRNA expression of Cyp3a11 in duodenum and jejunum. B: mRNA expression of Cyp3a11 and Oatp2 (Slco1a4) in liver. Results are normalised to vehicle treatment and displayed as mean values ± SD (n = 4). * $p < 0.05$ vs control. Note that basal expression levels of Cyp3a11 in duodenum, jejunum and liver were higher in *Pxr*-knockout mice compared to wild-type mice (1.5, 1.2, and 6.6-fold, respectively). Basal hepatic Oatp2 levels were 12-fold lower in *Pxr*-knockout mice compared to wild-type mice.

The induction of mouse Cyp3a11 has been attributed to various other transcription factors besides PXR, such as AhR, CAR, and Nrf2 (Cheng et al., 2005). Whether any of these transcription factors is indeed activated by glucocorticoids is, to our knowledge, still unknown. For human CYP3A5 (and CYP2C9) a direct effect of the GR on the induction of these genes has been shown (Schuetz et al., 1996), (Gerbal-Chaloin et al., 2002). A role for the GR in the mechanism of CYP3A4 induction has been discussed, although this gene contains no obvious consensus binding site for the GR (Pascussi et al., 2003). Data from GR-deficient mice suggest that the GR is not required for the induction of mouse CYP3A (Schuetz et al., 2000). Nevertheless, the GR seems to be indirectly involved in CYP induction by increasing the expression of PXR, RXR and CAR, thereby leading to a potentiation of xenobiotic-mediated CYP inductions (Huss and Kasper, 2000), (Pascussi et al., 2000). It may therefore be that, next to Pxr, also GR contributes to the budesonide-dependent expression changes we observed in Figure 27.

Since PXR is a transcription factor showing high expression in intestine and liver (Lehmann et al., 1998), we further analysed how oral drug treatment affected the expression of PXR target genes in liver (Figure 27b). There was a marked quantitative difference between the two glucocorticoids: dexamethasone induced the expression of hepatic Cyp3a11 mRNA 27-fold, whereas budesonide showed only a modest increase in the expression of this gene (less than 2-fold). In *Pxr*-knockout mice budesonide treatment did not influence hepatic Cyp3a11 expression at all, and dexamethasone showed a much reduced (albeit still significant) ~2-fold induction. We also measured the expression of the presumably more specific PXR target gene organic anion transporting polypeptide 2 (Oatp2, Slco1a4) (Cheng *et al.*, 2005). After drug treatment of wild type mice, hepatic expression levels of this gene were only induced by dexamethasone (4.5-fold induction, Figure 27b). Budesonide showed no effect on hepatic Oatp2 expression in wild-type mice, and - for unknown reasons - even downregulated Oatp2 in *Pxr*-knockout mice.

The apparent discrepancy in hepatic gene induction between the two investigated glucocorticoids might be attributable to their differences in systemic exposure, with only low amounts of budesonide reaching the liver compared to dexamethasone. Note, however, that our data do suggest that still some budesonide reaches the liver, in view of the effects of budesonide on hepatic gene expression (Figure 27b). One study that analysed budesonide degradation in tissue fractions from human liver and colon suggested that this drug is predominantly metabolised in

hepatic tissue (Cortijo *et al.*, 2001). On the other hand, two recent publications revealed that intestinal CYP3A4 expression also has a high impact on budesonide pharmacokinetics (Seidegard *et al.*, 2008; Ufer *et al.*, 2008). Regarding the pharmacokinetics of oral budesonide in mice not much is known, but the rates and routes of budesonide metabolism seem to be very similar in mice and humans, implying the relevance of mouse models in this respect (Edsbacker *et al.*, 1987). The data from our study suggest that budesonide has a strongly reduced induction capacity in the liver, possibly due to its high presystemic metabolism. However, locally in the intestine it exerts similar effects as a conventional glucocorticoid such as dexamethasone. Degradation of budesonide in enterocytes is probably not fast enough to significantly affect intestinal gene induction. Consequently, its pharmacological effects (whether GR- or PXR-mediated) seem to be mostly restricted to the intestinal tract, with a very low systemic component.

For the extrapolation of our results to the human situation, some aspects have to be taken into account. In our *in vivo* study we used high drug doses (25 mg/kg/d), comparable to studies where gene induction by glucocorticoids was investigated in mice. In patients suffering from chronic intestinal inflammation (e.g. Crohn`s Disease), usually an oral dose of 9 mg budesonide per day is applied (Spencer and McTavish, 1995), which is lower than the mouse dose, even after correction for metabolic weights. Another important factor is the way the drug is delivered. We suspended the drug in corn oil before oral administration to mice, allowing the drug to be absorbed along its way through the gastrointestinal tract. Patients, on the other hand, generally receive a controlled release formulation of the drug (Edsbacker *et al.*, 2002). This ensures that budesonide is mainly released in the distal small intestine and the colon, where the predominant sites of inflammation are located. Therefore, induction of CYP3A4 (or other PXR-target genes) might occur primarily at the site of drug release, where local concentrations can be very high. These high concentrations are necessary for PXR-mediated effects, given that it is a receptor with only low affinity for glucocorticoids (Sheppard, 2002).

When analysing clinical studies, there seems to be no particular evidence so far that budesonide is the cause of drug-drug interactions. However, since the degradation of budesonide itself is primarily CYP3A4/5-mediated (Jonsson et al., 1995), (Lu et al., 2008), induction of CYP3A4 by rifampicin resulted in virtually abolished budesonide plasma levels (Dilger et al., 2005). In this

respect it might be possible that budesonide could increase its own metabolism by inducing intestinal CYP3A4 expression. Indeed, peak plasma concentrations of the main metabolites 6β-hydroxybudesonide and 16α-hydroxyprednisolone were both found to be slightly increased after 7 days of budesonide treatment compared to single-dose administration (Dilger et al., 2006). It is therefore imaginable that the metabolism of a co-administered CYP3A4 substrate could be enhanced as well, provided that both drugs are absorbed in similar regions of the gastro-intestinal tract.

In conclusion, we showed that budesonide activates both human and mouse PXR *in vitro*. After oral administration, a local induction of PXR-target genes in the intestine could occur, whereas hepatic gene expression is most likely not much influenced. Therefore, the risk for budesonide-mediated drug interactions appears to be low, but cannot be ruled out. Only clinical studies can assess the possibility for drug interactions in humans.

4.3 Stable Transfection of hPXR in Caco-2 Cells

4.3.1 Abstract

Background and Purpose: Caco-2 cells represent a good model for intestinal absorption studies. Induction of PXR target genes such as P-gp and CYP3A4 is an important determinant of intestinal drug absorption. Caco-2 cells are not inducible by ligands of PXR. To reconstitute functional active PXR we aimed to establish a stable PXR-transfected Caco-2 cell system.

Experimental Approach: Caco-2 cells were stable transfected with the full ORF expression PXR plasmid. PXR mRNA expression of transfected clones was measured using real-time PCR, whereas PXR protein expression was shown by Western blot. Functional activity of PXR was assessed by measuring the induction of PXR target genes P-gp and CYP3A4. Changes in P-gp and CYP3A4 expression was measured using RT-PCR, Western blot analysis and measurement of functional P-gp activity.

Key Results: PXR mRNA and protein expression was increased in stable transfected PXR clones. The PXR target genes P-gp and CYP3A4 were significantly induced on mRNA level after induction with the PXR-ligand rifampicin. PXR-mediated induction of P-gp protein expression could not be quantified but Western blot analysis revealed a highly increased P-gp expression in PXR-transfected cells compared to wild-type cells. Induction of P-gp on functional level showed conflicting results. Efflux of the P-gp substrate R123 was increased after induction whereas remaining R123 did not confirm this finding.

Conclusions and Implications: We were able to generate a stable PXR-transfected Caco-2 cell line which expresses higher levels of P-gp than wild-type cells. However, the induction potencies of this system have to be investigated in further studies.

4.3.2 Introduction

The intestine constitutes a barrier function against xenobiotics and toxins. Intestinal epithelial cells form the first barrier for orally applied drugs. Beyond physicochemical properties of the drug itself, intestinal active extrusion and drug metabolism are important mechanisms that influence oral bioavailability of drugs. The impact of intestinal export-transport and metabolism in drug therapy has been extensively described in literature (Floren *et al.*, 1997), (Hebert *et al.*, 1992). Studies in patients receiving cyclosporine a (CsA) or tacrolimus have shown that gut metabolism and export-function significantly alter bioavailability of these drugs. CsA and tacrolimus both are substrates of the phase I metabolic enzyme cytochrome P450 3A4 (CYP3A4) and the export transporter P-glycoprotein (P-gp), which belongs to the ATP-binding-cassette transporters (ABC-transporter). CYP3A4 and P-gp share a broad overlap in substrate specificity. In parallel, they are both regulated by a large amount of drugs. Human pregnane X receptor (hPXR), a member of the orphan nuclear receptor family, was shown to be responsible for transcriptional regulation of P-gp and CYP3A4 (Bertilsson *et al.*, 1998), (Geick *et al.*, 2001). hPXR also called steroid and xenobiotic receptor (SXR) or pregnane-activated receptor (PAR) is encoded by the NR1I2 gene. Different isoforms of PXR are described, whereas PXR.1 is the most abundant splice variant. All of these isoforms contain a DNA-binding domain (DBD) which contains a zinc-finger motive at the N-terminal, and a ligand-binding domain (LBD) at the C-terminal (Kliewer et al., 1998), (Lamba *et al.*, 2004). The LBD domain is designed for binding of a broad range of ligands (Watkins *et al.*, 2001). Endogenous and synthetic steroids are known ligands of PXR but xenobiotics such as rifampicin, nifedipine, clotrimazole or paclitaxel also have been shown to activate hPXR (Kliewer *et al.*, 1998), (Blumberg *et al.*, 1998), (Lehmann *et al.*, 1998), (Synold *et al.*, 2001). Upon activation PXR builds a heterodimer with retinoid-X-receptor-α (RXRα) and translocates to the nucleus. In the nucleus the heterodimer binds to nuclear response elements on the promoter of the target gene. PXR:RXRα heterodimer binding sites on the promoter of CYP3A4 and P-gp were detected (Blumberg *et al.*, 1998), (Geick *et al.*, 2001). The following induction of CYP3A4 and P-gp then leads to increased metabolism or efflux of xenobiotics. This regulation is important in the intestine where orally applied drugs are absorbed. Caco-2 cells are a good *in vitro* model for the intestine (Hidalgo *et al.*, 1989). Unfortunately, Caco-2 cells express only low levels of endogenous PXR. Additionally, the known PXR activator rifampicin is not able

to induce PXR target genes in Caco-2 cells (Pfrunder *et al.*, 2003). In a previous study, we transiently transfected Caco-2 cells with PXR and could detect a slight induction of PXR target genes after induction with rifampicin (Maier *et al.*, 2007). The aim of the present study was to establish a stable model of PXR-transfected Caco-2 cells in order to have PXR-inducible Caco-2 cells which should represent a more similar model of the intestinal epithelium.

4.3.3 Methods

Materials

The Full ORF Expression vector of PXR (IOH34726-pT-Rex-DEST30) was purchased from the Deutsches Ressourcenzentrum für Genomforschung GmbH. Geneticin (50 mg/ml) was purchased from Invitrogen. Rifampicin (Sigma-Aldrich) was dissolved in dimethylsulfoxide (DMSO) and rhodamine 123 (Molecular Probes, Eugene, OR, USA) was dissolved in ethanol.

Cell Culture: see chapter 2.1.

Real-time RT-PCR: see chapter 2.2

Sequences for primers and probes are described in **Table 1**.

Western Blot Analysis: see chapter 2.3

Efflux Assay: see chapter 2.6

Transfection

The Full ORF Expression vectors of PXR (IOH34726-pT-Rex-DEST30) was purchased from the Deutsches Ressourcenzentrum für Genomforschung GmbH. The empty vector (-pPXR), which served as negative control, was obtained by cutting the PXR plasmid (+pPXR) with the restriction enzymes EcoRV and Mlu I followed by purification on a 0.5% agarose gel. The cut ends were blunt-ended with T4 DNA polymerase and self-circulated with T4 DNA ligase. For stable transfection, Caco-2 cells were seeded on 6-well-plates in Dulbecco's modified Eagle's medium with Glutamax-I, supplemented with 10% fetal bovine serum, 1% non-essential amino

acids, 1% sodium pyruvate, 50 µg/ml gentamycin. Transfection was started when cells reached a confluency of ~60%. Transfection was performed with Lipofectamine™ 2000 (Invitrogen) during 72 hours following the manufacturer's protocol using 4 µg Plasmid DNA and 10 µl Lipofectamine™ 2000 each in a volume of 250 µl Opti-MEM I (Invitrogen) and 2 ml DMEM without gentamycin per well. After 72 hours, cells were diluted 1:40 into 10 mm Petri dishes and the selection antibiotic geneticin (Invitrogen) was added in a concentration of 1.5 mg/ml. The appropriate concentration of the resistance antibiotic geneticin was previously determined by a killing curve in Caco-2 wt cells using different concentrations of geneticin over 10 days. Every 3-4 days DMEM with geneticin was replaced. After 2 weeks clones were picked using Scienceware® Cloning discs (Sigma-Aldrich) and further cultured in 24-well plates with a geneticin concentration of 1 mg/ml.

Each clone was cultured over 6 passages and mRNA expression of PXR was measured. Clones expressing high levels of PXR were used for induction studies with rifampicin. Induction of MDR1 and CYP3A4 mRNA expression was quantified using real-time RT-PCR. The clone with the best induction on mRNA level was chosen for further evaluation on protein and on functional level.

4.3.4 Results

Expression of PXR

Stable transfection of PXR into Caco-2 cells was performed. In order to measure the extent of transfection PXR mRNA expression was analysed in PXR-transfected clones compared to empty-vector-transfected Caco-2 cells. The expression of PXR mRNA in PXR-clones was about 30-fold higher compared to control cells (Figure 28A). In addition to mRNA expression PXR protein expression was detected using western blot analysis (Figure 28B). As shown in Figure 28B an increase in PXR expression was detected in PXR-transfected clones compared to wt Caco-2 cells. LS180 cells were used as positive control for PXR detection, as shown in Figure 28B basal PXR expression level is higher in LS180 cells compared to Caco-2 wt as well as stable PXR-transfected Caco-2 cells.

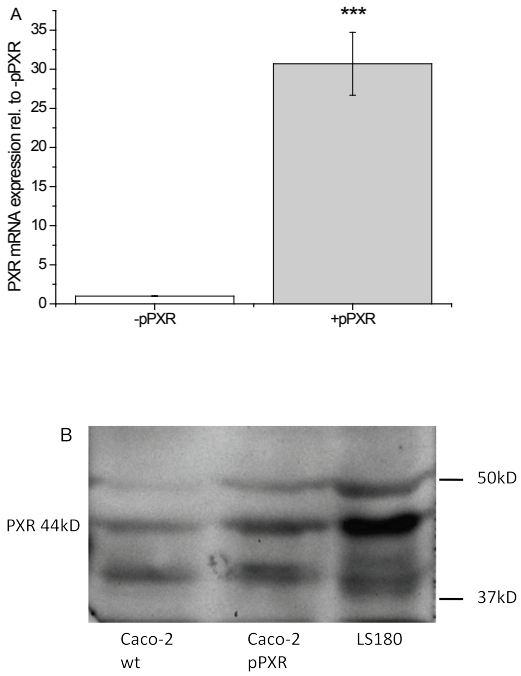

Figure 28 (A) mRNA expression of PXR in Caco-2 cells after transfection with empty vector (-pPXR) and PXR vector (+pPXR). mRNA expression was determined by quantitative real-time PCR. PXR expression is expressed relatively to empty-vector transfected cells. Data represent mean ± s.e.m. of 3 independent assays (n=9) ***$p< 0.001$ vs –pPXR. (B) Western blot analysis for PXR protein expression in Caco-2 wt, PXR-transfected clones and LS180 cells.

Induction of MDR1 and CYP3A4

In order to check functional activity of transfected PXR, induction studies with rifampicin were performed for 3 days. Expression of the PXR-target genes MDR1 (Figure 29A) and CYP3A4 (Figure 29B) was measured on mRNA level. In PXR-clones we observed a significant induction (ca 1.6 fold) of MDR1 mRNA expression after induction with rifampicin, while in empty-vector transfected clones, no alteration in MDR1 mRNA expression was observed. A similar effect was observed on CYP3A4 mRNA expression after rifampicin induction (ca 1.9 fold). However, regarding the low level of CYP3A4 expression in Caco-2 cells, this result has to be considered carefully. MDR1 Protein expression after induction with rifampicin was analysed using western blot technique (Figure 29C). Western blot was difficult to quantify and the assay was suboptimal. Unusually, P-gp protein expression was only poorly detectable in Caco-2 wild-type and LS180 cells. In Caco-2 wild-type cells P-gp expression did not alter after treatment with the inductor. A slight difference in P-gp expression after rifampicin treatment was detected in the positive control

LS180 cells. Unfortunately, in PXR-transfected Caco-2 cells, the inductor effect of rifampicin could not be quantified in this Western blot analysis. However, this Western blot analysis clearly showed that PXR-transfected Caco-2 cells have definitely a higher expression level of P-gp compared to wild-type cells even when lower amounts of protein were added.

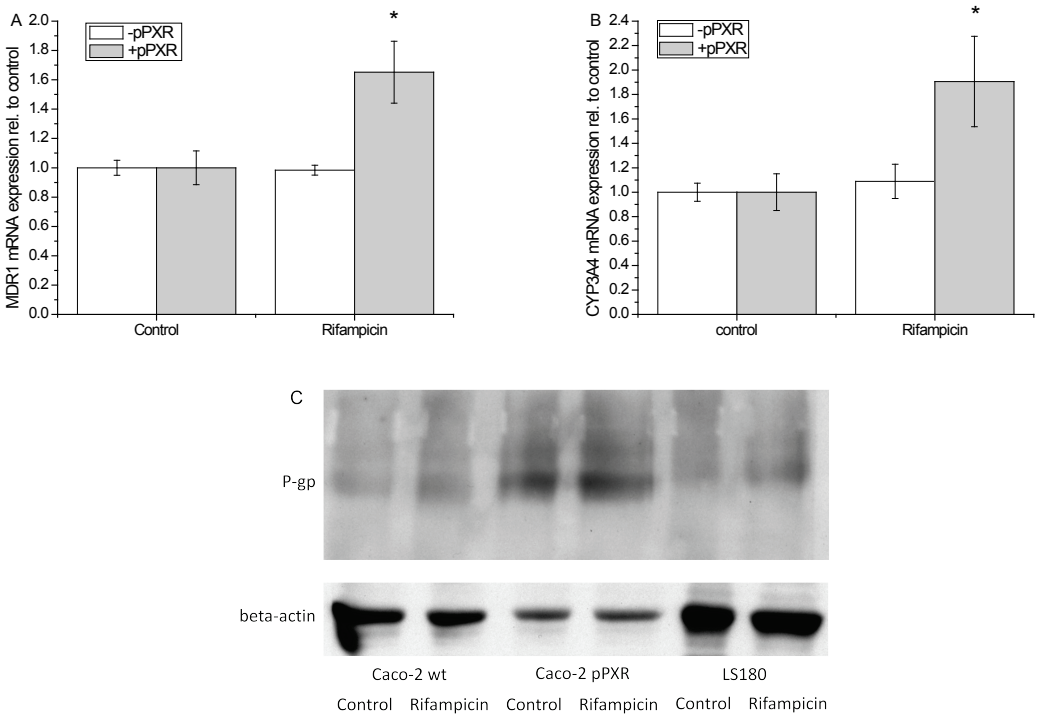

Figure 29 mRNA expression of PXR target genes MDR1 (A) and CYP3A4 (B) after 72 hours induction with rifampicin 10 µM in Caco-2 cells stable transfected with empty vector (-pPXR) and PXR vector (+pPXR). mRNA expression was determined by quantitative real-time PCR. Results are normalised to the respective control treatment (vehicle only). Data represent mean ± s.e.m. of 3-5 assays (n=6-15) *p<0.05 (C) Western blot analysis: Protein expression of P-gp after 72 hours induction with rifampicin or vehicle only in Caco-2 wild-type cells, PXR-clones and LS180 cells.

Functional Activity of P-gp

Functional activity of P-gp in Caco-2 wild-type, PXR-clones and LS180 cells was measured using Rhodamine (R123) as substrate of P-gp. Cells were induced for 3 days with rifampicin and the change of P-gp activity was measured using R123-Efflux-assay. Efflux and remaining accumulation of R123 after 1 hour efflux-period was measured compared to cells where P-gp

activity was inhibited with verapamil. More efflux and less remaining accumulation means higher P-gp activity. Efflux of R123 was elevated in Caco-2 PXR-clones and LS180 cells after rifampicin induction, while no change was detectable in Caco-2 wild-type cells (Figure 30A). However, remaining accumulation of R123 is slightly but not significantly lower (Figure 30B).

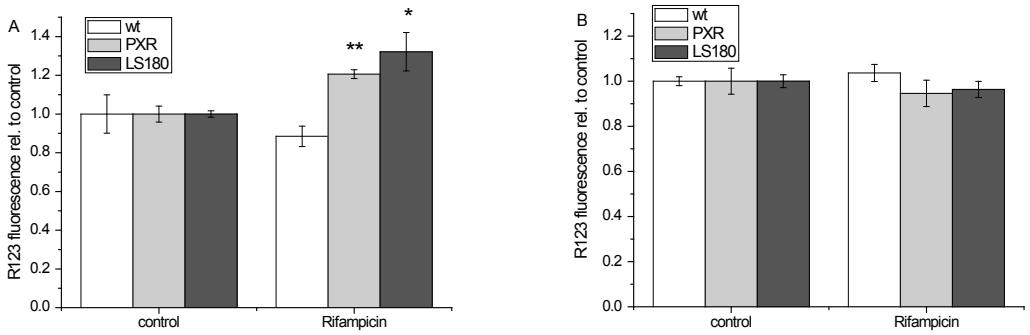

Figure 30 Functional activity of P-gp after 3 days induction with rifampicin 10 µM in Caco-2 wild-type, PXR-transfected Caco-2 cells and LS180 cells. Efflux-assay was performed with R123 (0.5 µM) for 1 hour. Efflux of R123 (A) and remaining substance (B) was measured. Data represent R123 fluorescence relative to verapamil (100 µM)-treated cells normalised to control. Results are expressed as mean±s.e.m. (n=5-6). *p<0.05, **p<0.01

4.3.5 Discussion

CYP3A4 and P-gp play an important role in drug absorption and metabolism. Intestinal expression of these two proteins can limit bioavailability of orally applied drugs. Previous studies demonstrated that CYP3A4 and P-gp are regulated via the orphan nuclear receptor PXR. PXR exhibits a broad range of ligands and consequently is activated by a large amount of drugs. PXR activation and further transcriptional regulation of CYP3A4 and P-gp possibly diminish bioavailability of administered drugs and drug-drug interaction could be the consequence. Intestinal models to assess this interaction potential *in vitro* are required. LS180 cells represent a good model for intestinal induction experiments (Pfrunder *et al.*, 2003). Caco-2 cells are an established model for intestinal drug-transport studies (Hidalgo *et al.*, 1989), since they form a tight monolayer, and the expression level of ABC-transporters is similar to that of human jejunal tissue (Taipalensuu *et al.*, 2001). Unfortunately, Caco-2 cells express only low levels of hPXR and PXR target genes CYP3A4 and P-gp are not inducible with rifampicin, suggesting non-functional PXR-expression in Caco-2 cells (Pfrunder *et al.*, 2003). Transfection of hPXR into Caco-2 cells

might serve as a good intestinal *in vitro* model for transport and induction in the intestine. In an earlier study, we transiently transfected PXR into Caco-2 cells and could observe a significant induction of MDR1 and CYP3A4 mRNA expression, which was still lower than the one observed in LS180 cells (Maier *et al.*, 2007). In this study, a stable PXR-transfected Caco-2 model was intended to establish.

PXR transfection led to a stable transfected cell line with a 35-fold increase in PXR mRNA expression compared to control cells. PXR protein expression was also increased after transfection but not to the same extent as mRNA. Functional activity of PXR was checked via induction of PXR target genes with rifampicin. The induction with rifampicin led to a significant increase in CYP3A4 and MDR1 mRNA expression. However this effect is lower than the induction effect on MDR1 and CYP3A4 in LS180 cells (data not shown). This might be explained by the fact that LS180 cells express a basic PXR level (Figure 28B) that is still higher than in stable transfected cells. Induction of P-gp protein after rifampicin treatment was hardly detectable in PXR-transfected Caco-2 cells and also in the positive control LS180 cells. Probably changes in P-gp protein expression were small and the sensitivity of the present assay apparently was not adequate.

Elevated functional activity of P-gp after rifampicin induction was assessed by rhodamine 123 efflux assays. Increased efflux of the P-gp substrate R123 was observed after induction in PXR-clones and LS180 cells. Unfortunately, residual accumulation of R123 after one hour efflux could not confirm the effects seen in the efflux. A small but not significant decrease in R123 accumulation after rifampicin induction was observed in PXR-transfected clones as well as in the positive control LS180 cells. Induction effects on the PXR target gene P-gp were not as pronounced as in LS180 cells as we showed in earlier studies. A possible explanation for this observation could be the different cell system. PXR-mediated induction requires co-factors such as RXR for heterodimerisation upon activation. Our Caco-2 cells express RXR mRNA, but possible differences in co-factors expression and function in Caco-2 cells compared to LS180 cells is not clarified and might play a role in the observed small induction of PXR target genes.

Transfection of hPXR into Caco-2 cells was earlier described by Korjamo et al. (Korjamo *et al.*, 2005), (Korjamo *et al.*, 2006). Comparable to our results, they only could observe small

inductions of P-gp in hPXR-transfected Caco-2 cells and these results were predominantly achieved after 7-14 days induction with rifampicin.

Taken together, Caco-2 cells stable transfected with PXR show increased levels of P-gp expression. However, functional induction of PXR-target genes by rifampicin is not as explicit as in LS180 cells and further evaluations are needed to establish a definite PXR-inducible Caco-2 cell system.

5 Curcuma longa

5.1 Effects of Curcuma-Extracts and Curcuminoids on Expression of Intestinal ABC-Transporters and Cytochrome P450

5.1.1 Abstract

Background and Purpose: *Curcuma longa L.* is a widely used spice. Its main ingredients, the curcuminoids, are used in the treatment of inflammatory diseases and cancer. Bioavailability of curcuminoids is low and huge amounts remain in the intestine. We therefore aimed to investigate the interaction potential with ATP-binding cassette transporters (ABC-transporters) and cytochromes P450 (CYPs) in an intestinal cell line (LS180). Intestinal ABC-transporters and CYP enzymes play a major role in drug absorption and consequently changes in their expression level could lead to interactions.

Experimental Approach: The intestinal LS180 cell line was incubated with different curcuma extracts, the single curcuminoids, curcumin, demethoxycurcumin and bisdemethoxycurcumin, and a curcuminoid mixture. Changes in mRNA expression of the ABC-transporters MDR1 and BCRP and the cytochromes CYP3A4 and 1A2 were measured by real-time RT-PCR.

Key Results: MDR1 mRNA expression was significantly but not relevant downregulated by the curcuminoids, whereas the extract had no significant effect. BCRP expression in our cell system was very low and therefore, we could not quantify any effects on BCRP mRNA expression. CYP3A4 and 1A2 mRNA expression did not alter significantly after treatment.

Conclusions and Implications: Curcuma extracts, the single curcuminoids and a curcuminoid-mixture have no relevant effect on the investigated ABC-transporters and CYPs mRNA expression in our cell system. Further studies are required to evaluate its effects *in vivo*.

5.1.2 Introduction

Curcuma longa L. (turmeric) belongs to the family of zingiberaceae. Its rhizome is widely used as spice and food-coloring agent. It also has a long history in traditional Indian medicine. The mainly investigated constituents of turmeric are the three curcuminoids curcumin, demethoxycurcumin and bisdemethoxycurcumin. They are supposed to be responsible for the biological activities of turmeric such as anti-inflammatory or antioxidant activities. Several *in vitro* studies showed that curcumin interacts with a variety of proteins such as transcriptional factors, inflammatory cytokines, enzymes, kinases, growth factors, receptors etc. Curcumin also regulates expression of different anti-inflammatory agents and studies with curcumin suggest that it has an antiproliferative potential. Clinical trials with curcumin were performed mainly in the field of anti-inflammatory or anti-cancer therapy (Goel *et al.*, 2008).

Unfortunately, curcumin has a low oral bioavailability mainly due to poor enteral absorption and rapid metabolism. Studies in rats and humans showed low plasma concentrations after oral application. Higher systemic concentrations were achieved after inhibition of glucuronidation (Shoba *et al.*, 1998). Studies in rats revealed that after oral intake most of the applied drug remains in the intestinal tract (Ravindranath and Chandrasekhara, 1980).

The intestine forms the first barrier for drugs or other xenobiotics. Physicochemical properties of the drug itself and active efflux-transport or metabolism mainly form this barrier. Among these efflux-transporters P-glycoprotein (P-gp, MDR1) is the most prominent. It belongs to the class of ATP-binding-cassette transporters (ABC-transporters) and has broad substrate specificity for different compounds such as anticancer drugs, antibiotics, antivirals or immunosuppressive agents. P-gp is regulated via the human pregnane X receptor (hPXR) (Geick *et al.*, 2001) Breast cancer resistance protein (BCRP) is another ABC-transporter with an important role in the intestine. BCRP is a half-transporter and is also highly expressed in the intestine. Its substrate specificity is partly overlapping with P-gp. The regulation of BCRP is yet not fully understood, but there is some evidence that upregulation of BCRP is mediated via the aryl hydrocarbon receptor (AhR) (Ebert *et al.*, 2005).

Important metabolising enzymes in the intestine are the cytochromes P450 (CYP). They belong to the haemoproteins and metabolise lipophilic substrates of diverse structures. CYPs are

important in oxidative, peroxidative and reductive metabolism of several endogenous compounds and xenobiotics. CYP3A4 plays a major role in intestinal metabolism of various drugs. It has a broad substrate specificity that overlaps with P-gp substrates to a large extent. This combination leads to an effective barrier for various drugs (Doherty and Charman, 2002). In parallel with P-gp, CYP3A4 also is regulated via hPXR (Bertilsson *et al.*, 1998). CYP1A2 metabolises preferentially arylamines and N-heterocyclics and its expression is regulated via AhR (Nebert and Russell, 2002).

Interactions with ABC-transporters or CYPs can lead to drug-drug interactions. Functional inhibition of ABC-transporters or CYPs by curcumin has been shown (Appiah-Opong *et al.*, 2007), (Chearwae *et al.*, 2004). It was further shown that the curcuminoids reduce MDR1 mRNA expression (Limtrakul *et al.*, 2004) and that curcumin increases BCRP mRNA (Ebert *et al.*, 2007).

In this study we investigated the effects of different curcuma extracts and the single curcuminoids on the expression of MDR1, BCRP and CYPs in an intestinal cell culture model. Additionally, effects on gene expression by the curcuminoid content in the extract were compared to the full extract and to pure curcuminoids.

5.1.3 Methods

Materials

Rifampicin and benzpyrene (Sigma Aldrich, Buchs, CH) were dissolved in dimethylsulfoxide (DMSO). Curcumin, demethoxycurcumin and bisdemethoxycurcumin were purchased from Chromadex (Irvine, CA, USA) and dissolved in ethanol. Curcuma sigma (Sigma-Aldrich) (contains about 70% curcumin and 30% demethoxy- and bisdemethoxycurcumin) was dissolved in ethanol. The different curcuma extract were received from Vitaplant AG (Witterswil, CH) and were dissolved in their individual extracting agent.

Cell Culture

LS180 cells were purchased from American Type Culture Collection (Manassas, NE, USA). They were cultured in Dulbecco's modified Eagle's medium with Glutamax-I, supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% sodium pyruvate and 50 µg/ml gentamycin (Invitrogen AG, Basel, Switzerland). Culture conditions were 37°C with humidified air and 5% CO_2. Cells were seeded in 12 well-dishes (3.8 cm^2 per well, BD Falcon AG, Allschwil, Switzerland) 3 days before starting the incubation with curcuma-extracts curcuminoid mixture and the single curcuminoids. Incubation was performed during 72 hours with medium change every 24 hours. Toxicity was tested in advance using sulforhodamine B staining (Skehan *et al.*, 1990). All curcuma-extracts, curcuma sigma and the single curcuminoids were used in a concentration of 10 µg/ml. Rifampicin was used at 10 µM.

Real time RT-PCR: see chapter 2.2

Sequences for primer and probes are listed in **Table 1**

5.1.4 Results

Curcuma Extracts

Curcuma extracts were produced and analysed by Vitaplant AG (Witterswil, CH). Extracting agents were composed of different concentration of ethanol:water. In Table 6 analysis on curcuminoids content is listed. Curcuma mixture was made by mixing single curcuminoids in the concentration of Curcuma 70 extract.

Table 6 Composition of curcuma extracts

	Extracting agent	Curcuminoids Total	Curcumin	Demethoxy-curcumin	Bisdemethoxy-curcumin
Curcuma 30	30% Ethanol	1.07%	0.52%	0.28%	0.27%
Curcuma 50	50% Ethanol	16.93%	8.12%	4.57%	4.24%
Curcuma 70	70% Ethanol	25.85%	15.61%	5.97%	4.27%
Curcuma 90	90% Ethanol	27.77%	16.65%	6.62%	4.50%
Curcuma 100	100% Ethanol	37.52%	22.39%	9.00%	6.13%

MDR1 and BCRP mRNA Expression

The effect of the different curcuma extracts and the single curcuminoids on MDR1 mRNA expression was investigated. LS180 were incubated with the different extracts and the single curcuminoids, rifampicin served as positive control for MDR1 induction. mRNA expression was analysed using real time RT-PCR. In Figure 31 we observed a strong induction of MDR1 by the positive control. No significant effect on MDR1 mRNA expression was observed in cells treated with the different extracts (Figure 31A). Curcumin, bisdemethoxycurcumin and the curcuma 70 mix (curcuminoid-mixture) showed a small but statistically significant downregulation of MDR1 mRNA expression (Figure 31B). BCRP is unfortunately only at very low levels expressed in LS180 cells; therefore, quantification was not possible.

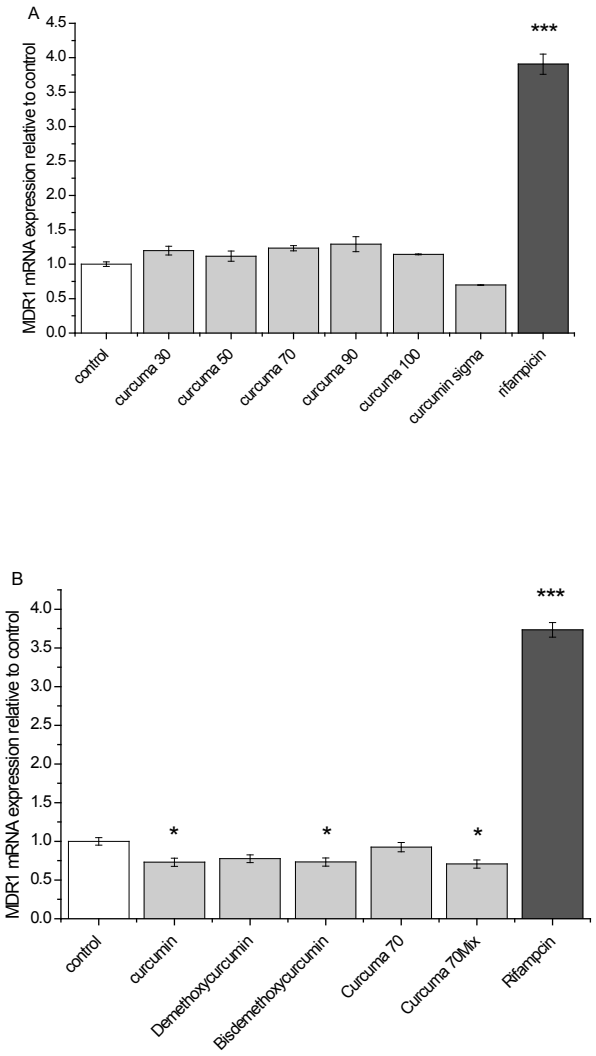

Figure 31 MDR1 mRNA expression in LS180 cells after 72 hours incubation with different curcuma extracts (A) and single curcuminoids (B). Rifampicin 10 µM served as positive control for induction. Data represent mean±s.e.m. (n=3). $^{*}p<0.05$, $^{***}p<0.001$ vs. control

CYP3A4 and CYP1A2 mRNA Expression

The mRNA expression of intestinal CYP3A4 after treatment with curcuma extracts was investigated. We could see no significant effect on intestinal CYP3A4 expression after treatment with curcuma extracts. Rifampicin served as positive control and showed a strong induction (Figure 32). mRNA expression of CYP1A2 after treatment with the extracts or the curcuminoids did not alter significantly (data not shown).

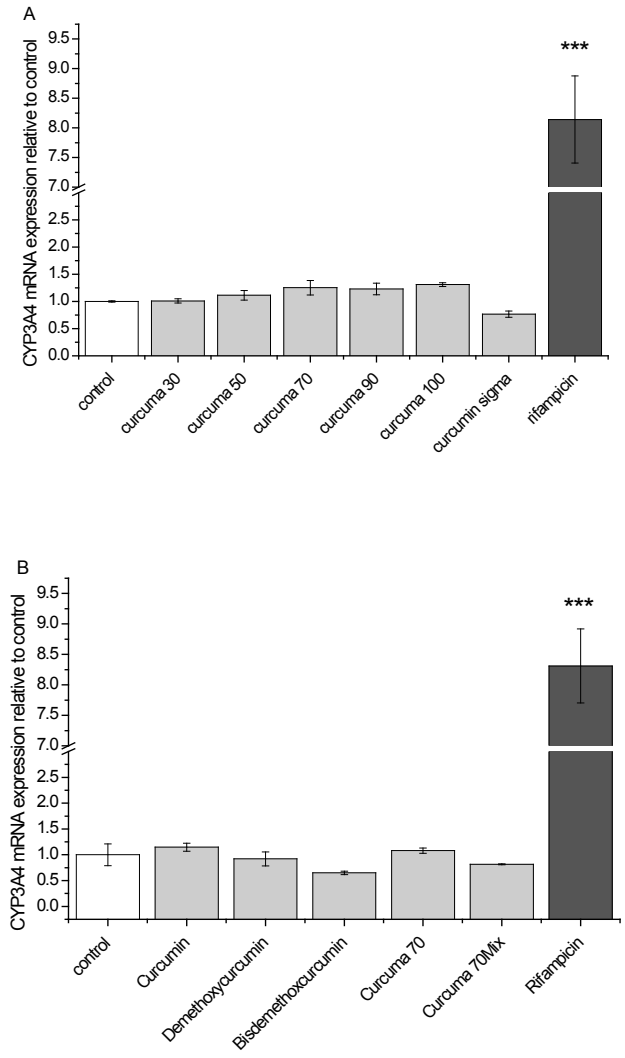

Figure 32 CYP3A4 mRNA expression in LS180 cells after 72 hours incubation with different curcuma extracts (A) and single curcuminoids (B). Rifampicin 10 µM served as positive control for induction. Data represent mean±s.e.m. (n=3). ***p<0.001 vs control.

5.1.5 Discussion

Extracts of curcuma longa L. are a widely used spice. Data with curcumin have been shown that it is hardly absorbed after oral administration and a large amount remains in the intestine (Ravindranath and Chandrasekhara, 1980). Regarding drug-drug interactions in the intestine, it is relevant to investigate whether curcumin has effects on ABC-transporters or CYPs expression. An induction of ABC-transporters and CYPs could decrease bioavailability of their substrates and on the other hand, a downregulation could increase their bioavailability or could impair the

natural barrier function of the intestine. Therefore, we investigated the effect of different curcuma extracts and the single curcuminoids on P-gp, BCRP and CYPs mRNA expression in the intestinal cell model LS180. Our data showed that neither the extracts nor the single curcuminoids had a relevant effect on ABC-transporters and CYPs mRNA expression.

Curcuma extracts containing different determined amounts of curcuminoids did not show the same effect on MDR1 mRNA expression compared to the single curcuminoids. Extracts had no significant effect while the single curcuminoids significantly downregulated MDR1 mRNA expression. This slightly differing result could be due to the concentrations of curcuminoids which are much smaller in the extract than in the single curcuminoids. Therefore, we mimicked one extract by composing a curcuminoid-mixture with the same concentration of curcumin, demethoxycurcumin and bisdemethoxycurcumin as it exists in the extract. Interestingly, the curcuminoid-mixture showed the same small downregulation of MDR1 mRNA expression as the single curcuminoids while the extract showed no alteration. Similar effects were observed in Caco-2 cells by Hou et al. Incubation with curcuma extracts induced MDR1 expression while curcumin showed a slight downregulation (Hou *et al.*, 2008). Further components of curcuma-extracts that are not yet evaluated possibly have also effects on regulation of MDR1 mRNA expression. Other studies with the single curcuminoids showed a downregulation of MDR1 mRNA and protein level with the most powerful effect using bisdemethoxycurcumin (Limtrakul *et al.*, 2004). On functional level, they concluded that curcumin is the most potent inhibitor of P-gp activity (Chearwae *et al.*, 2004).

Results on CYP3A4 mRNA expression revealed a similar pattern like MDR1, but in contrast to MDR1, no significant effects were achieved. Effects of the single curcuminoids on CYP3A4 mRNA expression are not yet described in literature. Hou et al. investigated different curcuma extracts and curcumin in Caco-2 cells and revealed that CYP3A4 activity and protein expression was reduced after 72 hours incubation. In contrast to this finding, they could not see a reduction in CYP3A4 mRNA expression, concluding that the effect on protein level might be due to reduced protein stability (Hou *et al.*, 2007). Another study in the intestinal cell culture model Caco-2 showed that curcumin efficiently inhibited P-gp and CYP3A4 activity (Zhang and Lim, 2008). *In vivo* data in Sprague-Dawley rats revealed that curcumin downregulated intestinal P-gp

and CYP3A4 protein and consequently substrates of P-gp and CYP3A4 reached lower AUC (Zhang *et al.*, 2007).

Taken these results together, changes in mRNA expression of MDR1 and CYPs after incubation with curcuma extracts and single curcuminoids are only small in our intestinal cell culture model. Interactions of curcuminoids and curcuma-extracts with P-gp and CYPs on mRNA expression level are described but further studies are indicated to identify its relevance *in vivo*.

5.2 Anti-inflammatory Effects of Curcuminoids and a Curcuma-extract in Intestinal Cells

5.2.1 Abstract

Background and Purpose: Curcuma was used in traditional Asian medicine as agent against inflammation. Several scientific data also indicate anti-inflammatory effects of the curcuminoids which are the main biological active components. Chronic inflammatory diseases of the intestine could be an indication for treatment with curcuminoids. We therefore investigated anti-inflammatory effects of a curcuma extract, the single curcuminoids curcumin, demethoxycurcumin and bisdemethoxycurcumin, and a curcuminoid mixture in an intestinal cell culture model.

Experimental Approach: LS180 cells were stimulated with TNF-α (tumor necrosis factor) in order to induce inflammatory conditions. Cells were concomitantly treated with a curcuma extract, curcuminoids and a curcuminoid mixture and effects on TNF-α, Il-8 (interleukin-8), iNOS (intrinsic nitric oxide synthase), 5-LOX (5-lipoxygenase), and COX-2 (cyclooxygenase-2) mRNA expression was measured by real-time RT-PCR. Direct inhibitory effects of the curcuma extract on COX-2 activity were measured by detecting synthesis of prostaglandin E2 (PGE2).

Key Results: Stimulation with TNF-α revealed an induction of all investigated pro-inflammatory proteins except for 5-LOX. This induction was significantly abolished for iNOS or COX-2 mRNA expression after treatment with the curcuminoids or curcumin, respectively. In stimulated cells, Il-8 mRNA expression was not altered after treatment with the curcuminoids and the extract. TNF-α mRNA expression did not vary significantly after treatment with the curcuminoids but treatments tended to downregulate the auto-induction by TNF-α. 5-LOX mRNA expression was downregulated after stimulation with TNF-α and treatment with curcuminoids. Additionally to mRNA expression, COX-2 activity was directly inhibited by the curcuma extract.

Conclusions and Implications: Anti-inflammatory effects of curcuminoids were shown by a decrease in mRNA expression of different inflammatory proteins. Direct inhibition of COX-2 by the curcuma extract could be demonstrated. Further studies on protein level and *in vivo* should be performed to verify the anti-inflammatory effects and to gain insight into the possible therapeutic mechanisms.

5.2.2 Introduction

Powdered rhizome of *Curcuma longa L.*, turmeric, is widely used in the food industry as spice or coloring agent. *Curcuma longa L.* grows primarily in southern Asia and therefore it is also used in traditional Chinese and Indian medicine. Turmeric has been used in traditional Asian medicine for treatment of inflammation (Ammon and Wahl, 1991). For many traditional medicines, there is sparse scientific information about its efficacy and security. Recent studies gave attention to curcuma extracts and curcuminoids, which are suggested to be responsible for the biological activity of turmeric. *In vitro* studies have shown that curcumin interacts with several molecular targets such as transcriptional factors (e.g. nuclear-factor-kappa-B (NFκB)), inflammatory cytokines, enzymes (e.g. cyclooxygenase-2 (COX-2), 5-lipoxygenase (5-LOX)). Preclinical studies with curcumin indicate that it has a chemopreventive potential in cancer. Clinical studies in humans show evidence that curcumin is safe and well tolerated. It exerts chemopreventive effects against multiple human cancers and has antioxidant, anti-inflammatory and anti-rheumatic properties (Goel *et al.*, 2008) (Aggarwal et al., 2008) (Aggarwal and Sung, 2009).

Inflammation is a process that involves many complex pathways. One of those pathways is associated with a large number of mediators, which initiate inflammatory response and which recruit and activate other cells to the site of inflammation. Some cytokines, including TNF-α, IL-1, IL-8, IL-12, IFN-γ among others, belong to those mediators. They are regulatory polypeptides and are produced after appropriate stimuli e.g. lipopolysaccharide (LPS) from gram negative bacteria. TNF-α and IL-1β are two cytokines that are involved in the initiation of inflammation These proteins exhibit inductory effects on other cytokines and are able to activate the arachidonic acid pathway. Key-enzymes of the arachidonic acid pathway are cyclooxygenase (COX), which produce prostaglandins (PG) and thromboxanes, and 5-lipoxygenase (5-LOX), which produce leuktrienes (LT). Metabolites of this pathway also participate in the inflammatory process. Effects of prostaglandins are local vasodilatation and sensitising of nociceptors, while leukotrienes lead to an increase in permeability of blood vessels. Several transcription factors such as NF-κB and AP-1 are known to be part of the production of cytokines and arachidonic acid metabolising enzyme COX-2.

Curcumin has been shown to have effects on several of these pro-inflammatory components. *In vitro* studies revealed that curcumin reduces TNF-α and IL-1β production and inhibits NF-κB activation (Chan, 1995). Curcumin has also been reported to reduce expression of inducible nitric oxide synthase (iNOS), an inflammation-induced enzyme that catalyses the production of nitric oxide (NO) (Chan *et al.*, 1998). Other *in vitro* experiments showed that curcumin reduces COX-2 expression and inhibits the catalytic activities of 5-LOX (Lantz *et al.*, 2005), (Hong *et al.*, 2004). Hidaka et al reported that curcumin could inhibit the production of IL-8, which is a chemotactic cytokine and is able to direct leukocyte migration, activate inflammatory responses and help tumour growth (Hidaka *et al.*, 2002).

Bioavailability of curcuminoids is very low; therefore the investigation of local effects of curcuminoids in the intestine is of interest. Ulcerative colitis and Crohn's disease are chronic inflammatory diseases of the intestine. Protective effects after curcumin treatment were observed in mouse models of ulcerative colitis (Salh *et al.*, 2003), (Ukil *et al.*, 2003).

In this study, we investigated effects of the 3 main curcuminoids, curcumin, demethoxycurcumin and bisdemethoxycurcumin, a curcuma extract and a curcuminoid-mixture on defined pro-inflammatory mediators in a human intestinal cell model.

5.2.3 Methods

Materials

Curcumin, demethoxycurcumin and bisdemethoxycurcumin were purchased from Chromadex (Irvine, CA, USA) and dissolved in ethanol. Curcuma 70 extract was produced by Vitaplant AG (Witterswil, CH) and dissolved in its extracting agent (see chapter 5.1.4) Curcuminoid mixture was made by mixing pure curcuminoids in the concentration of the extract (see Table 6). TNF-α was obtained from PeproTech EC Ltd (London, UK).

Cell Culture

The LS180 cell line was purchased from American Type Culture Collection (Manassas, NE, USA). LS180 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with Glutamax-I, supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acids, 1% sodium pyruvate, 50 µg/mol gentamycin (Invitrogen AG, Basel, Switzerland). Cells were seeded into 12-well plastic culture dishes (BD Falcon AG, Allschwil, Switzerland) and were maintained in a humified 37°C incubator with 5% carbon dioxide in air atmosphere. One day before starting incubation with curcuminoids, cells were starved in DMEM containing only 0.25% FBS. The first day of incubation, cells were stimulated with TNF-α (100 ng/ml) in DMEM containing 0.25% FBS. The treatment with curcuminoids, the curcuma 70 extract and the curcuma extract mixture was added to the culture medium concomitantly with the stimulus (TNF-α). After 24 hours, the incubation was continued for another 48 hours without stimulus. In this case, medium and treatment was changed every 24 hours.

Real-time RT-PCR: see chapter 2.2

Sequences for primers and probes are listed in **Table 1**.

COX-2 Assay

Activity of COX-2 enzyme was measured using the prostaglandine E2 –EIA (enzyme immune assay)-kit (Cayman Chemical, Michigan, USA). Curcuma 70 extract was incubated in different concentration for 15 minutes with the assay mixture (0.1 M Tris-HCl, 2 mM Phenol, 5 mM EDTA and Hematin) and COX-2 enzyme. As negative control, a heated sample was used where the enzyme was inactivated. Indomethacin was used as positive control of COX-2 inhibition. To start the reaction, arachidonic acid is added and the mixture is incubated for 3 minutes at room temperature. During this time production of PGE2 takes place. The reaction was stopped by adding acetic acid (1 N) and neutralising with NaOH (1 N) afterwards. The inhibition of COX-2 enzyme is determined by measuring quantitatively PGE2 with ELISA. PGE2 bound with acetylcholine-esterase (AChE) competes with the PGE2 produced by COX-2 for binding at the

PGE2 specific antibody for 24 hours. Afterwards acetylcholine-containing Ellman's reagent was added for 2 hours and absorbance was measured at 415 nm.

5.2.4 Results

mRNA Expression of Inflammatory Proteins

LS180 cells were stimulated with TNF-α to induce inflammation and treated with the 3 curcuminoids curcumin, demethoxycurcumin and bisdemethoxycurcumin, a curcuma extract and a curcuminoid-mixture, which mimics the curcuminoid content of the curcuma extract. mRNA expression of TNF-α, IL-8, iNOS, COX-2 and 5-LOX after 72 hours treatment was analysed using real-time RT-PCR. After the stimulus with TNF-α an induction of mRNA expression of all investigated genes was reached except for 5-LOX, where a downregulation was measured.

TNF-α mRNA expression was induced after stimulation with TNF-α (auto-induction). Treatment with the curcuminoids demethoxycurcumin and bisdemethoxycurcumin reduced this auto-induction about 50% but did not reach significance. Incubation with curcumin showed variable effects in our assays and the relation between stimulated and curcumin treated cells in TNF-α expression level can therefore not be well interpreted. Treatment with the extract or the curcuminoid-mixture did not significantly reduce the auto-induction of TNF-α (Figure 33A).

After treatment with the curcuminoids or the extract, IL-8 mRNA expression did not change significantly compared to the stimulation without treatment. Nearly all treatments investigated lead to a significant induced IL-8 mRNA expression compared to control (Figure 33B).

iNOS mRNA expression was significantly induced after stimulation with TNF-α, but after treatment with the curcuminoids this induction was significantly reduced and reached the level of not-stimulated cells (Figure 33C).

5-LOX mRNA expression was not induced after stimulation with TNF-α, it was even significantly downregulated compared to control. Additional treatment with the curcuminoids or the extract did not change 5-LOX mRNA expression compared to the stimulated cells, all were significantly downregulated compared to control (Figure 33D).

COX-2 mRNA expression was significantly induced in the TNF-α stimulated cells. Additional treatment with curcuminoids could again neutralise this induction down to the level of not-stimulated cells, but significance was only reached with curcumin (Figure 33E).

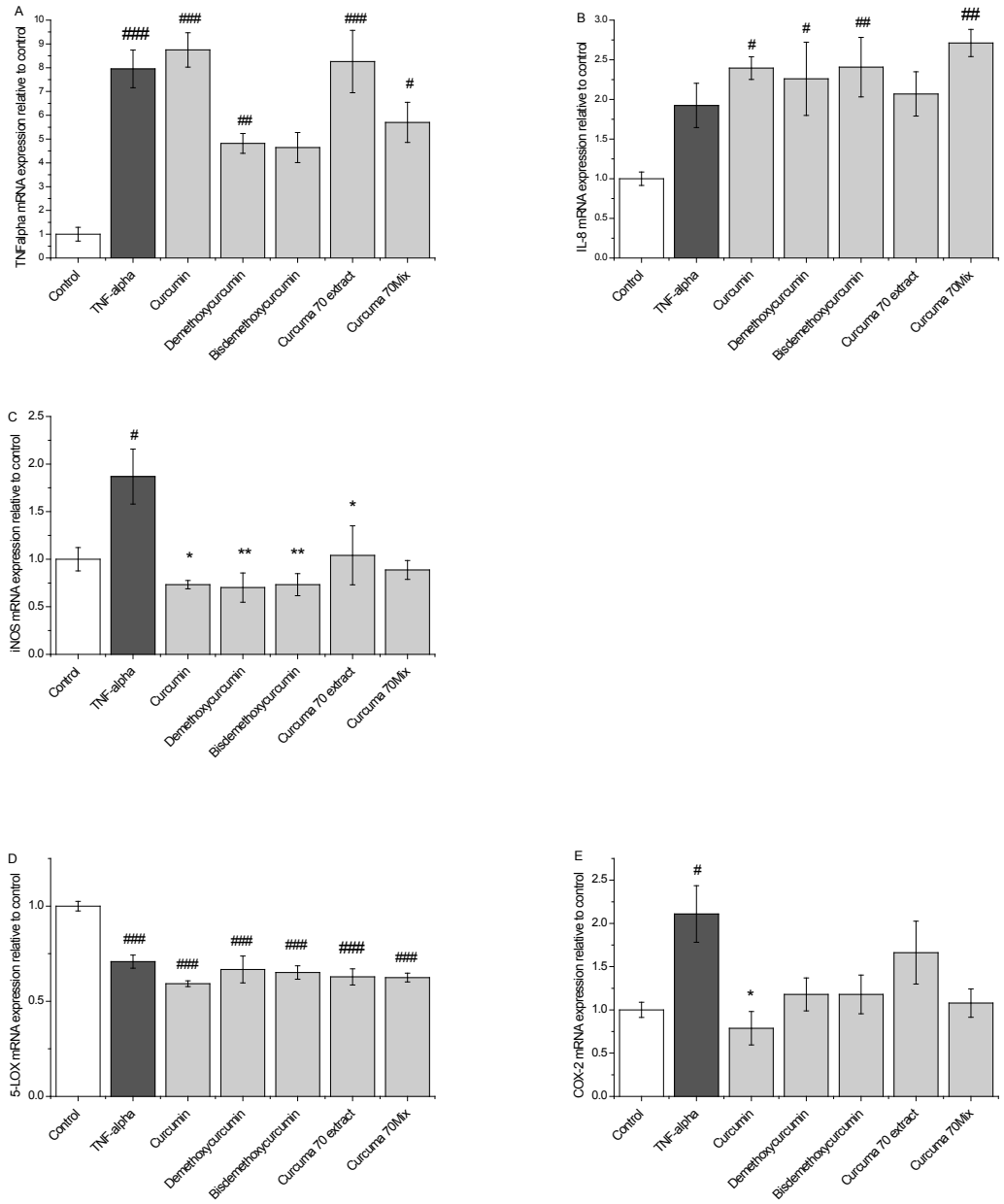

Figure 33 mRNA expression of TNF-α (A), IL-8 (B), iNOS (C), 5-LOX (D) and COX-2 (E) after stimulation with TNF-α and treatment with the indicated substances for 72 hours. Data represent mean ± s.e.m (n=3-6 except Figure 1A: DMC n=2). # $p<0.05$ ## $p<0.01$ ### $p<0.001$ compared to control, * $p<0.05$ ** $p<0.01$ compared to stimulation with TNF-α.

COX-2 Inhibition Assay

Using the PGE2-EIA kit, we measured COX-2 inhibition after addition of different concentration of curcuma 70 extract. COX-2 is responsible for the conversion of arachidonic acid into prostaglandins. A competitive enzyme immunoassay quantitatively gives information about the produced PGE2 and is therefore indicative for the inhibition of COX-2.

Indomethacin, an established COX-2 inhibitor, was used as positive control. The concentration of indomethacin where 50% of total enzyme activity was inhibited (IC_{50}-value) was 3 µM (1, 07 µg/ml). Curcuma 70 extract was measured in different concentrations and its IC_{50} was calculated at 45 µg/ml. (Figure 34)

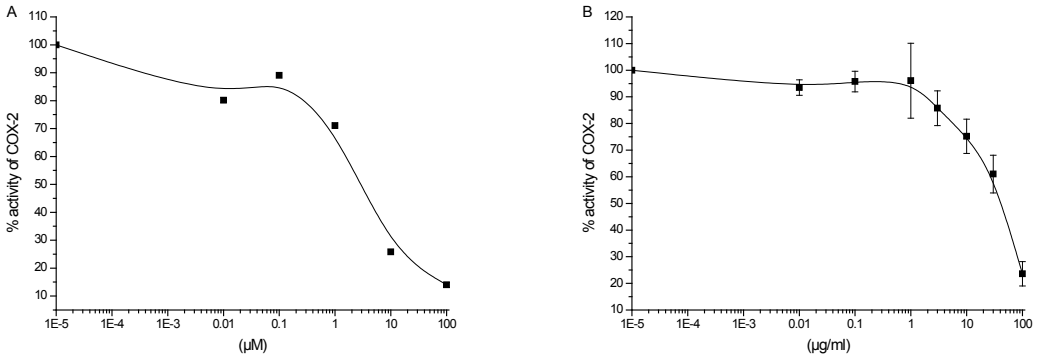

Figure 34 Percentage of COX-2 activity after addition of different concentrations indomethacin (A) and curcuma 70 extract (B). Data of curcuma 70 extract represent mean ± s.e.m (n=3)

5.2.5 Discussion

Powdered rhizome of Curcuma longa L., turmeric, is used in traditional Indian medicine to treat inflammations, wounds and tumours (Ammon and Wahl, 1991). Curcuminoids such as curcumin, demethoxycurcumin and bisdemethoxycurcumin are suggested to be responsible for its biological activities. Curcumin is the best characterised curcuminoid and showed several anti-inflammatory actions *in vitro*. Mechanistic insights into its anti-inflammatory action revealed interactions with the transcriptional factor NFκB and mitogen-activated-protein-kinases

(MAPK), which are responsible for transcription of several inflammatory mediators (Cho *et al.*, 2007).

We investigated anti-inflammatory effects of curcuminoids, a curcuma extract and a curcuminoid-mixture on mRNA expression of TNF-α, IL-8, iNOS, COX-2 and 5-LOX in the intestinal cell culture model LS180.

TNF-α is one of the inflammatory proteins which is responsible for initiation of inflammation and activation of arachidonic acid metabolism. Stimulation of cells with TNF-α led to an increase in TNF-α mRNA expression, which represents initiation of inflammation. Reduction of this induction suggests anti-inflammatory effects. The curcuminoids, the curcuma extract and the curcuminoid-mixture showed a tendency to reduce TNF-α mRNA expression but did not reach significance. Curcumin unfortunately did not show a consistent pattern in our experiments and its effects on TNF-α mRNA expression in LS180 cells has to be evaluated in further experiments. Nevertheless, it was shown in literature that curcumin could decrease TNF-α production and further inhibit nuclear translocation of NFκB (Chan, 1995), (Abe *et al.*, 1999). However, it has to be taken into account, that these data from literature measured TNF-α protein production from monocytes and functional activity of TNF-α while our data analysed the mRNA expression of TNF-α in an intestinal epithelial cell line.

IL-8, a cytokine which is produced by epithelial cells and induces chemotaxis, was also investigated in our cell system. Stimulation of TNF-α induced IL-8 mRNA expression but the curcuminoids and the extract could not reverse this effect. This finding is in accordance with the results of Cho et al. who could not detect an inhibitory effect on IL-8 production with curcumin treatment in TNF-α-stimulated keratinocytes (Cho *et al.*, 2007). However, the results seem to be contradictory to Kim et al. who investigated the effect of curcumin on IL-8 mRNA and protein expression in human umbilical vein endothelial cells (HUVECs). In contrast, they observed a downregulation on TNF-α -induced IL-8 mRNA expression after curcumin treatment (Kim *et al.*, 2007). The differences observed in these studies might be explained by the unequal cell system.

iNOS plays an important role in inflammation and tumour genesis through production of the free radical nitric oxide (NO). iNOS mRNA expression was induced after TNF-α stimulation

and additional treatment with the curcuminoids, the curcuma-extract and the curcuminoid-mixture could neutralise this induction. These data are in agreement with the results of Pan et al., who demonstrated that curcumin inhibits NFκB activation which is essential for iNOS induction (Pan *et al.*, 2000).

Unexpected results were observed at 5-LOX mRNA expression after stimulation with TNF-α. 5-LOX mRNA expression was significantly reduced after stimulation with TNF-α and curcuminoid treatment did not further change 5-LOX mRNA expression. In literature it was shown that addition of TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) to HL-60 cells could induce 5-LOX activity (Steinhilber *et al.*, 1993). For GM-CSF a significant augmentation of 5-LOX mRNA expression was observed 18 hours after incubation in human neutrophils (Stankova *et al.*, 1995). These data show increased 5-LOX activity by stimulation with TNF-α and GM-CSF. Additionally, after stimulation with GM-CSF increased 5-LOX mRNA expression was shown. We measured 5-LOX mRNA expression after stimulation with TNF-α which is not described by Stankova et al. and Steinhilber et al. Nevertheless, it is unclear, why we observed a downregulation in our cell system. A possible explanation why we cannot see an induction of 5-LOX mRNA expression after TNF-α -stimulation could be the short half-life of this protein. It was shown that half-life of 5-LOX in HL-60 is approximately 26 hours (Kargman and Rouzer, 1989). However, the downregulation of 5-LOX after stimulation remains to be elucidated.

COX-2, another enzyme of the arachidonic acid pathway, was clearly upregulated after TNF-α -stimulation and curcumin could significantly neutralise this induction. In addition to this transcriptional data, direct inhibitory effects on COX-2 activity showed that curcuma 70 extract could inhibit PGE2 production. It is reported that curcumin reduces mRNA and protein expression of COX-2 in an intestinal cell model. Following incubation with curcumin a reduced PGE2 production was measured (Goel *et al.*, 2001), (Zhang et al., 1999). Lantz et al. investigated a dichloremethane-methanol (1:1) extract of Curcuma longa L. and analysed its fractions on inhibition of PGE2 and TNF-α production in HL-60 cells. The fraction containing the curcuminoids provoked after 24 hours incubation an IC_{50} value of PGE2 production of 0.9 µg/ml, whereas the fractions containing the turmeric oils reached an IC_{50} of 0.084 µg/ml. Interestingly, curcumin induced a downregulation of COX-2 mRNA expression while turmeric

oils did not (Lantz *et al.*, 2005). These data indicate that besides COX-2 mRNA downregulation, components of the extract probably interact with COX-2 enzyme on another level. Our data show, that the curcuma-extract directly inhibits COX-2 activity. However, the IC_{50} value of PGE2 production was 45 µg/ml for the curcuma extract and is higher compared to indomethacin which achieved an IC_{50} value of 3 µM (1.07 µg/ml). Further inhibition experiments with the single curcuminoids on COX-2 activity are indicated. Analysing the effect of the curcuminoid-mixture, which mimics the curcuminoid content of the extract, on COX-2 activity would give information about the role of curcuminoids in direct COX-2 inhibition.

In conclusion, after stimulation with TNF-α, the curcuminoids could effectively downregulate different cytokines or enzymes taking part in the inflammatory response. The effects of the curcuma-extract were mostly weaker compared to the single curcuminoids. While the extract could directly inhibit COX-2 activity, inhibition by the single curcuminoids has to be analysed in further experiments. The effects found in our *in vitro* system have to be verified in an *in vivo* system to gain further insights into its therapeutic effects in inflammation.

6 Conclusion and Outlook

Drug absorption in the intestine is a fundamental requirement for oral drug therapy. Transport proteins and metabolising enzymes are important determinants of drug bioavailability. Decreased absorption of orally administered drugs due to efflux transport proteins is a well known phenomenon. P-gp and BCRP seem to play an important role since they exhibit high intestinal expression levels and are involved in the efflux transport of many drugs.

In this thesis, the impact of the drug transporters P-gp and BCRP on drug absorption was investigated. We established an intestinal *in vitro* model with selective and concomitant knock-down of P-gp and BCRP. Using this model, we were able to examine substrates of both, P-gp and BCRP, and to evaluate the impact of each transporter on absorption of these substrates. The tyrosine kinase inhibitor imatinib and the anti-retroviral drug abacavir were described as substrates of P-gp and BCRP. The evaluation of drug absorption in our cell system revealed that in the case of these two substrates probably P-gp plays a more important role than BCRP and possibly can compensate drug efflux transport when BCRP is inhibited. However, these results have to be evaluated in further experiments to rule out other interferences which possibly could affect our system.

Beside this inhibition of P-gp or BCRP, induction of these transporters might negatively influence drug absorption. Lower systemic available drug concentration due to higher export transport could be the consequence. Efflux transport was described as a cause for therapy resistance in several diseases such as cancer. Increased P-gp expression in steroid treated IBD-patients was also described and it is discussed whether this could be a possible cause of therapy resistance.

Budesonide, a frequently used glucocorticoid, regulated P-gp expression differently in two common intestinal cell lines LS180 and Caco-2. Induction of P-gp expression was observed in LS180 cells where the nuclear receptor PXR is functional active. Collaborative work revealed that budesonide is a ligand of PXR and has the potential to induce PXR target genes such as P-gp and CYP3A4 via this pathway. *In vivo* data in mice, however, showed only modest gene induction,

which mainly occurred in the intestine. These data indicate that budesonide has the potential to induce PXR-target genes in the intestine, but only to a slight extent. Therefore, risk for drug-interactions due to budesonide seems to be low but cannot be ruled out completely.

The well established intestinal cell line Caco-2 exhibits properties of small intestinal epithelium but lacks functional PXR expression. Therefore, we stably transfected PXR into Caco-2 cells. In our cell system, P-gp expression was constitutively increased and could be induced by the PXR ligand rifampicin. Since induction effects were not as pronounced as in LS180 cells, further evaluations are needed. However, after further evaluation, this system could serve as an *in vitro* intestinal cell culture model with combined properties for intestinal transport and induction studies.

Curcuma longa L. is a widely used spice and the curcuminoids, which account for its main constituents, are being investigated for treatment of cancer, inflammation and other diseases. The curcuma extracts and the curcuminoids did not change the expression of P-gp and CYP to a major extent. Thus, a curcuma-mediated risk for drug-interactions due to transcriptional regulation of ABC-transporters and CYPs seems to be low but has to be further evaluated *in vivo*. A downregulation of several TNF-α induced pro-inflammatory proteins after treatment with curcuminoids or curcuma extract suggest anti-inflammatory effects in the intestinal cell line LS180. Further investigations on anti-inflammatory effects in the intestine have to be performed *in vivo*. The efficacy of the curcuminoids or the curcuma extract in treatment of inflammatory diseases in the intestine remains to be elucidated.

In this thesis we provided data on transcriptional regulation and impact of ABC-transporters in intestinal cell lines. Newly generated Caco-2 clones with a specific knock-down of P-gp or BCRP mRNA expression were used to get knowledge of the impact of each transporter on transport of two different drugs. Topical effective anti-inflammatory drugs such as budesonide and curcuminoids were analysed on transcriptional level to investigate the interaction potential of ABC-transporters and CYPs. PXR was stably transfected into Caco-2 cells in order to have a tool for transcriptional interaction studies of P-gp and CYPs. However, our cell systems need further evaluations, nevertheless as a future perspective, screening of new drugs on transcriptional regulation and evaluations of the impact of P-gp- and BCRP-transport could be performed.

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