Expression of drug transporters in intestine and blood

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Abbreviations

A	apical
ABC	ATP binding cassette
ANOVA	analysis of variance
ASBT	apical sodium-dependent bile salt transporter
ATP	adenosine-5`-triphosphate
B	basolateral
BCRP	breast cancer resistance protein
BLAST	basic local alignment search tool
BSEP	bile salt export pump
bp	base pairs
Caco-2	human colon carcinoma cell line
CAR	constitutive androstane receptor
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CMFDA	5-chloromethylfluorescein diacetate
cMOAT	canalicular multispecific organic anion transporter
Ct	cycle threshold
CYP450	cytochrome P450
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNP-SG	2,4-dinitrophenyl-S-glutathione
ET-1	endothelin (isoform 1)
FCS	(heat inactivated) foetal calf serum
FD-4	FITC Dextran (MW 4000)
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GSH	reduced glutathione
HBSS	Hank´s balanced salt solution
HIV	human immunodeficiency virus
HPLC	high performance liquid chromatography
HUGO	human genome organisation
IBD	inflammatory bowel disease

kDa	kilodalton
LDL	low-density lipoprotein
LS180	human colon carcinoma cell line type LS180
MDCK	Madin-Darby Canine kidney
MDR	multi-drug resistance
MEM	minimum essential medium
MF-SG	methylfluorescein-glutathione
MMC	mitomycin C
mRNA	messenger ribonucleic acid
MRP	multi-drug resistance associated protein
MTX	methotrexate
OAT	organic anion transporter
OATP	organic anion transporting polypeptide
OCT	organic cation transporter
P(app)	apparent permeability coefficient
PCR	polymerase chain reaction
PGE2	prostaglandin E2
P-gp	P-glycoprotein
PMEA	9- [2-(phosphonomethoxy)ethyl] adenine (=adefovir)
PXR	pregnane X receptor
R123	rhodamine 123
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
SEM	standard error of the mean
SLC	solute carrier
SN-38	active metabolite of irinotecan
SNP	single nucleotide polymorphism
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride

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Summary

Proteins that are capable to transport molecules across membranes are fundamental for the accurate functioning of the body. Many diseases have their cause in a dysfunction of a particular transport protein. Membrane transporters are involved in the absorption, distribution, metabolism, and excretion of endogenous and ingested substances, but for numerous transport proteins their substrates and physiological roles are still unknown or hypothetical.

Most transporters exhibit a high specificity for their natural substrates. However, some transporters show broad substrate specificity, thereby translocating a large variety of substances including drugs. Consequently, the expression of so-called *drug transporters* can influence the pharmacokinetics of administered drugs by controlling their oral absorption, their distribution within the body, and their elimination through excretory organs. Furthermore, over-expression of particular drug transporters can lead to a decreased drug bioavailability. The reduced drug concentrations in blood and in tissues can even result in a phenotype of drug resistance. This phenomenon is often observed in patients with cancers. However, therapy resistance is also a well-known problem in other diseases such as inflammatory bowel disease (IBD). Approximately 50% of patients with Crohn`s disease and 20% of patients with ulcerative colitis require other therapeutic strategies due to inefficient steroid treatment. Many of these patients need surgery as a result of therapy resistance. But the underlying mechanisms of therapy resistance in IBD patients are poorly understood.

The aim of this thesis was to assess the general expression of transporters in humans. The main focus was the intestine as an important site of drug absorption. Furthermore, *in vitro* experiments using intestinal cell lines were performed to evaluate alterations in transporter expression by drugs and endogenous compounds. This knowledge can help to assess the impact of these transporters on 1) the oral bioavailability of drugs, 2) therapy resistance, 3) possible drug-drug interactions.

Initially, a method was developed to accurately quantify the expression of transporters using real-time PCR (TaqMan® analysis, chapter 2). Thus, a standard for each gene of interest was synthesized and quantified in order to compose standard curves with known amounts of PCR templates. Consequently, for each transporter the gene-expression could be expressed as

absolute mRNA transcript number. This method was used in all projects where mRNA expressions were analyzed.

The general expression of drug transporter mRNA along the human intestinal tract was studied in biopsies from 10-14 healthy volunteers (chapter 3). Biopsies were taken from the duodenum, the terminal ileum, and from the proximal to the distal colon (ascending, transverse, descending, and sigmoid colon). Site-specific mRNA expressions for MDR1 and MRP1-5 (chapter 3.1), BCRP (chapter 3.2), and ASBT (chapter 3.3) were shown. These data can be useful in developing new targeting strategies for enteral drug delivery. Additionally, the transporter expression obtained in these healthy control patients can be compared with the transporter expression in IBD patients in further studies. This might help to elucidate the role of transporters in IBD.

Using *in vitro* experiments, we investigated whether budesonide, an often-used glucocorticoid in patients with IBD, might affect the expression of drug transporters (chapter 4.1). A selective induction of MDR1 on mRNA and protein level was detected in a human intestinal cell line. Since budesonide is also a P-gp substrate, this induction might be one reason for the steroid resistance that is often observed in IBD patients treated with glucocorticoids.

Thalidomide is an “old” drug that is increasingly used as an adjuvant therapy in malignant and inflammatory diseases, including IBD. Therefore, this drug was screened for possible interactions with P-gp (chapter 4.2) and MRP2 (chapter 4.3) by performing induction-, inhibition-, and transport-assays. Thalidomide showed no potential for interactions regarding these two drug-efflux transporters.
Furthermore, a HPLC method for the determination of thalidomide enantiomers in blood was developed (chapter 5). This sensitive method can be applied in prospective clinical trials where the efficacy of thalidomide is further investigated.

In a study, including vasospastic persons with increased Endothelin-1 plasma levels, the expression of MDR1 and MRP1-5 in isolated blood mononuclear cells was determined (chapter 6). Vasospastic persons differed from healthy controls in their expression pattern of transporter proteins. They showed a significant decrease in their expression of MDR1, MRP2, and MRP5 mRNA when compared to controls. This might be an indirect effect of elevated ET-1 levels and this could explain the enhanced drug-sensitivity reported by these patients.

In a further project, the release of mitomycin C from collagen implants was determined using a newly developed HPLC method (chapter 7). In this study it was clearly shown that commercially available collagen implants could be loaded with MMC, and could subsequently release it. The pharmacokinetics of this relationship is determined *in vitro*.

Aim of the thesis

Our extensive goal is to reveal whether drug transporters are involved in therapy resistance observed in patients with inflammatory bowel disease. The contribution of the present thesis to that issue was the determination of the general expression of transporters along the intestinal tract. By providing data from healthy control subjects we will be able to conduct further studies including IBD patients. Moreover these data can elucidate the role of intestinal transporters in general drug absorption. Additionally, we intended to investigate the impact of IBD drugs on transporter expression and function using *in vitro* experiments. Results from intestinal cell lines should give indications about alterations of transporters during drug treatment. Overall, this knowledge will help to characterise the role of these transporters with regard to 1) oral bioavailability of drugs, 2) therapy resistance, and 3) possible drug-drug interactions.

In particular, we aimed to elaborate the following points:

- Establishment of a method for the absolute quantification of mRNA transcript abundances.
- Determination of the general drug transporter expression along the human intestine.
- Influence of IBD drugs on transporter expressions *in vitro*.
- Investigation of the absorption of thalidomide and interactions with P-gp and MRP2.
- Establishment of a HPLC method for the determination of thalidomide enantiomers in blood samples.

Beside our focus on IBD, we planned additional projects in collaboration with the University Eye Clinic of Basel. The following topics were deliberated:

- Analysis of drug transporter expression in vasospastic subjects.
- Determination of the *in vitro* release of mitomycin C from collagen implants.

1. Introduction

1.1 Drug absorption and transport proteins in the gastrointestinal tract

The gut represents an important interface between the organism and the external environment. Therefore, the gastrointestinal mucosa consists of special epithelial layers that display various features. They aid the digestion of food, absorb nutrients, export waste components and act as a selective barrier to protect the body from pathogens. Transcellular and paracellular fluxes are controlled by membrane pumps, ion channels and tight junctions, adapting permeability to physiological needs (Baumgart and Dignass, 2002). Due to the fact that the intestine is heavily colonized with bacteria, the epithelium plays a very active role in protecting the host from invading and damaging bacteria and endotoxin (Ding and Li, 2004).

For the majority of orally administered drugs, the small intestine represents the primary site of absorption. The most common mode of plasma membrane penetration is passive diffusion along a concentration gradient. However, many drugs exhibit a poor absorption after oral administration. Beside the physicochemical properties that influence oral bioavailability of drugs, poor absorption has also been attributed to metabolism and active efflux in the small intestine (Suzuki and Sugiyama, 2000). The enterocytes form a barrier to drug entry that exploits both drug-metabolising enzymes, e.g. the cytochrome P450 family, and drug export pumps, e.g. P-glycoprotein. A synergistic role of the cytochrome isoform 3A4 (CYP3A4) and P-glycoprotein has been suggested due to several facts: their coexistence in the intestine, the significant overlap of their substrate specificity and the poor bioavailability of substances that are substrates of both proteins (Zhang and Yuan, 2001). In addition to oxidative metabolism and drug efflux, conjugation reactions play an important role in the detoxification of xenobiotics in the small intestine (Lin et al., 1999a). After being conjugated (e.g. to glutathione-, or glucuronide-conjugates) these compounds can be excreted into the lumen by transporters with an affinity to organic anions such as the multi-drug resistance associated proteins (MRPs).

In general, transport proteins mediate the translocation of specific molecules across various membranes. Dependent on their local expression they control the absorption, distribution and excretion of endogenous compounds and exogenous xenobiotics in the organism. The translocation of their substrates can be either primary active using ATP hydrolysis as an energy source or secondary active using an existing cellular electrochemical gradient. Examples are the ATP-binding cassette transporters (ABC-transporters) or the solute carrier (SLC), respectively.

Numerous members of different transporter families are involved in the cellular uptake and efflux of drugs. Therefore, pharmacokinetics can be strongly influenced by the expression of these transporters, particularly in excretory organs (liver, kidney) and protective barriers (intestine, blood brain barrier). Consequently, both the knowledge of the accurate transporter expression in tissues and the knowledge whether drugs are substrates, inhibitors or inducers of individual transport proteins is of importance. This holds true especially during pharmacological development of novel classes of therapeutic compounds and in the development of new targeting strategies for drug delivery. As a result, potential drug-drug interactions could be prevented and thereby drug safety could be improved.

1.2 ABC transporters

The ATP-binding cassette (ABC) transporters are a large and diverse superfamily of proteins comprising around fifty members with many and varied functions. They share extensive sequence homology and domain organisation including the characteristic ATP-binding cassette, consisting of two nucleotide-binding domains. They are classified into seven subfamilies (ABCA to ABCG). In this thesis the family members that are known to mediate drug transport were of particular interest, since these proteins can have a major impact on drug disposition. All of these are located in the plasma membrane where they can extrude a variety of structurally diverse drugs, drug conjugates and metabolites. Export of these compounds occurs in an active, ATP-dependent manner, and can take place against considerable concentration gradients.

The first member discovered in 1976 (Juliano and Ling, 1976) was P-glycoprotein (MDR1, ABCC1). This protein appeared to be overexpressed in tumor cells with a multi-drug resistance phenotype where it conferred resistance to many unrelated cytotoxic drugs. Later the existence of the multi-drug resistance associated proteins (MRPs, ABCC) was revealed. Some of these transporters are also relevant for drug transport, as well as the recently discovered ABC transporter breast cancer resistance protein (BCRP, ABCG2). Although many ABC transporters have been identified as drug-resistance proteins in cancer therapy, they are all expressed in normal tissues transporting endogenous substrates or protecting the organism from natural cytotoxins (Gottesman et al., 2002).

1.2.1 MDR1 (ABCB1)

P-glycoprotein (P-gp), the gene product of MDR1, is possibly the best-studied ABC drug efflux transporter to date. The protein has a molecular weight of 170 kDa and 12 transmembrane domains and two nucleotide-binding sites. P-gp is a transporter with extreme wide substrate

specificity and therefore many unrelated substances were identified as P-gp substrates. However, a tendency towards organic compounds with cationic or amphiphatic nature could be determined (Schinkel and Jonker, 2003). P-gp is assumed to represent a protective mechanism against potentially toxic xenobiotics. The high expression in solid tumours indicates the pivotal role of P-gp in resistance to anticancer therapy. But its general expression in the apical membrane of normal tissues such as intestine, kidney, liver, and blood-brain barrier is also of great significance due to its excretory and barrier role. There, P-gp mediates the efflux of xenobiotics and toxins into the intestinal lumen, urine, bile, and blood.

Drug-drug interactions may occur when the activity of the transporter is altered by one drug resulting in a change of the clearance of another drug that is a P-gp substrate. It has been reported that P-gp inhibitors such as verapamil, itraconazole, ritonavir, and talinolol increased the plasma concentrations of the P-gp substrate digoxin due to inhibition of P-gp mediated efflux (Verschraagen et al., 1999; Westphal et al., 2000a; Angirasa and Koch, 2002; Ding et al., 2004). Additionally, P-gp has also been shown to be inducible *in vitro* and *in vivo* by xenobiotics such as rifampicin (Westphal et al., 2000b), phenobarbital (Lu et al., 2004), dexamethasone (Fardel et al., 1993), and herbal extracts from St. John's wort (Zhou et al., 2004). Increased P-gp expression can therefore lead to subtherapeutic concentrations of concomitantly administered substrates. Moreover, genetic variants (single nucleotide polymorphisms, SNPs) can alter P-gp expression and function. To date 28 SNPs have been identified on the MDR1 gene, whereas 11 SNPs resulted in an amino acid exchange (Schwab et al., 2003a). So far, only the C3435T polymorphism, which does not influence the amino acid sequence, was associated with an altered P-gp expression and function. On average, the TT homozygotes have a lower level of intestinal P-gp resulting in an increase of digoxin plasma levels, compared to the CC genotype group (Hoffmeyer et al., 2000). C3435T is also reported to be a risk factor for certain class of diseases including inflammatory bowel disease, Parkinson's disease and renal epithelial tumour (Sakaeda et al., 2004).

1.2.2 ABCB4 (MDR3)

A cDNA corresponding to a further human P-glycoprotein gene was found in liver (Van der Bliek et al., 1987). The protein has 80% amino acid homology with the product of the human MDR1 gene. It was designated MDR3 since it corresponds to the third of the P-glycoprotein genes mapped in hamster. MDR3 is mainly expressed in the bile canalicular membrane of the liver, but is also found in the heart, muscle and in B cells. The murine mdr2 gene (homolog of human MDR3) was found to function as a lipid translocase or flippase. It was demonstrated that mdr2

expression caused an enhancement of phosphatidylcholine translocation (Ruetz and Gros, 1994). Biliary secretion of lipids is an important physiological event; not only for the disposal of cholesterol from the body, but also for the protection of cells lining the biliary tree against bile salts (Elferink and Groen, 1999). Patients with progressive familial intrahepatic cholestasis (PFIC) type 3 have a mutation in the MDR3 gene, which leads to a disruption of biliary lipid secretion (de Vree et al., 1998). Recent work has shown that MDR3 has significant drug transport activity and that this transport is inhibited by MDR1 inhibitors (Smith et al., 2000). Whether MDR3 also functions *in vivo* as a transporter of some drugs remains to be seen (Borst et al., 2000).

1.2.3 ABCC1-6 (MRP1-6)

The multi-drug resistance associated proteins (MRPs) are a further important group of human ABC transporters that are relevant for drug transport. All of them possess the characteristic ATP-binding cassette motive but they vary in the number of their transmembrane domains. So far, this subfamily includes nine members (MRP1-9). In contrast to P-gp, MRPs work mainly as transporters of amphiphatic organic anions. Therefore, they are capable to extrude drug conjugates, such as glucuronide-, glutathione-, and sulphate-conjugates out of cells.

MRP1 (ABCC1) is ubiquitously expressed in the body. It is localised on the basolateral membrane of epithelial cell layers, and its substrates are therefore transported towards the basolateral side. Physiological important substrates for MRP1 include glutathione S-conjugates such as leukotriene C4, as well as bilirubin glucuronides (Keppler et al., 1998). In addition, anionic drugs and drugs conjugated to glutathione like methotrexate or arsenite are also transported by MRP1 (Bakos et al., 2000; Vernhet et al., 2000).

MRP2 (ABCC2) is localised in the apical membrane of cells from liver, intestine, and kidney where it plays a central role in detoxification by secreting metabolites into bile, intestinal lumen and urine (Schaub et al., 1997; Fromm et al., 2000). The substrate specificity of MRP2 is similar to that of MRP1, and includes glutathion conjugates, billirubin glucuronides, and a number of drugs and their conjugated drug metabolites (Jedlitschky et al., 1997; Kawabe et al., 1999). These drugs include temocaprilat, irinotecan, SN-38, arsenite, cisplatin, methotrexate, vincristine, saquinavir, and ceftriaxone (Kusuhara and Sugiyama, 2002; Dietrich et al., 2003). Patients with Dubin-Johnson syndrome have a fully deficient MRP2 gene. The absence of this transporter in the hepatocyte canicular membrane leads to impaired biliary secretion of glutathione, glutathione

conjugates, and bilirubin glucuronides (Paulusma et al., 1997). Similar to MDR1, MRP2 seems to be inducible by rifampicin treatment (Fromm et al., 2000).

MRP3 (ABCC3), like MRP1, is present in the basolateral membrane of polarized cells, mainly in liver, intestine and kidney (Scheffer et al., 2002b). MRP3 transports a wide range of bile salts and seems to be involved in their reabsorption (Hirohashi et al., 2000). MRP3-transfection of cell lines conferred resistance to epipodophyllotoxins, vincristine and methotrexate (Kool et al., 1999). Therefore, MRP3 may also contribute to a toxicological defence function by excreting a range of toxic substances from various epithelial cell types.

For **MRP4** (ABCC4), there are no definite data concerning cellular localization or tissue distribution. For instance, it has been reported that MRP4 is located on the basolateral membrane of prostate cells (Lee et al., 2000), whereas another study showed MRP4 expression on the apical membrane of kidney cells (van Aubel et al., 2002). The significance of MRP4 in drug transport is at present unclear as well. However, an over-expression of MRP4 severely impaired the antiviral efficacy of adefovir, azidothymidine and of other nucleoside analogs in cell lines (Schuetz et al., 1999). Other substrates include folic acid, bile acids, methotrexate and 6-mercaptopurine (Wielinga et al., 2002; Chan et al., 2004). A physiological role of MRP4 might be the release of prostaglandins from cells (Reid et al., 2003).

MRP5 is widely expressed throughout most tissues. Like MRP4, it has an affinity to nucleotide-based substrates. A study demonstrated that MRP5 transports the cyclic nucleotides cAMP and cGMP (Jedlitschky et al., 2000), but the physiological function of this transporter remains to be elucidated. There are no reports at present, which could suggest a role for MRP5 in drug disposition. Experiments with transfected cells showed enhanced efflux of DNP-SG (2,4-dinitrophenyl-S-glutathione), adefovir, and the purine analogues 6-mercaptopurine and thioguanine (Wijnholds et al., 2000).

MRP6 (ABCC6) expression occurs mainly in kidney, liver and to a lower extends in several other tissues (Scheffer et al., 2002a). Analysis of the drug sensitivity of MRP6-transfected cells revealed low levels of resistance to several natural product agents, including etoposide, teniposide, doxorubicin, and daunorubicin (Belinsky et al., 2002). Mutations in the MRP6 gene are the genetic basis of pseudoxanthoma elasticum, a disease that affects elastin fibers in the skin, retina, and

blood vessels (Ringpfeil et al., 2000). However, both the physiological function and potential involvement of MRP6 in drug resistance are still unclear.

1.2.4 ABCG2 (BCRP)

Human breast cancer resistance protein (BCRP, ABCG2) belongs to the ABC drug efflux transporters and was first discovered in breast cancer cells (Doyle et al., 1998). It can render tumor cells resistant to the anticancer drugs topotecan, mitoxantrone, doxorubicin, and daunorubicin (Jonker et al., 2000). Structurally, BCRP is a half-transporter (one nucleotide-binding domain, 6 transmembrane domains) and it seems very likely that it functions as a homodimer (Ozvegy et al., 2001). Whether BCRP can also function as a heterodimer with other half-transporters of the ABCG class is not known. In humans, BCRP is expressed in placenta, breast, ovary, intestine, and liver. BCRP mediates apically directed drug transport, appears to reduce drug bioavailability, and protects fetuses against drugs (Jonker et al., 2000).

1.3 Solute carrier (SLC)

The SLC (Solute Carrier) series includes ion coupled transporters, facilitated transportes, and exchangers. The genes encoding these transporters are divided into 43 gene families (SLC1-43, according to the HUGO Gene Nomenclature Committee) and include 298 transporter genes at present (Hediger et al., 2004). These SLC membrane proteins use cellular chemical and/or electrical gradients to move molecules across cell membranes, whereas Na^+ is the favoured cation to move solutes into cells and anion exchange moves solutes out of cells. Physiologically, they transport many endogenous substances such as amino acids, glucose, bicarbonate, bile acids, ascorbic acid, urea or fatty acids. However, members of this superfamily can also be involved in drug transport and play a role in drug disposition. Many of them are expressed in important organs for drug disposition such as kidney, liver, and intestine. Relevant transporters are members of the organic anion transportes (OATs), the organic anion transporting proteins (OATPs), the organic cation transporters (OCTs, OCTNs), and the concentrative nucleoside transporters (CNTs). In this thesis we also investigated the expression and regulation of the apical sodium dependent bile acid transporter (ASBT, SLC10A2). This transporter, mainly located in the ileum, is responsible for bile acid uptake and therefore contributes substantially to the enterohepatic recycling of bile salts.

1.4 Regulation of transporter expression by nuclear receptors

Nuclear receptors such as the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) play important roles in protecting the body against toxic xenobiotics. They act as activators of detoxifying proteins (e.g. cytochrome P450 enzymes or transporters). The mechanism initially involves an interaction between the receptor and a specific ligand (Kliewer et al., 1999). Ligand binding induces a conformational change within the receptor that facilitates binding of co-activator proteins (e.g. RXR). This heterodimer regulates the transcription of the target gene by binding to specific DNA response elements (Renaud and Moras, 2000).

PXR and CAR are highly expressed in liver and intestine, where they can be activated by a broad spectrum of lipophilic xenobiotics that include drugs such as rifampicin, dexamethasone, phenobarbital, troglitazone, and St. John's wort (Jones et al., 2000; Kullak-Ublick and Becker, 2003). PXR and CAR stimulate the expression of similar sets of genes including those encoding phase I and II enzymes and transporters, which are collectively involved in the metabolism and excretion of lipophilic substances from the body. PXR and CAR are thus important "xenosensors" that mediate drug-induced activation of the detoxifying transport and enzyme systems in liver and intestine (Kullak-Ublick and Becker, 2003). Transporters activated by these receptors include P-gp, MRP2, MRP3, and OATP2 (Kast et al., 2002; Staudinger et al., 2003; Wang and LeCluyse, 2003).

Although PXR and CAR protect the body from xenobiotics, their activation by drugs represents the molecular basis for an important class of drug-drug interactions. Assays that detect PXR activation during drug development are used to predict and prevent these drug-drug interactions (Moore and Kliewer, 2000). Concerning transporters, most cases of such interactions are related to elevated P-gp expressions that lead to a decrease of the plasma level of concomitantly administered P-gp substrates. It was demonstrated that paclitaxel activates PXR leading to enhanced P-gp mediated drug clearance. In contrast, docetaxel did not activate PXR and displayed superior pharmacokinetic properties. Moreover, ET-743, another potent antineoplastic agent, suppressed MDR1 transcription by acting as an inhibitor of PXR (Synold et al., 2001). These findings demonstrate how the molecular activities of nuclear receptors can control drug clearance.

1.5 Inflammatory bowel disease (IBD) and transporters

Crohn's disease and ulcerative colitis, summarised as inflammatory bowel disease (IBD), are states of chronic intestinal inflammation, whereas the inflammation occurs in different parts of the gut. The incidence is 6-11 cases per 100000 people per year (Neurath and Schurmann, 2000). The pathogenesis of both diseases is still unclear. However, a loss of tolerance against the enteric flora and a generalized enhanced reactivity against intestinal bacterial antigens may be the central events in IBD (Wen and Fiocchi, 2004).

In the colon of patients with ulcerative colitis, Langmann and co-workers showed a down-regulated expression of genes involved in intestinal detoxification, including MDR1 and pregnane X receptor (PXR) (Langmann et al., 2004). Furthermore, mice deficient for mdr1a spontaneously develop colitis similar to IBD in humans (Panwala et al., 1998). Schwab and co-workers revealed that a mutation in the MDR1 gene is associated with the susceptibility for IBD in humans (Schwab et al., 2003b). This single nucleotide polymorphism (C3435T) leads to a decreased intestinal P-gp expression in patients with the TT genotype (Hoffmeyer et al., 2000). These are convincing arguments for the role of transporters in the development of IBD by a defective detoxification.

Nonetheless, another great problem concerning IBD is therapy failure. Many patients with IBD experience steroid dependence or steroid resistance. A study showed that elevated expression of P-glycoprotein (MDR1) in enterocytes and in peripheral blood lymphocytes is associated with poor response to medical therapy (Farrell et al., 2000).

In conclusion, the results from recent studies indicate that transporters, especially P-gp, could be involved in the events of inflammatory bowel disease. P-gp expression, however, seems to have a different impact on development and therapeutic efficacy of the disease.

2. Absolute quantification of transporter mRNA expression (quantitative real-time RT-PCR)

2.1 Introduction

Cellular alterations of gene expression can be analysed on different levels. Changes of the mRNA level, the protein level, and protein function can be a measure for a regulated gene expression. Reverse transcription polymerase chain reaction (RT-PCR) is the most common method for analysing mRNA expression patterns and comparing mRNA levels in different samples. In this thesis, several studies were performed where gene expressions in human tissues or cell lines were measured using real time RT-PCR (TaqMan®). By using real-time RT-PCR the specific products generated during each cycle of the PCR can be reliably measured and these are directly proportionate to the amount of template prior to the start of the PCR process. But before real-time PCR amplification could be performed, the isolated cellular mRNA had to be reverse transcribed into cDNA. The cDNA was subsequently quantified with TaqMan® analysis using the standard curve method. Therefore we used external standards that comprised known amounts of specific cDNA fragments of the gene of interest. Consequently, the unknown amount of cDNA in the analysed samples could be expressed as absolute transcript numbers of the corresponding gene.

2.2 Generation of cDNA standards for absolute mRNA quantification

In order to generate standard curves we used gene-specific cDNA fragments with known concentrations as standards. These standards serve as a template during the real-time PCR because they cover the TaqMan primer/probe area and therefore they are amplified similar to the cellular reverse transcribed mRNA of the appropriate gene. Standards were obtained by classical PCR amplification using primers that anneal outside the area where the TaqMan primers anneal on the template. Since MDR1, MRP1-5, Villin, and ASBT are expressed in Caco-2 cells and BCRP is expressed in BB19 cells, we used reverse transcribed RNA of these cell lines as a template for classical PCR amplification. For the gene-specific PCR we used 25 ng cDNA per 25µL reaction volume including each primer at a concentration of 300 nM. The primers (Table 2.1) were designed using the primer express software 2.0 (Applied Biosystems) and were manufactured by Invitrogen (Basel, Switzerland). The components of the PCR reaction (AmpliTaq Gold, 10x PCR buffer, dNTPs, $MgCl_2$) were purchased form Applied Biosystems. Thermal cycling was conducted using a Mastercycler personal from Eppendorf (Hamburg, Germany) and an annealing temperature of 55°C was used. The PCR products (Table 2.2) were purified by running

a 1.5% agarose gel (TAE buffer, 100V, 50 min) and by a subsequent gel extraction (gel extraction kit, Qiagen). When cDNA yields were too low the PCR amplification was repeated using the purified product of the first PCR as a template.

The obtained standards were quantified using the PicoGreen® dsDNA quantitation kit according to the manufactureres protocol (Molecular Probes, Eugene, OR). The PicoGreen® reagent is an ultrasensitive fluorescent nucleic acid stain for quantitating double-stranded DNA using bacteriophage lambda DNA as a standard. The amount of cDNA in the sample was expressed as ng DNA per mL.

Additionally, the purified and quantified PCR products were analysed by sequencing (Microsynth GmbH, Balgach, Switzerland). The received sequences were aligned to the genes of interest using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) in order to confirm the identity of the PCR products. For further calculations the molecular weights of the cDNA fragments (Table 2.2) were determined on the basis of the corresponding sequence with the help of a biopolymer calculator (http://paris.chem.yale.edu/extinct.frames.html).

primer	sequence	start	length	Tm
MDR1 forward	5`-ACAGTCCAGCTGATGCAGAGG-3`	1730	21 bp	59.1 °C
MDR1 reverse	5`-CCTTATCCAGAGCCACCTGAAC-3`	2150	22 bp	58.7 °C
MRP1 forward	5`-CACACTGAATGGCATCACCTTC-3`	2173	22 bp	59.1 °C
MRP1 reverse	5`-CCTTCTCGCCAATCTCTGTCC-3`	2489	21 bp	59.8 °C
MRP2 forward	5`-CCAATCTACTCTCACTTCAGCGAGA-3`	3509	25 bp	60.0 °C
MRP2 reverse	5`-AGATCCAGCTCAGGTCGGTACC-3`	3981	22 bp	60.5 °C
MRP3 forward	5`-TCTATGCAGCCACATCACGG-3`	3419	20 bp	59.3 °C
MRP3 reverse	5`-GTCACCTGCAAGGAGTAGGACAC-3`	3746	23 bp	58.8 °C
MRP4 forward	5`-AAGTGAACAACCTCCAGTTCCA-3`	2026	22 bp	57.3 °C
MRP4 reverse	5`-CCGGAGCTTTCAGAATTGAC-3`	2543	20 bp	56.1 °C
MRP5 forward	5`-CTAGAGAGACTGTGGCAAGAAGAGC-3`	570	25 bp	59.0 °C
MRP5 reverse	5`-AAATGCCATGGTTAGGATGGC-3`	902	21 bp	59.6 °C
Villin forward	5`-AGAAAGCCAATGAGCAGGAGAA-3`	926	22 bp	59.1 °C
Villin reverse	5`-ATGGATGTGGCATCGAACTTC-3`	1163	21 bp	58.5 °C
BCRP forward	5'-TTTCAGCCGTGGAACTCTTT-3'	1529	20 bp	56.2 °C
BCRP reverse	5'-TGAGTCCTGGGCAGAAGTTT-3'	1990	20 bp	56.0 °C
ASBT forward	5`-CATCTCTGGTTGCTCTCGTTGTTC-3`	1098	24 bp	61.1 °C
ASBT reverse	5`-TGATGTCTACTTTTCGTCAGGTTGAA-3`	1651	26 bp	60.0 °C

Table 2.1: Sequence, starting position, length (base pairs), and melting temperature of the primers that were used for the generation of gene-specific cDNA standards.

amplicon	gene name	accession number	length	molecular weight
MDR1	ABCB1	NM_000927	421 bp	130377.2 g/mol
MRP1	ABCC1	NM_004996	317 bp	97797.4 g/mol
MRP2	ABCC2	NM_000392	473 bp	145684.6 g/mol
MRP3	ABCC3	NM_020038	328 bp	100912.6 g/mol
MRP4	ABCC4	NM_005845	518 bp	159304.6 g/mol
MRP5	ABCC5	NM_005688	333 bp	102594.6 g/mol
Villin	VIL1	NM_007127	238 bp	73486.6 g/mol
BCRP	ABCG2	NM_004827	462 bp	142064.4 g/mol
ASBT	SLC10A2	NM_000452	554 bp	170455.8 g/mol

Table 2.2: Gene name, gene bank accession number, length (base pairs) and molecular weight of the PCR amplicons that were used as standards for TaqMan analysis.

2.3 Standard curve method

A standard curve for each gene on each plate is essential for accurate quantification of mRNA transcript numbers. The standard curves were generated by a serial dilution of cDNA standard solutions with known amount of PCR template. However, the starting amount for the PCR had to be evaluated in order to obtain curves that span the range above and below the amount of the unknown samples. Therefore, the quantified standard solutions were first analysed in TaqMan assays and then adapted by further dilutions (= **standard dilution** in equation 1) so that the obtained curves were adequate.

Linear standard curves were composed by plotting the Ct values of the standards against the log of their corresponding serial dilution factor. Slope and Y-intercept of the standard curve line were then calculated by linear regression. By measuring the Ct value of the unknown sample under the same conditions, its corresponding serial dilution factor (= **X** in equation 1) could then be determined.

Based on this serial dilution factor (X) the number of cDNA molecules of the analysed gene in the sample (transcript number) could be estimated. Therefore, the number of cDNA fragments in the applied standard solution (standard 1) was calculated and subsequently multiplied with the serial dilution factor (X) of the sample. Usually, the transcript number is normalised to 1 µg RNA. The following equation (equation 1) shows how the transcript number per µg RNA was calculated.

$$\text{Transcript number per µg total RNA} = \frac{\mathbf{C \times V \times N \times X}}{\mathbf{\text{standard dilution} \times MW \times 1x10^{12}}}$$

Equation 1: **C** (ng/mL) is the concentration of the standard determined with the PicoGreen® assay. **V** (µL) is the volume of sample cDNA that contains 1 µg of reverse transcribed RNA. This is 100 µL for the common cDNA concentration of 10 ng/µL. **N** is Avogadro`s number ($6.022x10^{23}$ molecules per mol). **X** is the serial dilution factor of the sample determined with the standard curve. The **standard dilution** describes how-fold the standard 1 has been diluted for adapting the standard curve. **MW** (g/mol) is the molecular weight of the standard. **$1x10^{12}$** accounts for conversions of units.

2.4 Real-time PCR (TaqMan® assay)

The 5`nuclease assay or TaqMan® assay is a highly sensitive method to determine mRNA levels quantitatively. This method uses a target specific oligonucleotide, the TaqMan probe, which anneals between the forward and reverse primer sites. The probe carries a reporter dye on the 5` end (6-carboxy-fluorescein) and a quencher dye on the 3` end (6-carboxy-tetramethyl-rhodamine). As long as the probe is intact the fluorescence of the reporter dye is suppressed by the quencher dye. However, during the PCR the DNA polymerase (Taq polymerase) cleaves the probe due to its 5`-3` exonuclease activity. Now, a fluorescent signal is generated because the reporter dye is separated from the quencher dye. Consequently, there is an increase of fluorescence after each PCR cycle. With the ability to measure the PCR products as they are accumulating, in "real time," it is possible to measure the amount of PCR product at a point in which the reaction is still in the exponential range. It is only during this exponential phase of the PCR reaction that it is possible to extrapolate back to determine the starting amount of template. During the exponential phase in real-time PCR experiments, a fluorescence signal threshold is determined at which point all samples can be compared. Therefore, the number of PCR cycles required to generate enough fluorescent signal to reach this threshold is defined as the *cycle threshold*, or Ct. These Ct values are directly proportionate to the amount of starting template and are the basis for calculating mRNA expression levels. The *baseline* is defined as the PCR cycles in which a signal is accumulating but is beneath the limits of detection of the instrument.

TaqMan experiments were carried out either on a Gene Amp 5700 Sequence Detector using 96 well plates with total reaction volumes of 25 µL, or on a 7900HT Sequence Detection System using 384 well plates with total reaction volumes of 10 µL (all Applied Biosystems, Rotkreuz, Switzerland). PCR conditions were throughout 10 min 95°C followed by 40 cycles of 15 s 95°C and 1 min 60°C. TaqMan Universal PCR Mastermix (Applied Biosystems) was used. Each reaction contained 1 ng/µL cDNA and the concentrations of primers and probes were 900 nM and 225 nM, respectively. Primers and probes (Table 2.3) were designed according to the guidelines of Applied Biosystems with help of the Primer Express 2.0 software. Primers were synthesized by Invitrogen (Basel, Switzerland), probes by Eurogentec (Seraing, Belgium).

Gene	Probe	start	length	Tm
MDR1	5`-AAGCTGTCAAGGAAGCCAATGCCTATGACTT-3`	1929	31 bp	69.0 °C
MRP1	5`-CCTCCACTTTGTCCATCTCAGCCAAGAG-3`	2267	28 bp	69.0 °C
MRP2	5`-CTCAATATCACACAAACCCTGAACTGGCTG-3`	3773	30 bp	68.0 °C
MRP3	5`-CCAACCGGTGGCTGAGCATCG-3`	3608	21 bp	69.0 °C
MRP4	5`-CAAACCGAAGACTCTGAGAAGGTACGATTCCT-3`	2094	32 bp	68.4 °C
MRP5	5`-CTGACGGAAATCGTGCGGTCTTGG-3`	804	24 bp	69.0 °C
Villin	5`-TCATCAAAGCCAAGCAGTACCCACCAAG-3`	977	28 bp	69.2 °C
BCRP	5`-CCATTGCATCTTGGCTGTCATGGCTT-3`	1883	26 bp	69.4 °C
ASBT	5`-TTCAGCTCTCCTTCACTCCTGAGGAGCTC-3`	1419	29 bp	69.0 °C

Gene	Forward Primer	start	length	Tm
MDR1	5`-CTGTATTGTTTGCCACCACGA-3`	1854	21 bp	58.0 °C
MRP1	5`-GGGCTGCGGAAAGTCGT-3`	2236	17 bp	58.0 °C
MRP2	5`-ACTGTTGGCTTTGTTCTGTCCA-3`	3746	22 bp	58.4 °C
MRP3	5`-GGTGGATGCCAACCAGAGAA-3`	3567	20 bp	59.0 °C
MRP4	5`-AAGTGAACAACCTCCAGTTCCAG-3`	2026	23 bp	58.3 °C
MRP5	5`-CTGCAGTACAGCTTGTTGTTAGTGC-3`	768	25 bp	59.0 °C
Villin	5`-CATGAGCCATGCGCTGAAC-3`	957	19 bp	59.9 °C
BCRP	5`-CAGGTCTGTTGGTCAATCTCACA-3`	1859	23 bp	58.7 °C
ASBT	5`-ACGCAGCTATGTTCCACCATC-3`	1397	21 bp	59.0 °C

Gene	Reverse Primer	start	length	Tm
MDR1	5`-AGGGTGTCAAATTTATGAGGCAGT-3`	1992	24 bp	59.0 °C
MRP1	5`-AGCCCTTGATAGCCACGTG-3`	2315	19 bp	57.0 °C
MRP2	5`-CAACAGCCACAATGTTGGTCTCTA-3`	3845	24 bp	60.0 °C
MRP3	5`-GCAGTTCCCCACGAACTCC-3`	3651	19 bp	59.0 °C
MRP4	5`-GGCTCTCCAGAGCACCATCT-3`	2144	20 bp	58.0 °C
MRP5	5`-TCGGTAATTCAATGCCCAAGTC-3`	860	22 bp	59.8 °C
Villin	5`-TCATTCTGCACCTCCACCTGT-3`	1028	21 bp	59.2 °C
BCRP	5`-TCCATATCGTGGAATGCTGAAG-3`	1936	22 bp	58.7 °C
ASBT	5`-GCGGGAAGGTGAATACGACA-3`	1469	20 bp	60.0 °C

Table 2.3: Sequence, starting position, length (base pairs), and melting temperature of the primers and probes that were used for TaqMan analysis.

3. Determination of transporter expression in human intestinal biopsies

3.1 Mapping of multidrug resistance gene 1 and multidrug resistance-associated protein isoform 1 to 5 mRNA expression along the human intestinal tract

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3.1.1 Abstract

Efflux transporters such as P-glycoprotein and multidrug resistance-associated proteins (MRPs) in the intestinal wall restrict intestinal drug transport. To overcome this limitation for enteral drug absorption, galenical targeting approaches have been proposed for site-specific luminal drug release in segments of the gut, where expression of the respective absorption-limiting transporter is minimal. Therefore, expression of multidrug resistance gene 1 (MDR1) and MRP1-5 was systematically investigated in 10 healthy subjects. Biopsies were taken from different segments of the gastrointestinal tract (from duodenum, terminal ileum as well as ascending, transverse, descending, and sigmoid colon). Gene expression was investigated by quantitative real-time PCR (TaqMan). MRP3 appeared to be the most abundantly expressed transporter in the investigated parts of the human intestine, except for the terminal ileum, where MDR1 showed the highest expression. The ranking of transporter gene expression in the duodenum was MRP3>>MDR1>MRP2>MRP5>MRP4>MRP1. In the terminal ileum the ranking order was as follows: MDR1>MRP3>>MRP1≈MRP5≈MRP4>MRP2. In all segments of the colon (ascending, transverse, descending, and sigmoid colon), the transporter gene expression showed the following order: MRP3>>MDR1> MRP4≈MRP5>MRP1>>MRP2. We have shown, for the first time, systematic site-specific expression of MDR1 and MRP along the gastrointestinal tract in humans. All transporters showed alterations in their expression levels from the duodenum to sigmoid colon. The most pronounced changes were observed for MRP2 with high levels in the small intestine and hardly any expression in colonic segments. This knowledge may be useful to develop new targeting strategies for enteral drug delivery.

3.1.2 Introduction

Efflux transporters in the intestinal wall form a barrier to cellular accumulation of toxins as well as to drug absorption (Schinkel, 1997). Important efflux proteins in the gut are P-glycoprotein [gene product of the multidrug resistance 1 (MDR1) gene] and multidrug resistance-associated protein (MRP) transporters. They belong to the superfamily of ATP-binding cassette (ABC) transporters. ABC transporters mediate the translocation of a wide variety of substances across cellular membranes using ATP hydrolysis (Horio et al., 1991; Senior et al., 1995). The expression of ABC transporter genes is widespread throughout many tissues, most notably in excretory sites such as the liver, kidney, blood-brain barrier, and intestine. Therefore, they play a critical role in absorption and tissue distribution of orally administered drugs (Schuetz et al., 1998; Ambudkar et al., 1999). Due to their broad substrate specificity, they may influence the pharmacokinetics of many

chemically unrelated substances (e.g., HIV drugs, anticancer drugs, endogenous compounds) (Lee et al., 1997; Schinkel, 1998; Schuetz et al., 1999; Borst et al., 2000). MDR1 preferentially extrudes large hydrophobic, positively charged molecules, whereas the members of the MRP family extrude both hydrophobic uncharged molecules and water-soluble anionic compounds.

There is little knowledge about the expression pattern of those ABC transporters along the human intestine. Taipalensuu investigated gene expression of 10 ABC transporters in jejunal biopsies from healthy subjects (Taipalensuu et al., 2001). The highest expression was shown for breast cancer resistance protein and MRP2. Nakamura investigated the expression of three ABC transporters in duodenal and colorectal tissues in humans (Nakamura et al., 2002). In comparison to duodenum, in colon they found a decrease in MDR1 expression, equal levels of MRP1, and a strong decrease in MRP2 expression. However, this comparison was not obtained in the same subjects. Therefore, the intraindividual expression differences between these transporters could not be assessed.

Knowledge of the topographical distribution may be important for the development of specific galenical targeting approaches, which may be utilized to improve intestinal absorption of drugs. Therefore, in this study, the expression of MDR1 and MRP1-5 genes was investigated in the human intestine of 10 healthy subjects.

3.1.3 Materials and methods

Intestinal biopsies

Intestinal biopsies were obtained from a group of 10 healthy subjects (5 female, 5 male, aged 50–76 years, average age 62 years, no medication), which served as a control group in a clinical study designed to investigate the regional expression of different genes in patients with inflammatory bowel disease. The study protocol included specifically the investigation of drug-transporting proteins and was approved by the local ethical committee. Informed consent was obtained from all subjects prior to inclusion. No macroscopically pathological findings were observed during endoscopies in these subjects. Three to four biopsies were obtained from duodenum, terminal ileum, ascending colon, transverse colon, descending colon, and sigmoid colon (Figure 3.1). Due to low enterocyte content, duodenal biopsies from one subject had to be discarded, leading to nine duodenal samples.

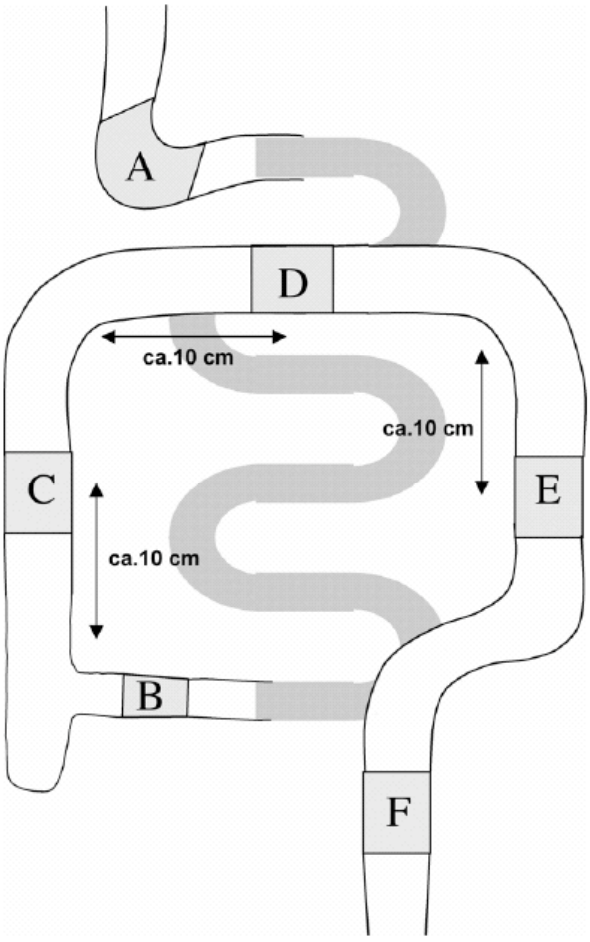

Figure 3.1 Schematic overview of biopsy sampling. Samples were taken from the duodenum (A), terminal ileum (B), ascending colon (C), transverse colon (D), descending colon (E), and sigmoid colon (F).

Preparation of Samples

The samples were immediately submerged in a tube with RNAlater (Ambion, Austin, TX) and stored at 80°C until further processing. For RNA isolation, two biopsies from each intestinal region were homogenized for 30 s (Polytron PT 2100; Kinematika AG, Littau, Switzerland) and RNA was extracted using the RNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany) following the instructions provided by the manufacturer. RNA was quantified with a GeneQuant photometer (Pfizer, Inc., Täby, Sweden). After DNase I digestion (Invitrogen, Basel, Switzerland), 1.5 µg of total RNA was reverse-transcribed by SuperScript (Invitrogen) according to the manufacturer's protocol, using random hexamers as primers.

TaqMan analysis was described in chapter 2.

Sequences of primers/probes are displayed in table 2.3.

Normalization to villin expression

For each sample, the number of transporter transcripts (MDR1, MRP1–5) and the number of villin transcripts were determined. By calculating the ratio of transporter/villin mRNA, the transporter expression was normalized. Enterocytes represent only a small fraction of the cells obtained in an intestinal biopsy. Determination of villin, an enterocyte-specific, constitutively expressed protein, can be used to control for the variation of enterocyte content in biopsy (Lown et al., 1994). Therefore, transporter mRNA concentrations were expressed as a ratio with the villin levels of the same samples. These villin-corrected values provide a relative measure of enterocyte concentration (Lown et al., 1997). Results with this approach have already been published (Taipalensuu et al., 2001; Mouly and Paine, 2003).

Statistical analysis

Gene expression was compared between the different intestinal segments by analysis of variance. In the case of significant differences, all segments were compared with the expression in duodenum using two-sided Dunnett's multicomparison *t* test. The level of significance was $P<0.05$. Comparisons were performed using SPSS for Windows software (version 11.0; SPSS Inc., Chicago, IL).

3.1.4 Results

There was a considerable interindividual variability of transporter gene expression amounting on average to 34% (CV%). Figure 3.2 displays the expression and ranking of all transporters in the analyzed tissues normalized to villin. MRP3 appeared to be the most abundantly expressed transporter in the investigated parts of the human intestine, except for the terminal ileum where MDR1 showed the highest expression. The ranking of transporter gene expression in the duodenum was MRP3 >> MDR1 > MRP2 > MRP5 > MRP4 > MRP1. In the terminal ileum the ranking order was as follows: MDR1 > MRP3 >> MRP1 ≈ MRP5 ≈ MRP4 > MRP2. In all segments of the colon (ascending, transverse, descending, and sigmoid colon), the transporter expression showed the following order: MRP3 >> MDR1 > MRP4 ≈ MRP5 > MRP1 >> MRP2.

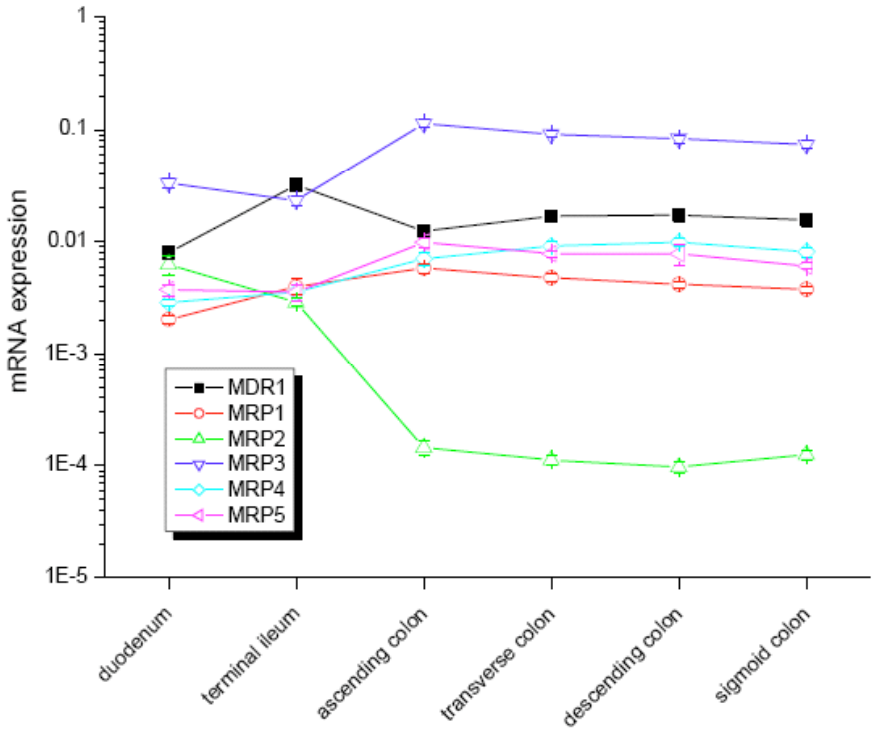

Figure 3.2: Expression of all investigated transporters in the analyzed tissues normalized to villin expression. Data represent means (± SEM) of biopsies from 10 health subjects, except duodenum, where biopsies from 9 subjects were used.

Figure 3.3 shows the expression pattern of each individual transporter from the duodenum to the sigmoid colon normalized to villin. Compared with the duodenum, the expression of MDR1 was 4-fold higher in the terminal ileum and approximately 2-fold higher in the colonic segments. MRP1 exhibited a 2- to 3-fold higher expression in both the terminal ileum and colon compared with duodenum. MRP2 showed highest expression in the duodenum, half-levels in the terminal ileum, and hardly any MRP2 transcripts in each colonic segment. MRP3, MRP4, and MRP5 exhibited a similar expression pattern with equal levels in the duodenum and terminal ileum, but a 2- to 3-fold increase in the colon. Within the colon, MRP1, MRP3, and MRP5 showed an expression pattern with decreasing levels from proximal to distal, whereas MDR1, MRP2, and MRP4 levels remained rather constant.

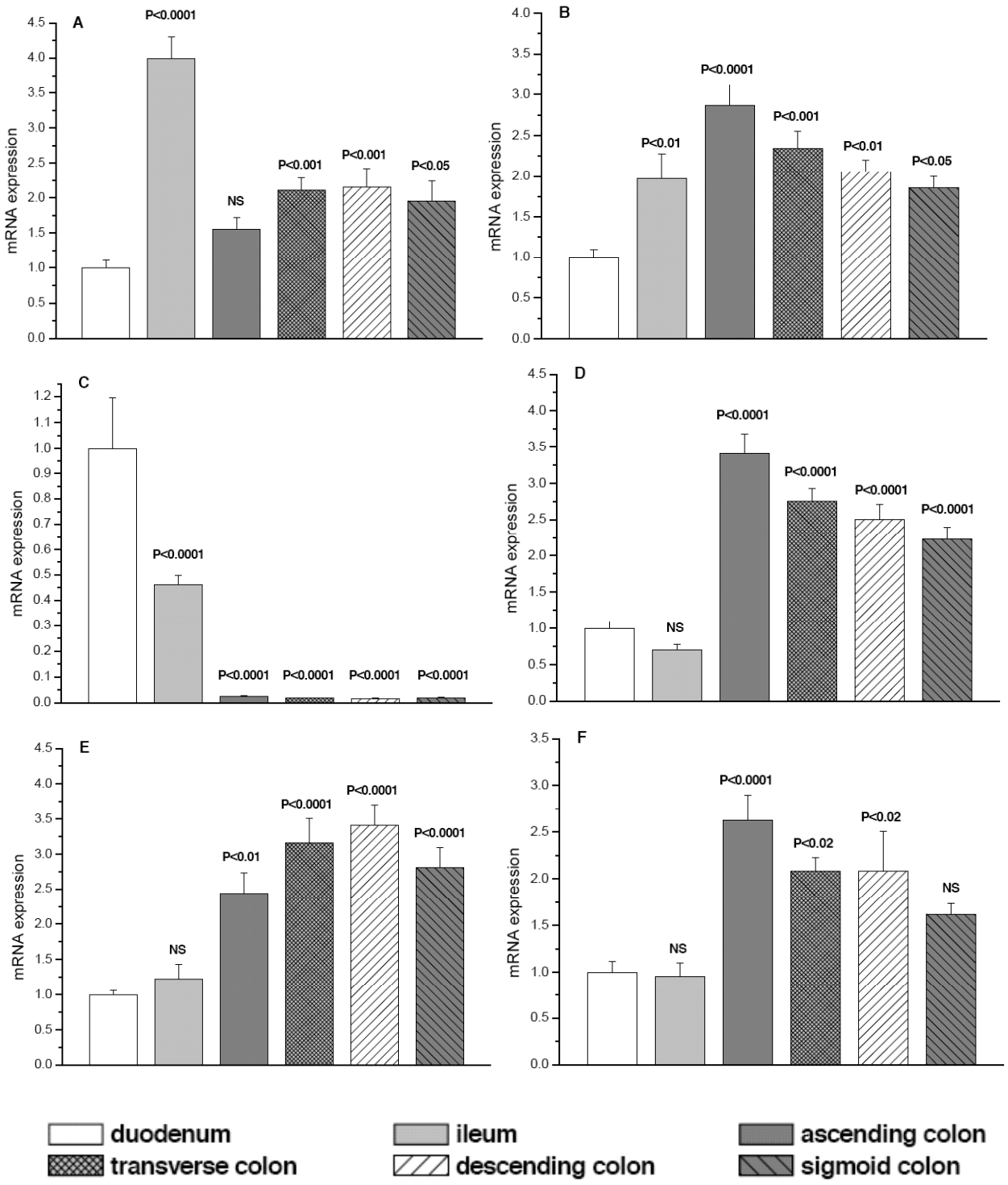

Figure 3.3 (A – F): Transporter specific gene expression in different gut segments normalized to the villin expression. **A**: MDR1, **B**: MRP1, **C**: MRP2, **D**: MRP3 **E**: MRP4 and **F**: MRP5. Data represent means (± SEM) of biopsies from 10 health subjects, except duodenum, where biopsies from 9 subjects were used.

3.1.5 Discussion

Only little information is available about the expression of ABC transporters along the intestinal tract. Available information relates mainly to MDR1 and MRP2 expression (Dietrich et al., 2003; Lindell et al., 2003). Furthermore, previous studies have focused on isolated parts of the intestine (Taipalensuu et al., 2001; Lindell et al., 2003), on animal models (Achira et al., 2002; Takara et al., 2003), or on cancer cells (Nakamura et al., 2002; Li et al., 2003; Pfrunder et al., 2003a). Here, we present a systematic investigation of multidrug resistance protein mRNA expression in various parts of the human intestine from proximal to distal within the same subject. One drawback of the study is the lack of samples from the jejunum, an important site for drug absorption. The subjects in our study underwent combined gastroscopy and colonoscopy procedures for screening of gastrointestinal cancer. Therefore, an additional jejunoscopy was not performed. However, Taipalensuu and co-workers focused on the human jejunum and found a transporter expression with the following ranking: MRP2 > MDR1 ≈ MRP3 > MRP5 ≈ MRP1 > MRP4 (Taipalensuu et al., 2001). Besides the high MRP2 levels, the transporter expression pattern in the jejunum shows strong similarity to the pattern we found in the terminal ileum, which is conclusive because of the proximity of these tissues.

It is suggested that MDR1 physiologically functions as a gatekeeper against xenobiotics in the gut. The bioavailability of many drugs is reduced due to MDR1 efflux. MDR1 shows extremely broad substrate specificity, including anticancer agents, antibiotics, antivirals, calcium channel blockers, and immunosuppressants. With respect to the expression of MDR1 in the human intestine, an increase from proximal to distal was stated, with the highest expression levels documented in the colon (Fricker et al., 1996; Dietrich et al., 2003; Chan et al., 2004). In mice, however, Chianale and co-workers found the highest levels of mdr3 mRNA in the ileum (Chianale et al., 1995). In the rat intestine, the P-glycoprotein-mediated drug efflux showed highest activity in the ileum as well (Stephens et al., 2001). We could also demonstrate, in humans, higher MDR1 mRNA levels in the terminal ileum compared with the duodenum. These results are consistent with human data from Mouly and Pain, who reported an increase in P-glycoprotein from duodenum to ileum (Mouly and Paine, 2003). Additionally, our results indicate the highest MDR1 expression in the terminal ileum within the investigated segments of the human intestine. It appeared to be 4-fold higher in the terminal ileum compared with the duodenum and 2-fold higher compared with the colon. Moreover, MDR1 was the most abundantly expressed transporter in the terminal ileum compared with all other ABC transporters that were analyzed in this study.

MRP1 showed the lowest variation in mRNA levels within the intestinal tract. This is in good agreement with the fact that MRP1 is expressed ubiquitously. Physiologically important substrates

for MRP1 include glutathione *S*-conjugates such as leukotriene C4, as well as bilirubin glucuronides (Keppler et al., 1998). In addition, anionic drugs and drugs conjugated to glutathione, like methotrexate or arsenite, are also transported by MRP1 (Bakos et al., 2000; Vernhet et al., 2000).

A previous study revealed that MRP2 is the ABC transporter with the highest expression besides breast cancer resistance protein in the human jejunum (Taipalensuu et al., 2001). We found relatively low MRP2 levels in the human duodenum and even lower levels in the terminal ileum, but almost no MRP2 expression in the entire colon. These results were also found in the rat intestine (Mottino et al., 2000; Rost et al., 2002), but up to now, they were not confirmed in humans. Our results are also consistent with the expression pattern of glutathione *S*-transferase in the human gastrointestinal tract mucosa (Coles et al., 2002). This phase II metabolizing enzyme provides the conjugated compounds for subsequent export by MRP2 or MRP1. The substrate specificity of MRP2 is similar to that of MRP1, and includes glutathione conjugates, bilirubin glucuronides, and a number of drugs and their conjugated drug metabolites (Jedlitschky et al., 1997; Kawabe et al., 1999). These drugs include pravastatin, temocaprilat, irinotecan, SN-38, arsenite, cisplatin, methotrexate, vincristine, saquinavir, and ceftriaxone (Kusuhara and Sugiyama, 2002; Dietrich et al., 2003). Regarding the amount of drugs transported by MRP2, a drug targeting which circumvents absorption sites with high MRP2 expression would be of benefit, especially for drugs with low bioavailability.

MRP3 transports a wide range of bile salts and seems to be involved in their reabsorption (Hirohashi et al., 2000). MRP3 transfection of cell lines conferred resistance to epipodophyllotoxins, vincristine and methotrexate (Kool et al., 1999). For MRP3, Rost and co-workers showed low expression in the rat duodenum and high expression in the ileum and colon (Rost et al., 2002). Our human data indicate low MRP3 levels in the duodenum as well as in the terminal ileum but also high expression in the colon. Within the colon, MRP3 expression diminished slightly from proximal to distal segments. This reduction in transporter expression from ascending to sigmoid colon was observed for MRP1, MRP3, and MRP5. Interestingly, all of these transporters are located on the basolateral membrane. For MDR1, MRP2, and MRP4, probably located on the apical membrane (Chan et al., 2004) we observed rather constant expression levels throughout the entire colon.

With respect to MRP4, we found equal expression levels in the duodenum and the terminal ileum but a 3-fold increase in the colon. To our knowledge, there is no previous publication on the MRP4 expression in the colon. The significance of MRP4 in drug transport is at present unclear. However, an overexpression of MRP4 severely impaired the antiviral efficacy of adefovir,

azidothymidine, and other nucleoside analogs in cell lines (Schuetz et al., 1999). Other substrates include folic acid, bile acids, methotrexate, and 6-mercaptopurine (Wielinga et al., 2002; Chan et al., 2004). A physiological role of MRP4 might be the release of prostaglandins from cells (Reid et al., 2003).

MRP5 expression appeared to be concordant to MRP4 expression with low levels in the duodenum and the terminal ileum, but a 2-fold increase in the different colon segments. Both transporters have an affinity to nucleotide-based substrates. There are no reports, at present, which could suggest a role for MRP5 in intestinal drug disposition. Experiments with transfected cells showed enhanced efflux of 2,4-dinitrophenyl-*S*-glutathione, adefovir, and the purine analogs 6-mercaptopurine and thioguanine (Wijnholds et al., 2000). Jedlitschky and co-workers demonstrated that MRP5 transports the cyclic nucleotides cAMP and cGMP (Jedlitschky et al., 2000), but the physiological function of this transporter remains to be elucidated.

Although our results indicate significant changes of MDR1 and MRP1-5 gene expression in investigated parts of the human intestine, this does not necessarily correlate with protein expression or function. Additional studies regarding the effect of expression on protein levels are therefore required.

The impact of these transporters should be evaluated for drugs permeating epithelial barriers, especially during pharmacological development of novel classes of therapeutic compounds. Selectivity of inhibitors, in particular, for human efflux transporters located at the apical mucosal membrane (such as MDR1, MRP2, and MRP4), remains to be examined, and further studies are required. Therefore, the knowledge of the transporter expression throughout the human intestine might be of special value.

Conclusion

We have shown, for the first time, systematic site-specific expression of MDR1 and MRP isoforms along the gastrointestinal tract in humans. All transporters showed alterations in their expression levels from the duodenum to the sigmoid colon. The most pronounced changes were observed for MRP2, with high levels in the small intestine and hardly any expression in colonic segments. This knowledge may be useful to develop new targeting strategies for enteral drug delivery.

Acknowledgments

We thank Mrs. Ursula Behrens for excellent technical assistance. We thank the nurses of the Department of Gastroenterology for excellent technical assistance during biopsies.

3.2 Breast cancer resistance protein (BCRP) mRNA expression in the human intestinal tract

3.2.1 Abstract

Human breast cancer resistance protein (BCRP/ABCG2) is an ABC-transporter that is present on the luminal membrane of intestinal epithelial cells and restricts absorption of anticancer drugs such as methotrexate, topotecan, mitoxantrone, and doxorubicin. The exact anatomic distribution of BCRP along the gastrointestinal tract has, however, not been determined before. The aim of this study was therefore to investigate its mRNA expression pattern along the intestine in 14 healthy subjects (7 women, 7 men). Furthermore, duodenal mRNA expression of BCRP was compared with MDR1. Since previous animal studies observed sex specific differences in BCRP expression we analyzed intestinal BCRP expression with respect to sex. Biopsies were taken from different gut segments (duodenum, terminal ileum and ascending, transverse, descending, and sigmoid colon). Gene expression was assessed by quantitative real-time RT-PCR (TaqMan). BCRP mRNA expression decreased continuously from the proximal to the distal parts of the intestine. Compared to duodenum, the expression decreased to 93.7 percent in terminal ileum, to 75.8 percent in ascending colon, to 66.6 percent in transverse colon, to 62.8 percent in descending colon, and to 50.1 percent in sigmoid colon. BCRP expression was comparable to MDR1 expression in the duodenum. Differences in BCRP expression related to sex were not observed. These findings represent the first systematic site-specific analysis of BCRP expression along the intestinal tract. This knowledge might be important to develop target strategies for orally administered anticancer drugs.

3.2.2 Introduction

BCRP (ABCG2) is a half-transporter that belongs to the G subfamily of ATP-binding cassette (ABC) transporters. BCRP needs the dimerisation to homodimers for proper function (Ozvegy et al., 2001). However, it is possible that heterodimeric forms of BCRP combined with another half transporter exist in mammalian systems (Nakanishi et al., 2003). Similar to MDR1, BCRP was detected and cloned from multi-drug resistant tumor cells (Doyle et al., 1998; Miyake et al., 1999; Scheffer et al., 2000) It displays a wide substrate specificity and mediates the energy-dependent translocation of various anticancer drugs such as methotrexate, daunorubicin, doxorubicin, mitoxantrone, SN38, and topoisomerase inhibitors (such as topotecan) across cellular

membranes (Doyle et al., 1998; Schellens et al., 2000; Nakanishi et al., 2003; Volk and Schneider, 2003).

BCRP expression was detected mainly in excretory organs, e.g. in cannalicular membranes of the liver, in epithelial cells of the small intestine, colon, kidney and lung, as well as in the blood-brain barrier and the placenta (Maliepaard et al., 2001). Its localization indicates an important role in the protection of tissues against xenobiotics. Particularly, the expression of BCRP in epithelial cells of the intestine implies, that BCRP might be an important transporter limiting the absorption of orally administered drugs and ingested toxins.

BCRP knock-out mice were found to be healthy and showed no major pathological alterations. However, they become extremely sensitive to the dietary chlorophyll-breakdown product pheophorbide a, resulting in severe, sometimes lethal phototoxic lesions on light-exposed skin (Jonker et al., 2002). These data provide a striking illustration of the importance of drug transporters in the protection of the body from toxicity of normal food constituents. Another example is PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine), a dietary carcinogen present in baked food and cigarette smoke. Its absorption and its hepatobiliary and intestinal elimination are clearly affected by Bcrp1 in mice (van Herwaarden et al., 2003). Furthermore, BCRP transport is selectively inhibited by the fungal toxin fumitremorgin (van Loevezijn et al., 2001).

Differences in the transport of endogenous and xenobiotic compounds associated with sex have been reported previously for several transport proteins (Salphati and Benet, 1998; Urakami et al., 1999; Buist et al., 2002; Cerrutti et al., 2002; Buist and Klaassen, 2004). Recently, gender associated differences for Bcrp have also been described (Tanaka et al., 2005). A higher expression of Bcrp mRNA in the kidney of male rats and in the liver of male mice compared to females has been observed. These gender differences were attributed to the suppressive effect of estradiol in rats and to the inductive effect of testosterone in mice, respectively.

Up to know, there is little knowledge about the expression pattern of BCRP along the human intestine. This knowledge, however, might be important for the development of specific galenical targeting approaches, which may be utilized to improve intestinal absorption of BCRP substrates such as anticancer drugs. Therefore, the expression of BCRP was investigated in the human intestine of 14 healthy subjects (see chapter 3.1.3) and its duodenal expression was compared with that of MDR1. We further determined, whether there are sex-related differences in human BCRP mRNA expression along the intestinal tract that might lead to pharmacokinetic variations in drug absorption.

3.2.3 Materials and methods

Preparation of intestinal biopsies was described in chapter 3.1.3.

TaqMan analysis and primer/probe sequences were shown in chapter 2.

Statistical analysis

Gene expression was compared between the different intestinal segments, between BCRP and MDR1, and between male and female by analysis of variance. In the case of significant differences between intestinal segments, all segments were compared with the expression in duodenum using two-sided t-test. The level of significance was P =0.05.

3.2.4 Results and discussion

There is information available about the tissue distribution of BCRP in animal species such as rat and mice (Tanaka et al., 2005) as well as in humans (Maliepaard et al., 2001). Beside other tissues, human BCRP is expressed in the apical membranes of small intestinal and colonic epithelial cells, where it might limit the bioavailability of toxic compounds. However, nothing is known about the site-specific localization of BCRP along the gastro-intestinal tract, which might be important for the development of specific galenic formulations of anticancer drugs. Here, we present for the first time a systematic analysis of the site-specific expression of BCRP along the human intestinal tract.

Figure 3.4 shows the mRNA expression pattern of BCRP from the duodenum to the sigmoid colon. Maximal expression was found in the duodenum and decreasing levels were found towards the rectum. In the terminal ileum BCRP mRNA expression is reduced to 93.7 per cent compared to duodenum. In the colonic segments BCRP mRNA expression is continuously decreasing from proximal to distal. In ascending colon the BCRP level is reduced to 75.8 percent, in transverse colon to 66.6 percent, in descending colon to 62.8 percent, and in sigmoid colon to 50.1 percent compared to duodenum, respectively.

Human jejunum was not investigated in our study due to ethical reasons. Taipalensuu and co-workers found high BCRP mRNA expression in jejunum (Taipalensuu et al., 2001). They showed even a 3.4-fold higher expression compared to the MDR1 gene, another important ABC-transporter of xenobiotics in the intestine. Data in rat have shown that the level of Bcrp gene expression is higher in the jejunum compared to duodenum (Tanaka et al., 2005). However, it is

not trivial to conclude from animal data on human, because species differences have been described even between rodents. Whereas rats expressed high levels of Bcrp in the ileum, the ileal level of Bcrp mRNA in mice was rather low. Nevertheless one is tempted to speculate, that BCRP expression level might be maximal in the human jejunum as well.

Differences in the membrane transport of xenobiotics and endogenous compounds caused by different levels of sexual hormones such as testosterone and estradiol have been described previously in several animal studies with rats (Urakami et al., 2000; Kobayashi et al., 2002). These gender related differences in membrane transport includes several transporters such as organic cation transporters, organic anion transporters, and multidrug resistance proteins Mdr1a, Mdr1b and Mdr2. Recently, sex-related differences of Bcrp expression levels in rodents have also been described (Tanaka et al., 2005). Male rats exhibited higher expression levels in kidney and liver compared to female rats. In our study, we found no significant differences in the expression level of BCRP mRNA between males and females, neither in duodenum and terminal ileum, nor in all colonic segments of the intestinal tract. We therefore conclude, that sexual hormones have most probably no effect on the expression pattern of BCRP in the human intestine.

To estimate the impact of BCRP for detoxification, we compared the level of BCRP mRNA with the level of MDR1 mRNA in the duodenum. Figure 3.5 shows that both MDR1 and BCRP mRNA is expressed in the same range in the duodenum, with a slight but significant lower expression of BCRP ($p<0.05$). However, since both transporters are highly expressed in the small intestine and both transporters have a broad and overlapping substrate specificity, these findings indicate that along with MDR1, BCRP might play an important role for limiting the absorption of orally administered anticancer drugs and ingested toxins.

In conclusion, these findings represent the first systematic site-specific analysis of BCRP expression along the human intestinal tract. We showed that BCRP expression significantly decreased from the distal to the proximal parts of the gut and we observed no gender-specific differences. This knowledge might be important for the development of target strategies for orally administered anticancer drugs.

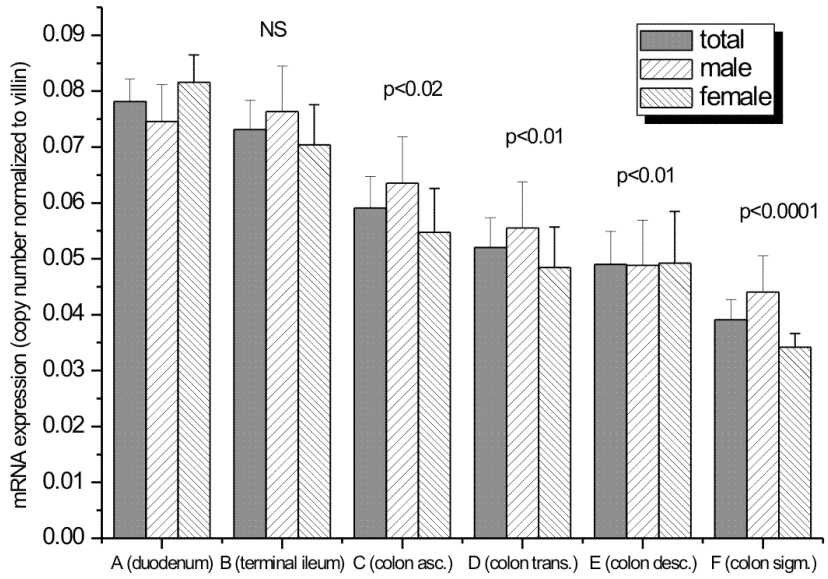

Figure 3.4: Expression of BCRP mRNA normalized to villin in different gut segments. Data represent means (± SEM) of biopsies from 14 healthy subjects (7 males, 7 females), except terminal ileum, where biopsies from 13 subjects (6 males, 7 females) were used.

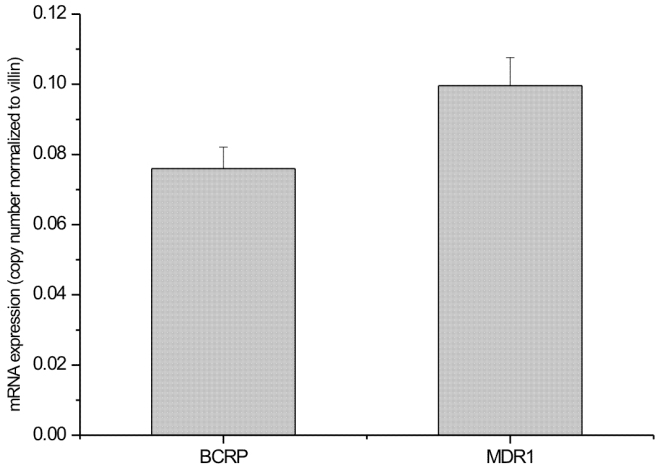

Figure 3.5: Expression of BCRP mRNA and MDR1 mRNA in human duodenum normalized to the expression of villin. Data represent means (± SEM) of biopsies from 14 healthy subjects.

3.3 Mapping of the apical sodium-dependent bile salt transporter (ASBT) in the human intestine and evaluation of gender-specific differences

3.3.1 Introduction

Bile acids are essential for normal intestinal lipid digestion and absorption. In the small intestine, bile acids emulsify dietary fats and lipid-soluble vitamins and regulate pancreatic secretion and the release of gastrointestinal peptides (Koop et al., 1996). But rather than being excreted, the majority of bile acids undergo enterohepatic circulation (Wong et al., 1996). They are reabsorbed from the intestine and reach the liver via the portal circulation. In the liver, bile acids are quantitatively extracted and resecreted into bile. The small amount of fecal bile salt loss (~5% of the total) is balanced by hepatic conversion of cholesterol to bile salts, a process representing an important route for the elimination of cholesterol from the body (Vlahcevic et al., 1999).

The enterohepatic circulation is dependent on several transport systems (Dawson and Oelkers, 1995; Trauner and Boyer, 2003). The hepatocellular bile salt uptake is mediated predominantly by the Na+/taurocholate cotransport polypeptide (NTCP) and by the organic anion-transporting polypetides (OATP-C in humans) located at the sinusoidal plasma membrane of hepatocytes. Bile salt secretion into bile is mediated by the bile salt export pump (BSEP) located on the canalicular membrane of hepatocytes. After their passage through the small intestine, bile salts are actively reabsorbed in the terminal ileum, where the apical sodium-dependent bile salt transporter (ASBT) has been identified as the responsible uptake protein.

ASBT (SLC10A2) is a 348-amino acid membrane glycoprotein with an apparent molecular mass of approximately 50 kDa (Wong et al., 1995). It transports conjugated and unconjugated bile acids with a high efficiency (Craddock et al., 1998). Subjects with mutations in the ASBT gene that lead to dysfunctional protein suffer from congenital diarrhea and steatorrhea, due to bile acid malabsorption (Oelkers et al., 1997). Pharmacological inhibition of ASBT can also lead to interruption of enterohepatic circulation of bile acids with changes in the cholesterol and bile acids homeostasis (Lewis et al., 1995; Huff et al., 2002; West et al., 2002).

To the current knowledge, ASBT is expressed at high levels on the apical brush border membrane in terminal ileum and at lower levels in renal proximal tubules (Craddock et al., 1998) and in cholangiocytes (Lazaridis et al., 1997). Regarding the human intestine, the knowledge of ASBT expression is restricted to the terminal ileum. This prompted us to compare ileal ASBT expression

with its expression in the duodenum and in different parts of the colon. Therefore, 14 healthy subjects (7 women, 7 men) who were undergoing a combined gastroscopy and colonoscopy were enrolled in this study (see chapter 3.1.3). Furthermore we determined gender-specific differences in the ASBT expression within our study population. Additionally, *in vitro* experiments were performed to evaluate the impact of sex hormones on ASBT expression in Caco-2 cells.

3.3.2 Materials and methods

Preparation of intestinal biopsies was described in chapter 3.1.3.

TaqMan analysis and primer/probe sequences were shown in chapter 2.

Incubation of Caco-2 cells with sex hormones

Caco-2 cells (used between passage 46-50) were purchased from ATCC (Manassas, USA) and were cultured in Dulbecco's MEM with Glutamax-I, supplemented with 10% fetal calf serum, 1% non-essential amino acids, 1% sodium pyruvate, and 50 µg/ml gentamycin. Cells were seeded into 12-well plastic culture dishes (3.8 cm^2/well, BD Falcon AG, Allschwil, Switzerland) and were maintained in a humidified 37°C incubator with 5% carbon dioxide in air atmosphere. After cells were grown to confluence incubations were performed for 72 h including daily change of the media. They were treated in triplicates with 0.1, 1, and 10 µM ethinylestradiol, 10 and 50 ng/mL progesterone, and 10, 20, and 25 µM budesonide (all from Sigma-Aldrich, St. Luis, MO, USA). Budesonide was used as a positive control for ASBT induction. The compounds were dissolved in dimethyl sulfoxide (DMSO), whereas the final DMSO concentration did not exceed 1%. After 72 hours cells were disintegrated by adding lysis buffer RLT (Qiagen, Hilden, Germany) and homogenized by using QIAshredder columns (Qiagen). Total RNA was extracted using the RNeasy Mini Kit (Qiagen). After DNaseI digestion (Gibco, Life Technologies, Basel Switzerland) 0.75 µg of total RNA was reversed transcribed by Superscript (Gibco) according to the manufacturer's protocol using random hexamers as primers (Applied Biosystems, Rotkreuz, Switzerland).

3.3.3 Results

Expression of ASBT mRNA in the human intestine

The expression pattern of ASBT mRNA in the human intestine was studied in 14 control subjects (Figure 3.6). Results were normalized to villin expression (ASBT mRNA / villin mRNA). We observed a pronounced ASBT expression in the terminal ileum, about 6-fold less expression in the duodenum and hardly any expression in the colon. The normalized ASBT expression (arbitrary units ± SEM) was 171.8 (± 20.3) in the duodenum, 1010 (± 330) in the terminal ileum, 8.3 (± 5) in the ascending colon, 4.9 (± 0.9) in the transverse colon, 4.8 (± 1.7) in the descending colon, and 1.1 (± 0.2) in the sigmoidal colon.

Gender-specific ASBT expression in the terminal ileum

We analyzed the data for sex-related differences. Only the biopsies obtained from terminal ileum showed gender-specific differences concerning their ASBT mRNA expression (Figure 3.7). Normalized ileal ASBT expression (± SEM) in women was 1426.8 (± 576.8), in men 533 (± 114.6). Women exhibited a 2.7-fold higher mean ASBT expression than men, however, the difference was not statistically significant.

Impact of sex hormones on the expression of ASBT in Caco-2 cells

Incubation with 0.1, 1, and 10 µM ethinylestradiol and 10 and 50 ng/mL progesterone for 72 h did not change the amount of ASBT mRNA in Caco-2 cells (Figure 3.8). Budesonide, as a positive control, showed a dose-dependent induction. ASBT mRNA amount increased 1.47-, 1.72-, and 1.82-fold compared to control cells using budesonide concentrations of 10, 20, and 25 µM, respectively.

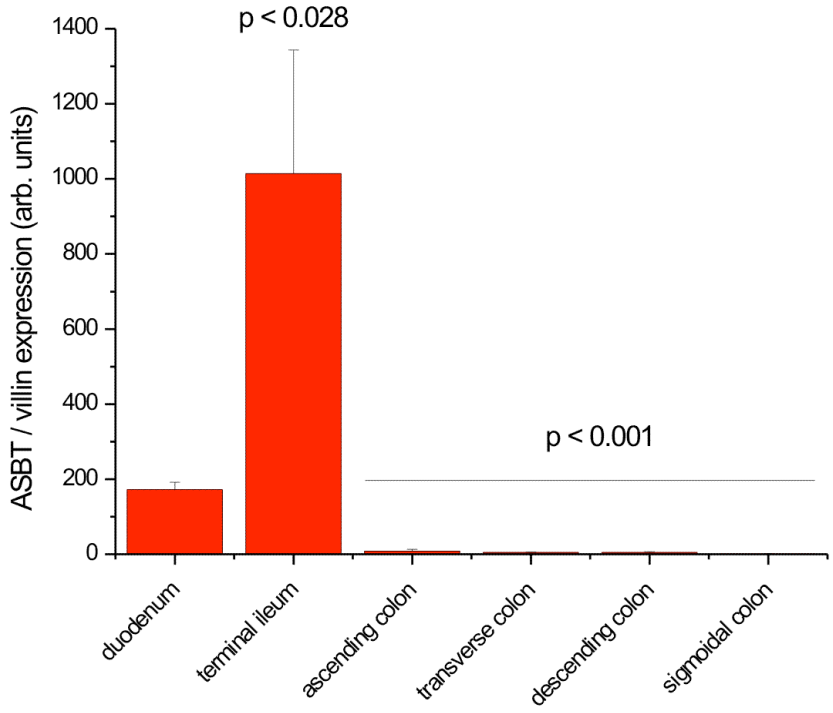

Figure 3.6: ASBT mRNA expression in different gut segments normalized to villlin expression. Data represent means (±SEM) of biopsies from 14 healthy subjects.

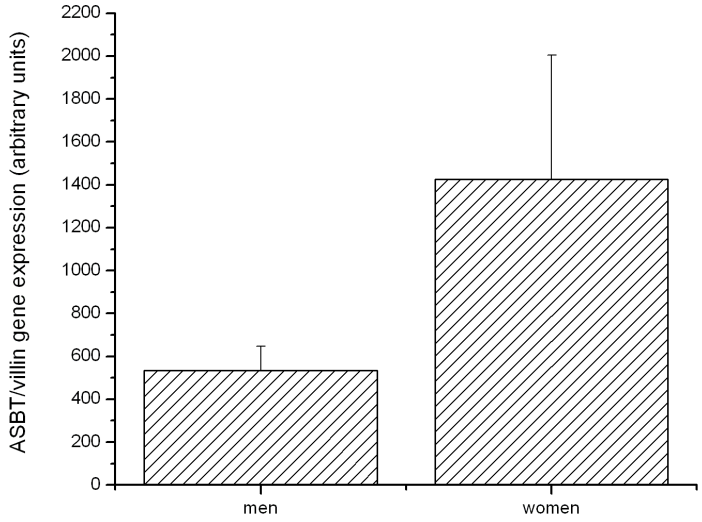

Figure 3.7: ASBT mRNA expression normalized to villin (±SEM). Biopsies were obtained from the terminal ileum of 6 men and 7 women. Difference is not significant.

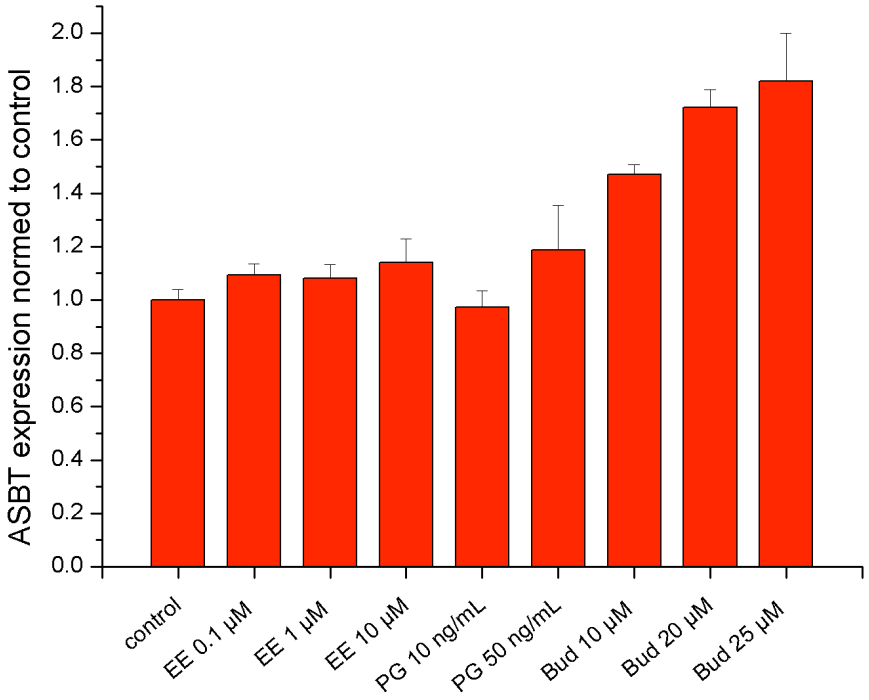

Figure 3.8: ASBT mRNA expression in Caco-2 cells normed to control. Cells were treated for 72 h with ethinylestradiol (EE), progesterone (PG), and budesonide (Bud). Values represent mean (±SEM).

3.3.4 Discussion

Our results indicate that human ASBT mRNA is expressed in the small intestine, predominantly in the terminal ileum but also, to a lower extent, in the duodenum. In the colonic segments only slight expression levels were observed. Since ASBT is seen as a potential target in pharmacotherapy (Hagenbuch and Dawson, 2004), data about the precise anatomic expression of this transporter are of relevance.

It was shown that the inhibition of intestinal reabsorption of bile salts results in an increase of hepatic cholesterol demand, leading to elevated low-density lipoprotein (LDL) receptor levels and decreased plasma LDL (Huff et al., 2002; West et al., 2002; Li et al., 2004). The use of binding resins was an early therapeutic strategy to increase fecal bile salt loss. Alternatively, high-affinity inhibitors of ASBT have been developed. Therefore, the knowledge of the anatomical distribution of this transporter can be of value, particularly regarding possible side effects of such inhibitors. Clinical trials have to assess whether ASBT inhibitors have advantages compared to the established cholesterol-lowering therapy (e.g. the use of statins).

Another potential role of ASBT could be a drug targeting, using the specificity of this transporter for bile salts combined with its high expression in the terminal ileum. ASBT has a very narrow substrate specificity encompassing only conjugated and unconjugated bile salts with negligible uptake of other organic anions (Craddock et al., 1998). Since ASBT shows very high expression in the terminal ileum compared to other tissues, drugs conjugated to bile salts could be targeted to ileocytes. This could be relevant for patients with Crohn's disease in whom mainly the ileum is affected.

Sexual hormones have been attributed to gender-differences in the expression of transporters in several animal studies with rats (Urakami et al., 2000; Cerrutti et al., 2002; Kobayashi et al., 2002; Buist and Klaassen, 2004). These transporters include organic cation transporters (OCTs), organic anion transporters (OATs), multidrug resistance proteins (MDRs), as well as the breast cancer resistance protein (BCRP). Our study showed gender-related differences in the expression of ASBT in the terminal ileum of humans. However, due to the small sample size and high inter-individual variability these differences were not significant. Potential factors for the observed trend towards elevated expression of ASBT in women were investigated in further *in vitro* experiments. The role of sex hormones or hormone replacement therapy on the expression of ASBT was addressed.

Caco-2 cells are known to express ASBT and Neimark and co-workers showed that in this cell line ASBT expression can be regulated by bile salts (Neimark et al., 2004). Therefore, we chose Caco-2 cells to investigate ASBT regulation by sex hormones. As a positive control budesonide was used since this glucocorticoid induced ASBT mRNA expression in ileal biopsies from healthy volunteers (Jung et al., 2004). Here we showed a dose-dependent induction in Caco-2 cells by budesonide. Sex hormones, such as ethinylestradiol and progesterone, however, did not change the expression levels of ASBT. Therefore, these hormones seem not to have a direct effect on the transcription of ASBT and further studies are required.

In conclusion, we showed for the first time that ASBT mRNA is expressed in the duodenum of the human intestine, however, to a lesser extent than in the terminal ileum. Colonic tissues showed only slight expression levels. Furthermore, gender-specific ASBT expression was observed in the terminal ileum, with a trend towards higher expression levels in women. Our *in vitro* data in Caco-2 cells did not provide evidence for a putative inductive effect of sex hormones on ASBT expression.

4. Evaluation of drug transporter interactions *in vitro*

4.1 Budesonide induces the expression of P-glycoptotein (MDR1) in an *in vitro* model of the intestinal mucosa

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Submitted to:
Inflammatory Bowel Disease

4.1.1 Abstract

Steroid resistance and inadequate response to glucocorticoid therapy are common problems in patients with inflammatory bowel disease (IBD). The underlying mechanisms are poorly understood. The involvement of P-glycoprotein, the product of the multidrug resistance gene MDR1, in the disposition of steroids has been described. Therefore, increased expression of this efflux transporter, located in the apical membrane of enterocytes, could lead to subtherapeutic steroid concentrations. Here, we investigated the hypothesis that steroids can induce MDR1 expression in the intestine using LS180 cells, an established model of the intestinal mucosa. Cells were treated with budesonide at a concentration of 1, 10, and 50 µM. As a positive control for MDR1 induction 10 µM rifampicin was used. Total RNA was extracted after incubating the cells for 6 h and 72 h, whereas proteins were extracted after 3 and 5 days of incubation. Real-time RT-PCR and Western blot analysis were used to determine mRNA and protein levels, respectively. Budesonide induced MDR1 mRNA expression in a dose-dependent manner at concentrations of 1, 10, and 50 µM. The effects relative to control cells were higher after an incubation time of 72 h (1.56-, 3.40-, 5.53-fold increase, respectively) compared to 6 h (1.00-, 1.71-, 2.87-fold increase, respectively). MDR1 protein levels increased substantially after 3 and 5 days of incubation with 50 µM budesonide compared to control cells. In conclusion, in this *in vitro* system, MDR1 expression is induced by budesonide, both at the mRNA and the protein level. We propose that the effect of budesonide on MDR1 expression represents one mechanism of steroid resistance in patients with IBD.

4.1.2 Introduction

Glucocorticoids are effective in the treatment of inflammatory bowel disease (IBD) by inhibiting the chronic intestinal inflammation (Malchow et al., 1984; Hanauer, 2004). However, inadequate response to glucocorticoids and steroid resistance are well known problems in these patients. Approximately 50% of patients with Crohn`s disease and 20% of patients with ulcerative colitis require other therapeutic strategies as a result of inefficient steroid treatment (Farrell and Kelleher, 2003). The underlying mechanisms of the glucocorticoid resistance in these patients are poorly understood.

The involvement of P-glycoprotein (P-gp), the product of the multidrug resistance gene MDR1, in the disposition of steroids has been previously described (Ueda et al., 1992; Schinkel et al., 1995). P-gp transports glucocorticoids and many other unrelated drugs out of the target cell through ATP-dependent efflux. Beside other tissues P-gp is expressed on the surface of peripheral blood lymphocytes (PBL) (Coon et al., 1991) and on the apical membrane of intestinal

epithelial cells (Zimmermann et al., 2004), both of which are putative targets of pharmacotherapy in IBD. It has been shown that IBD patients with constitutive high MDR1 expression in these cells exhibit a poor response to glucocorticoid treatment (Farrell et al., 2000). Furthermore, glucocorticoids themselves are known to induce P-gp expression demonstrated in PBL of glucocorticoid treated patients with ulcerative colitis (Hirano et al., 2004) and in the intestinal cells of dexamethasone treated rats (Yumoto et al., 2001). Therefore, increased expression of this efflux transporter by glucocorticoid treatment could lead to subtherapeutic drug concentrations in the target cells and diminished anti-inflammatory activity.

More recently clinical interest has focused on budesonide (Figure 4.1), a new synthetic glucocorticoid, for the treatment of active IBD. Budesonide is metabolized extensively presystemically in the intestinal wall and liver leading to a low systemical bioavailability (Schwab and Klotz, 2001). This results in a topical antiinflammatory effect on intestinal tissue, with decreased systemic glucocorticoid adverse effects (Hofer, 2003). Some glucocorticoids are known to be substrates of P-gp but only recently this was also shown for budesonide (Dilger et al., 2004). Along the same line P-gp expression was increased after topical budesonide treatment in the nasal mucosa of 5 patients (Henriksson et al., 1997).

Since there are no data available about P-gp induction in the human intestine by glucocorticoids including budesonide, we performed a study investigating P-gp mRNA and protein levels after a dose- and time-dependent incubation of LS180 cells with budesonide. LS180 cells are derived from human colon carcinoma cells and are an established intestinal *in vitro* system to measure P-gp induction (Schuetz et al., 1996; Pfrunder et al., 2003a). We hypothesised that budesonide might induce MDR1 expression in these human intestinal cells and that this effect might explain one mechanism of steroid resistance in patients with IBD.

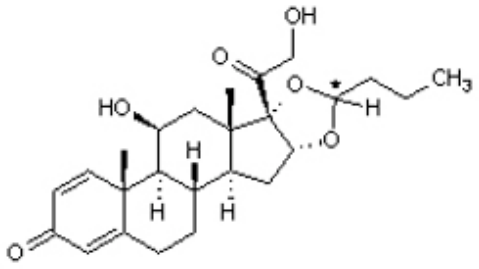


Figure 4.1: Structure of budesonide
Molecular Weight 430.53, Molecular Formula $C_{25}H_{34}O_6$
(from: http://www.pharmaceutical-technology.com/projects/astrazeneca/astrazeneca3.html)

4.1.3 Materials and methods

Cell culture

The LS180 cell line (used between passage 45 and 48) was purchased from ATCC (Manassas, USA). Cells were cultured in Dulbecco's MEM with Glutamax-I, supplemented with 10% (v/v) fetal calf serum, 1% non essential amino acids, 1% sodium pyruvate, 50 µg/ml gentamycin (Invitrogen AG, Basel, Switzerland). Cells were seeded into 6 well plastic culture dishes (9.2cm2/well, BD Falcon AG, Allschwil, Switzerland) and were maintained in a humidified 37°C incubator with a 5% carbon dioxide in air atmosphere. After the cells had reached confluence they were treated with 1, 10, and 50 µM budesonide (Sigma-Aldrich, St. Luis, MO, USA). As a positive control for MDR1 induction 10 µM rifampicin (Fluka Chemie AG, Buchs, Switzerland) was used in parallel. The compounds were dissolved in dimethyl sulfoxide (DMSO), whereas the final DMSO concentration did not exceed 1%.

Preparation of cells

After 6 and 72 hours of incubation (n=3) with pure medium, budesonide (1, 10, 50 µM), and rifampicin (10 µM) the cells were disintegrated by adding lysis buffer RLT (Qiagen, Hilden, Germany) and homogenized by using QIAshredder columns (Qiagen). Total RNA was extracted using the RNeasy Mini Kit (Qiagen). RNA was quantified with a GeneQuant photometer (Pharmacia, Uppsala, Sweden). The purity of the RNA preparations was high as demonstrated by the 260nm/280 nm ratio (range 1.8-2.0). After DNaseI digestion (Gibco, Life Technologies, Basel Switzerland) 0.75 µg of total RNA was reversed transcribed by Superscript (Gibco) according to the manufacturer's protocol using random hexamers as primers (Applied Biosystems, Rotkreuz, Switzerland).

TaqMan analysis and sequences of primers/probes were shown in chapter 2.

Western Blot Analysis

Cells were incubated in triplicates for 3 and 5 days with 50 µM budesonide and with medium as a control. Then proteins were extracted with protein extraction buffer (20 mM Tris-HCl, 1% Igepal CA-630, 0.5 mM sodium orthovanadate) including 1 mM phenylmethylsulfonyl fluorid (Sigma-Aldrich) and protease inhibitor mix (8 µM leupeptin hemisulfate, 5 µM bestatine hydrochloride, 2 µg/ml aprotinin, 6 µM E-64, 1 µM pepstatin A). 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, 75 µg of total protein extract was mixed with Laemmli sample buffer (Bio Rad Laboratories, Reinach, CH) and transferred to the polyacrylamide gel. Gel electrophoresis was performed with a Mini Protean 3 Electrophoresis Cell (Bio Rad) applying 80 mA for 40 min for the stacking gel (4% polyacrylamide) and 120 mA for 1 hour for the separating gel (6.5% polyacrylamide). After electrophoresis, proteins were blotted to the nitrocellulose membrane (250 mA for 2.5 hours) using a Mini Trans-Blot Cell (Bio Rad). The transfer buffer contained 25 mM Tris-HCl, 193 mM glycine, and 20% methanol. 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 for 45 minutes (0.05% Tween in PBS), the membrane was incubated for 2 hours at 37 °C with the primary, mouse anti-human antibody C219 against P-gp, 0.1 mg/ml (Alexis Corporation, Lausen, CH) diluted 1:50 in PBS containing 0.05% Tween, 1% bovine serum albumine (BSA) and 1% milk powder. After the first incubation the membrane was washed 3 times and then incubated with the secondary, horseradish peroxidase-conjugated, rabbit anti-mouse IgG (Amersham, Buckinghamshire, UK) diluted 1:1000. Secondary antibody incubation was performed for 1 hour at room temperature. Membranes were washed, and P-gp detection was performed with the enhanced chemiluminescence system (ECL-Detection-Kit, Amersham). The molecular weight was identified by using molecular weight Kaleidoscope TM Standard (Bio Rad).

4.1.4 Results

MDR1 mRNA expression in LS180 cells was induced by budesonide in a dose- and time-dependent manner (Figure 4.2). After an incubation time of 6 hours budesonide at concentrations of 1, 10, and 50 µM increased the MDR1 mRNA expression relative to control cells (mean ± SEM) 1.00-fold (±0.22), 1.71-fold (±0.18), and 2.87-fold (±0.24), respectively. After an incubation time of 72 hours the relative increase was 1.56-fold (±0.12), 3.40-fold (±0.58), and 5.53-fold (±0.24), respectively. Rifampicin (10 µM), as a positive control for P-gp induction, increased MDR1 mRNA expression 2.90-fold (±0.08) and 13.74-fold (±2.77) after incubation times of 6 and 72 hours, respectively. Western blot analysis revealed a substantial increase in MDR1 protein levels after 3 and 5 days of incubation with 50 µM budesonide compared to control cells (Figure 4.3).

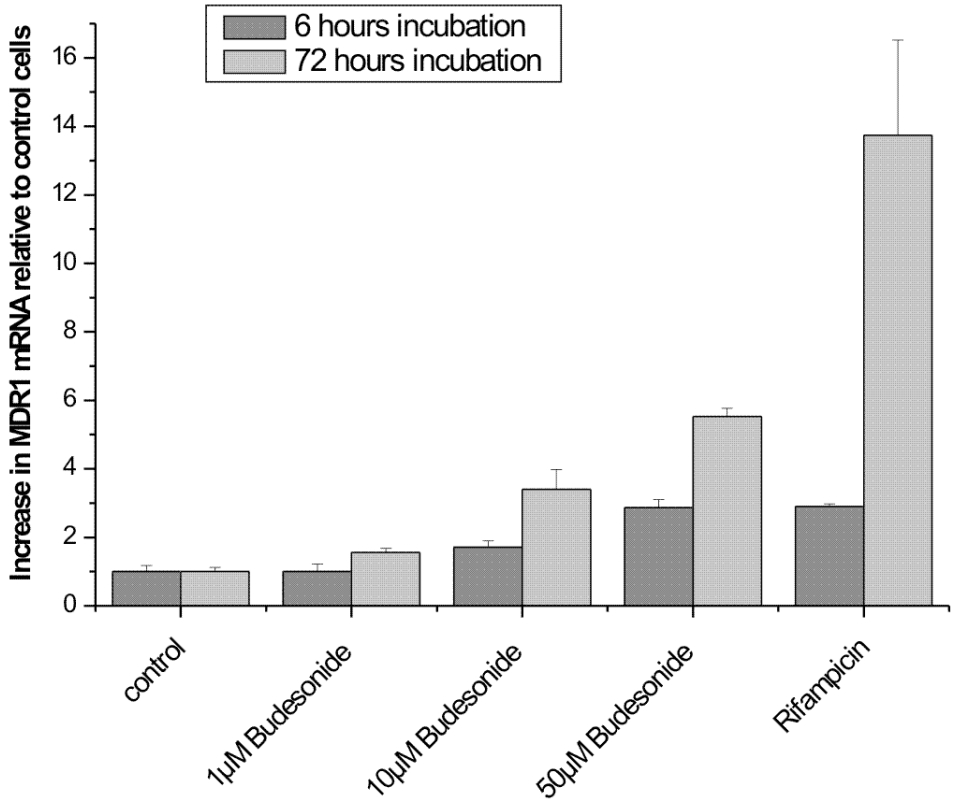


Figure 4.2: Expression of MDR1 mRNA in LS180 cells treated with medium (control), 1, 10, and 50 µM budesonide and 10 µM rifampicin for 6 and 72 hours. Values are normalized to control cells and represent mean ± SEM (n=3).

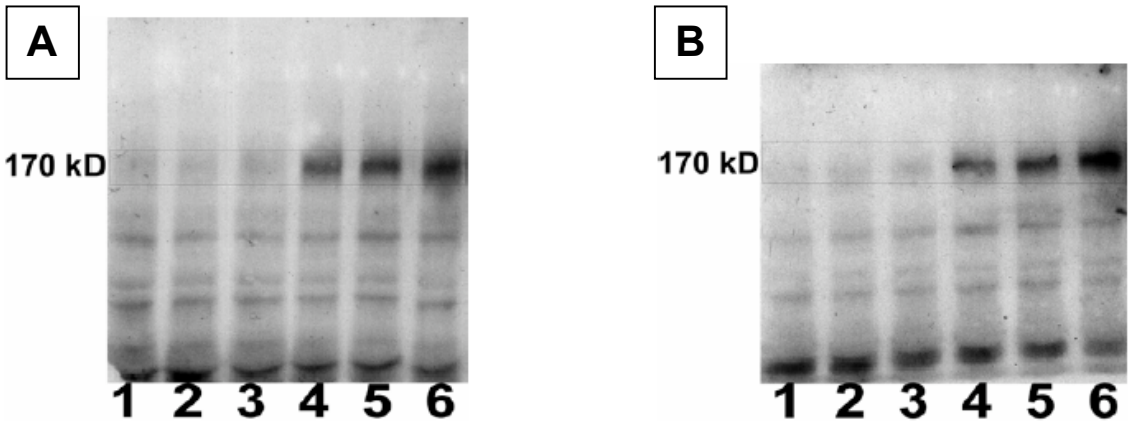


Figure 4.3: Western blot analysis of P-gp in LS180 cells with monoclonal antibody C219. Incubation times were 3 days (A) and 5 days (B). Columns 1-3 represent cells treated with medium and columns 4-6 represent cells treated with 50 µM budesonide.

4.1.5 Discussion

In this study we have shown that budesonide induces the expression of MDR1 in an *in vitro* model of the human intestine. So far, nothing is known about an effect of budesonide on intestinal P-gp expression. In one study, increased P-gp content was documented in the nasal mucosa of 5 patients after topical budesonide treatment (Henriksson et al., 1997). With respect to IBD patients, the effect of glucocorticoid treatment on the P-gp expression in enterocytes would be of special interest. There are several publications showing that dexamethasone induces P-gp expression in the intestine of rats (Yumoto et al., 2001; Perloff et al., 2004). Furthermore, drug interactions with the P-gp substrates cyclosporin A, indinavir and tacrolimus in dexamethasone treated rats have been reported (Lin et al., 1999b; Shimada et al., 2002; Yokogawa et al., 2002). Extrapolating these findings to budesonide, a similar effect could be assumed. The present results confirmed P-gp induction by budesonide in human intestinal cells.

The cells line we chose for this study were LS180 cells, a human colon carcinoma cell line. It has been shown before that several drugs such as rifampicin, reserpine, phenobarbital and verapamil can increase P-gp mRNA and protein content in this human cell line (Schuetz et al., 1996). *In vivo*, rifampicin treatment increased the P-gp content also in human duodenal biopsies (Greiner et al., 1999). Since LS180 cells behave like human intestinal cells with respect to P-gp induction, we used 10 µM rifampicin as a positive control in our experiments. The increase in MDR1 mRNA with 10 µM rifampicin and 50 µM budesonide was equal after 6 hours (about 2.9-fold increase) but after 72 hours 10 µM rifampicin showed a substantial stronger effect than 50 µM budesonide (13.74-fold versus 5.53-fold increase, respectively). Therefore, in vivo drug interactions that were reported for rifampicin (Greiner et al., 1999; Westphal et al., 2000b; Hamman et al., 2001) would be probably less pronounced for budesonide. The applied budesonide concentrations of 1, 10, and 50 µM are high, but we infer that they could be relevant local concentrations in the gut lumen when the drug is applied rectally as an enema. The P-gp expression in the small intestine would, however, most likely not be affected after rectal administration of budesonide and therefore interactions with drugs absorbed in the small intestine are not likely.

Other authors (Jung et al., 2004) revealed that budesonide intake increases the expression of the apical sodium dependent bile acid transporter (ASBT) in ileal biopsies of healthy subjects. They also showed that the ASBT gene was transactivated by the glucocorticoid receptor. The authors of the study claimed that ASBT induction by budesonide could be beneficial in patients with Crohn`s disease who exhibit reduced ASBT expression and suffer from bile acid malabsorption. Unfortunately, they did not examine P-gp expression in these biopsies. To the current knowledge,

MDR1 seems to be transactivated by the pregnane X receptor (PXR) (Geick et al., 2001). Consequently, budesonide seems to have an affinity to both the glucocorticoid receptor and PXR.
IBD patients with general high MDR1 expression in peripheral blood lymphocytes and in intestinal epithelial cells exhibit a poor response to glucocorticoid treatment (Farrell et al., 2000). The results of this study also showed a stable MDR1 expression in PBL among patients with IBD and controls regardless of disease activity or glucocorticoid therapy. The authors did, however, not investigate MDR1 expression in enterocytes in their patients. If patients with generally elevated MDR1 levels experience an additional transient induction through glucocorticoid treatment, this could result in a state of steroid resistance through markedly enhanced P-gp efflux.
On the other hand, knockout mice deficient for mdr1a P-gp developed an inflammation of the large intestine similar to inflammatory bowel disease (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 the colon (Langmann et al., 2004). Reinforcement of the intestinal barrier against bacterial toxins by increased P-gp expression could therefore be a further beneficial effect of steroid treatment.
In conclusion, budesonide increases the expression of P-gp in an *in vitro* model of the human mucosa. In patients with generally high MDR1 expression budesonide treatment could induce a state of steroid resistance due to strongly elevated intestinal P-gp levels. In these patients, the administration of steroids that show less pronounced MDR1 induction might reduce the incidence of steroid resistance. However, this hypothesis warrants further studies.

Acknowledgement
We thank Ursula Behrens for excellent technical assistance.

4.2 Thalidomide's potential for interactions with P-glycoprotein (MDR1)

4.2.1 Abstract

BACKGROUND: There is growing clinical interest in thalidomide for the treatment of various disorders due to its anti-inflammatory, immunomodulatory, and anti-angiogenic properties. In numerous clinical trials thalidomide is used as an adjunct to standard therapy. Therefore, clinicians should be aware of all possible drug-drug interactions that might occur with this drug. P-glycoprotein (P-gp), a drug efflux transporter that is expressed in many tissues, is the cause of several drug-drug interactions. Competition for substrate binding or up-regulation of this transporter can lead to significant changes concerning efficacy or safety of the therapy. In this study we investigated thalidomide's potential to cause drug-drug interactions on the level of P-gp. METHODS: LS180 cells were incubated with thalidomide for 72 h in order to determine P-gp induction using real-time RT-PCR. A human leukaemia cell line over-expressing MDR1 (CCRF-CEM/MDR1) was used to measure uptake of rhodamine 123, a P-gp substrate, in the presence of thalidomide. Dose-dependent and bi-directional transport of thalidomide through Caco-2 cell monolayers was performed to assess site-directed permeability. Transport rates were determined using HPLC including chiral separation of the thalidomide enantiomers. RESULTS: Thalidomide did not induce P-gp expression in LS180 cells. The uptake of rhodamine 123 in CCRF cells over-expressing MDR1 was not influenced by co-incubation with thalidomide. The transport through Caco-2 monolayers was linear and the permeability was similar for both directions. No differences between the thalidomide enantiomers were observed. CONCLUSIONS: Our study indicates that thalidomide is neither a substrate, nor an inhibitor or an inducer of P-gp. Therefore, P-gp related drug-drug interactions with thalidomide are not likely.

4.2.2 Introduction

Thalidomide was widely used in Europe as a sedative-hypnotic drug (Contergan®) since 1956. In the early 1960s it was withdrawn from the market due to its obvious teratogenic effects. Later, in 1965, it was reported to show remarkable efficacy against erythema nodosum leprosum (Sheskin, 1965). The recent renewed interest in thalidomide is based on its potential to treat inflammatory and autoimmune disorders and to its antiangiogenic activity (Meierhofer and Wiedermann, 2003). Thalidomide's anti-inflammatory and immunomodulatory effects of have been explained by a degradation of mRNA encoding tumour necrosis factor α (TNF-α) in monocytes (Moreira et al., 1993). Furthermore, thalidomide seems to inhibit the production of the cancer-associated growth factor interleukin 6 (Rowland et al., 1998; Kedar et al., 2004) and the production of several other cytokines (Meierhofer et al., 2001). Clinical applications of thalidomide include aphthous ulcers, Behçet`s syndrome, rheumatoid arthritis, graft-versus-host disease, and inflammatory bowel disease. In addition, malignant diseases such as haematological cancers, prostate cancer, and renal-cell carcinoma have been treated with thalidomide (Singhal and Mehta, 2002; Franks et al., 2004).

Thalidomide (α-phthalidomidoglutarimide) is a neutral racemic compound derived from glutamic acid. It is applied in equimolar amounts of (+)-(R)- and (-)-(S)-enantiomers that rapidly interconvert at physiological pH (Eriksson et al., 1998b). The parent compound undergoes spontaneous hydrolysis in aqueous solution at pH 7, leading to twelve hydrolysis products (Eriksson et al., 2001). This nonenzymatic hydrolysis is the main break-down mechanism of thalidomide in the body, whereas hepatic metabolism seems to play a minor role (Schumacher et al., 1965). Therefore, only low concentrations of 5-hydroxythalidomide were detectable in a human study (Eriksson et al., 1998a). Additionally, it was shown *in vitro* that thalidomide is a poor substrate for cytochrome P-450 isoenzymes and that it does not inhibit the metabolism of CYP-specific substrates (Teo et al., 2000). These results suggest that thalidomide is not involved in clinically important drug-drug interactions caused by an inhibition of cytochrome P-450 metabolism.

However, interactions may also occur at the level of drug transporters such as P-glycoprotein (P-gp), the gene product of MDR1. This protein appeared to be overexpressed in tumour cells with a multi-drug resistance phenotype, where it conferred resistance to many unrelated cytotoxic drugs (Juliano and Ling, 1976). P-gp is an efflux pump, located on the apical membrane of cells. It actively extrudes a variety of substances and presumably functions as a biological barrier against xenobiotics and pathogens (Ambudkar et al., 1999). Beside tumour cells, P-gp is generally expressed in many tissues such as intestine, kidney, liver, and blood brain barrier, where it is involved in the absorption, distribution and elimination of many drugs (Thiebaut et al., 1987;

Cordon-Cardo et al., 1990). Due to its broad substrate specificity it influences the pharmacokinetics of numerous unrelated substances, such as HIV drugs, anticancer drugs and endogenous compounds. (Lee et al., 1998; Borst et al., 2000; Takara et al., 2002). Therefore, drugs that induce P-gp expression or inhibit its function can alter the pharmacokinetics of concomitantly administered P-gp substrates. Many cases of drug-drug interactions on the basis of these mechanisms have already been described (Lin, 2003). For a drug that is a P-gp substrate, the outcome of P-gp inhibition can be elevated plasma concentrations, whereas P-gp induction can lead to decreased plasma concentrations. This can result in side effects or ineffective therapy, respectively.

Due to its antiangiogenic activity, thalidomide is being tested in patients who suffer from various cancers (Fanelli et al., 2003). In tumour cells, P-gp is often overexpressed, making them resistant to P-gp substrates (Juliano and Ling, 1976). Whether thalidomide is a substrate of this transporter could therefore be of interest. Moreover, many drugs used in cancer therapy, such as actinomycin C, etoposide, teniposide, paclitaxel, docetaxel, and topotecan, as well as glucocorticoids and morphine are P-gp substrates. Therefore, the coadministration of a potential inhibitor or inducer of P-gp could influence the therapy due to the described mechanisms.

Since thalidomide is mostly used as an adjuvant therapy, clinicians should be aware of all possible drug-drug interactions. So far, there is nothing known about the affinity of thalidomide to transporter proteins. In this study, it was investigated whether this drug has a potential for interactions on the level of P-gp. Experiments were performed to determine the effect of thalidomide on the function and expression of this clinically important drug efflux transporter. This knowledge can help to assess the risk of P-gp related drug-drug interactions during therapies where thalidomide is applied. Moreover, it might further elucidate the process of thalidomide absorption.

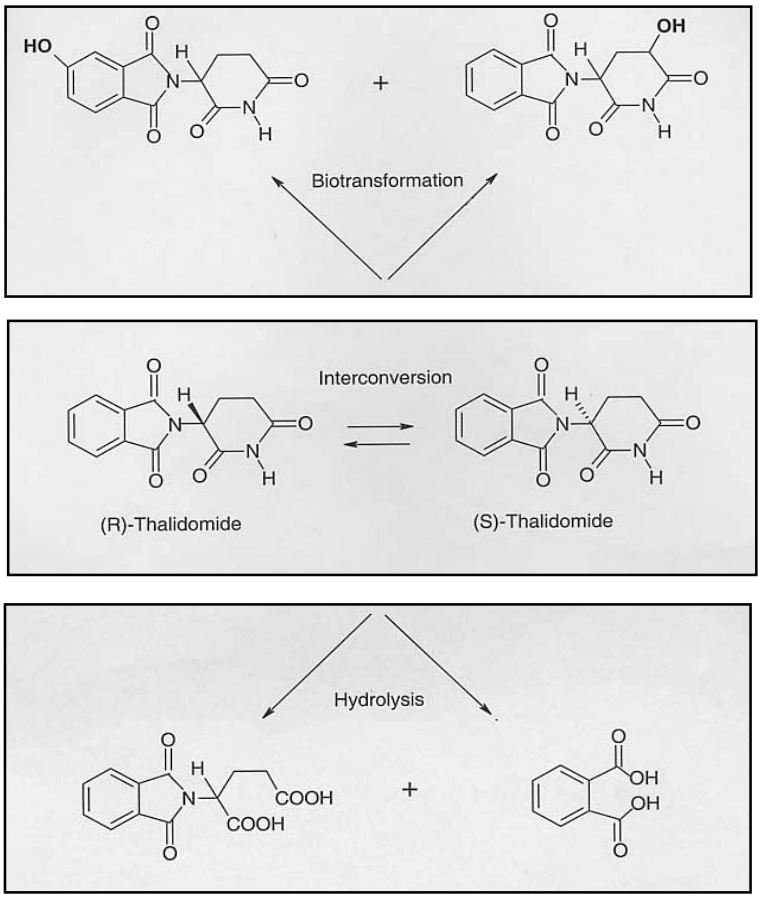


Figure 4.4: The pharmacokinetics of thalidomide: Biotransformation by cytochrome P-450 enzymes (negligible). Interconversion of the enantiomers (albumin and pH-dependent). Hydrolysis of the amide bonds (pH-dependent).

4.2.3 Materials and methods

Determination of MDR1 mRNA induction with quantitative real-time RT-PCR

(+/-)-Thalidomide and rifampicin (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) were dissolved in dimethyl sulfoxide (DMSO). LS180 cell line (used between passage 40 and 45) was purchased from ATCC (Manassas, USA). Cells were cultured in Dulbecco's MEM with Glutamax-I, supplemented with 10% (v/v) fetal calf serum, 1% non essential amino acids, 1% sodium pyruvate and 50μg/ml gentamycin. Cultures were maintained in a humidified 37°C incubator with a 5% carbon dioxide in air atmosphere. The cells were seeded into 6 well plastic culture dishes (9.2cm^2/well) and after they had reached confluence they were treated with 10 and 100 μM thalidomide, and 10 μM rifampicin for 72h. Medium was changed two times per day. At the end of

the culture period total RNA was extracted using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was quantified with a GeneQuant photometer (Pharmacia, Uppsala, Sweden). The purity of the RNA preparations was high as demonstrated by the 260nm/280nm ratio (range 1.8-2.0). After DNaseI digestion (Gibco, Life Technologies, Basel Switzerland) 1μg of total RNA was reversed transcribed by Superscript (Gibco, Life Technologies, Basel Switzerland) according to the manufacturer's protocol using random hexamers as primers.

TaqMan analysis and sequences of primers/probes were shown in chapter 2.
All samples were run in triplicates. Results are expressed as ratios of MDR1 expression to GAPDH expression.

Rhodamine 123 uptake in CCRF-CEM/MDR1 cells
CCRF-CEM/MDR1 cells (human leukaemic T-lymphocytes over-expressing MDR1) were a gift from Altana Pharma Ltd. (Konstanz, Germany). The cells are growing in suspension and were cultured in RPMI 1640 medium with Glutamax-I, supplemented with 10% FCS and 50 µg/mL gentamycin. Cells were continuously cultured in the presence of 1 µg/ml vincristine under 5% CO_2 / 95% air atmosphere at 37°C. One day before the experiment, cells were grown in vincristine-free medium.
Two million CCRF-CEM/MDR1 cells per milliliter were pre-incubated at 37°C for 10 min in the presence of 10, 100 and 300 µM thalidomide and 100µM verapamil (both Sigma-Aldrich Chemie, Schnelldorf, Germany), followed by an incubation with additional 5 µM rhodamine 123 (Molecular Probes, Eugene, OR). After 15 min, rhodamine 123 uptake was stopped by transferring samples on ice. Cells were washed three times in the presence of the previous applied drug at 4°C and lysed in 1 % Triton X-100. Aliquots were transferred into Optiplate-96 plates (Packard, Zürich, Switzerland) and fluorescence of the lysate was analyzed with a HTS 7000 Plus Bio Assay Reader (Perkin Elmer Ltd., Buckinghamshire, UK) with 485 nm excitation and 535 nm emission filters. Prior to the assay, possible quenching of rhodamine 123 due to thalidomide or verapamil was checked for all concentrations used in the uptake experiment.

Transport of (+/-)-thalidomide across Caco-2 cell monolayers
Caco-2 cells (used between passage 54-59) were purchased from ATCC (Manassas, USA). They were cultured in Dulbecco's MEM with Glutamax-I, supplemented with 10% fetal calf serum, 1% non-essential amino acids, 1% sodium pyruvate and 50 μg/ml gentamycin. Cells were seeded with a density of 6 x 10^4 cells/cm^2 onto Snapwell polycarbonate membrane filters (12 mm

diameter, 0.4 µm pore size; Costar, Cambridge, MA) and were cultured in standard six well cluster plates in a humidified 37°C incubator with a 5% carbon dioxide in air atmosphere. 16 days after seeding, the membranes with the cell monolayer were placed into a diffusion chamber (Costar, Cambridge, MA). The experiments were performed at 37°C with sample volumes of 4 mL per compartment. Cells were washed twice and equilibrated for 10 min with preheated transport buffer (HBSS + 1mM Pyruvate).

Bi-directional transport was initiated by adding transport buffer containing 100 µM thalidomide to the donor chamber (apical or basolateral side of the cell monolayer). The transport buffer was removed from the acceptor compartment after 5, 10, 15 and 30 minutes and replaced with fresh buffer. For the investigation of dose-dependent transport 10, 50, 100, and 250 µM thalidomide was added to the apical compartment. After 10 minutes the buffer in the basolateral compartment was removed for analysis.

The drawn samples were instantly acidified with citrate buffer (pH 1.5) and frozen until further processing. This approach prevents interconversion and hydrolysis of thalidomide enantiomers (Eriksson and Bjorkman, 1997). The paracellular marker fluorescein isothiocyanate dextran (FITC dextran, Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) was used for monitoring the monolayer integrity after each experiment. FITC dextran transport was measured in a fluorescence reader (Perkin Elmer HTS Soft 7000 Plus).

The transported amount of thalidomide in the transport buffer samples was determined by enantioselective HPLC analysis. Therefore, samples were extracted with dichloromethane : hexane (1:1, v/v), the extraction solvent was evaporated and the residue was re-dissolved in methanol for the injection into the HPLC system (Merck, Switzerland). The separation of the thalidomide enantiomers was performed with a chiral column (Chiralcel OJ, 250 x 46 mm, Daicel Chemical Industries). Anthracene (Riedel-de Haen, Seelze, Germany) served as internal standard. The mobile phase consisted of ethanol / hexane (65:35, v/v) with a flow rate of 0.85 mL/min at 40°C. Thalidomide enantiomers were quantified with UV-detection at 220 nm using external standard curves. All solvents were of HPLC quality (LiChrosolv®, Merck, Switzerland).

For the investigation of bi-directional transport, the apparent permeability values (P_{app}) were calculated as $\Delta Q/\Delta t \times 1/A \times 1/C_0$. $\Delta Q/\Delta t$ was determined by plotting the amount of transported thalidomide enantiomer (µmol) as a function of time (s). Time points were 5, 10, 15, and 30 min. The slope of the line was calculated using linear regression. A is the surface of the filter (cm^2) and C_0 is the thalidomide concentration (µM) in the donor compartment.

For the determination of dose-dependent transport, the transported amount of each thalidomide enantiomer (µmol) after 10 min was plotted against the applied concentration. The correlation coefficients were determined by linear regression.

4.2.4 Results

In LS180 cells, thalidomide treatment had no effect on MDR1 expression. Incubation with 10 and 100 µM thalidomide for 72 hours did not change the MDR1 mRNA transcript number compared to control (Figure 4.5). Rifampicin, as a positive control for MDR1 induction, increased the expression 2.5-fold. The expression of the housekeeping gene GAPDH was not altered under any of the treatments (data not shown).

The uptake of the P-gp substrate rhodamine 123 in CCRF-CEM/MDR1 cells was not significantly changed by thalidomide treatment compared to control (Figure 4.6). Thalidomide concentrations of 10, 100 and 300 µM did not influence P-gp function, whereas verapamil showed strong P-gp inhibition as the intracellular fluorescence of rhodamine increased about 5-fold. At all applied concentrations thalidomide and verapamil exhibited no quenching regarding the fluorescence of rhodamine 123 (data not shown).

The bi-directional transport of 100 µM thalidomide across Caco-2 cell monolayers indicates that the permeability (P_{app}) from A to B side was similar to that from B to A side (Figure 4.7). For (+)-thalidomide the P_{app} values (cm/s) were $6.89x10^{-5}$ and $5.70x10^{5}$, for (-)-thalidomide $6.90x10^{-5}$ and $5.67x10^{-5}$, respectively. There was a trend towards increased transport from A to B side (not significant, $p>0.1$). The enantiomers showed no differences in their permeability. The ratios P_{app} (B to A) over P_{app} (A to B), as a measure for active transport, were 0.826 and 0.822 for (+)- and (-)-thalidomide, respectively. Dose-dependent transport of 10, 50, 100, and 250 µM thalidomide after 10 min appeared to be linear. The correlation coefficients were >0.999 for both enantiomers (Figure 4.8).

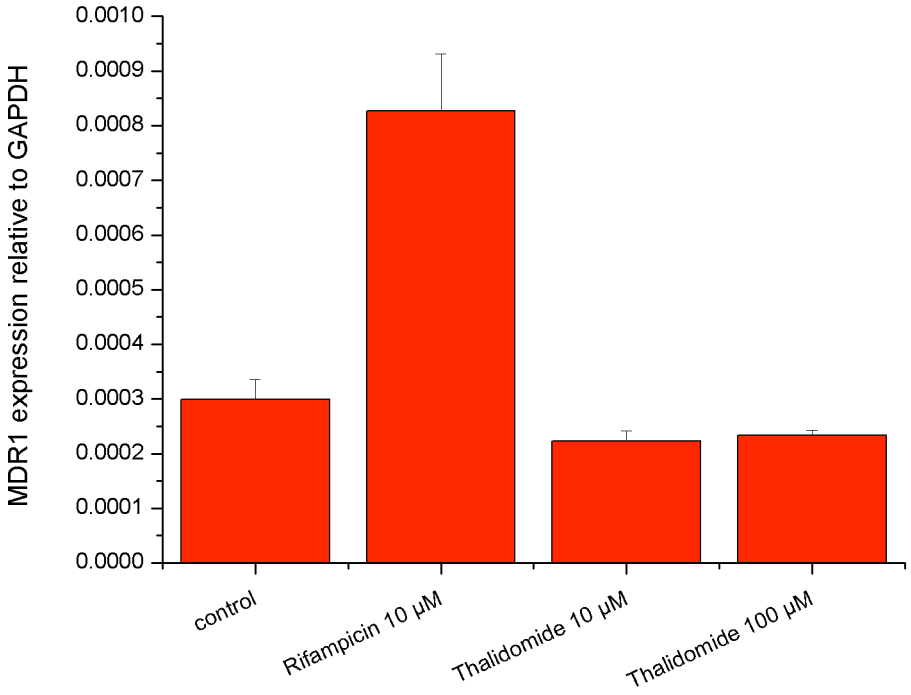


Figure 4.5: Expression of MDR1 in LS180 cells. Cells were incubated for 72h. Rifampicin served as a positive control for MDR1 induction. Values represent mean expression ratios (MDR1 expression / GAPDH expression) ± SEM.

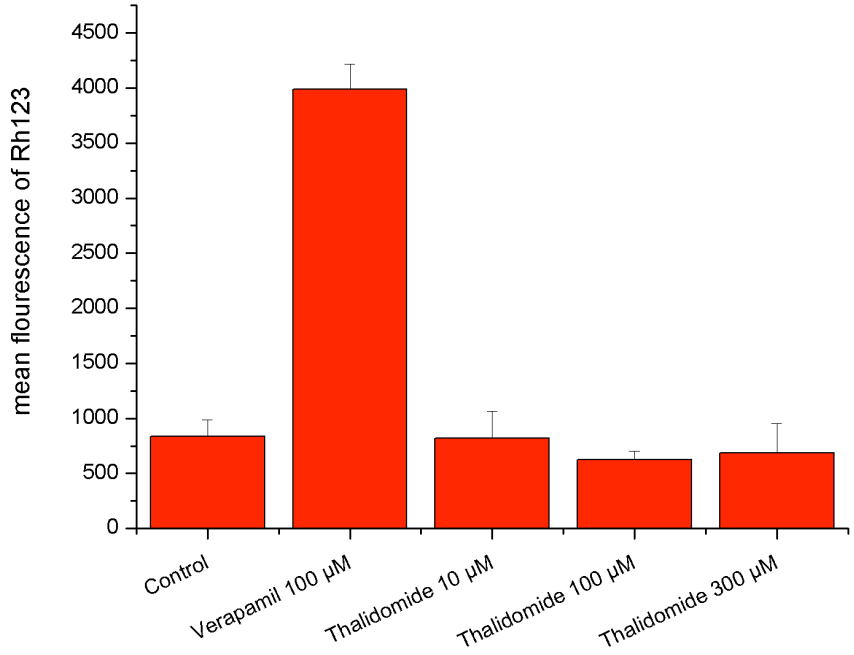


Figure 4.6: Uptake of R123 in CCRF/MDR1 cells. Verapamil was applied as a P-gp inhibitor. Values represent mean intracellular R123 fluorescence ± SEM.

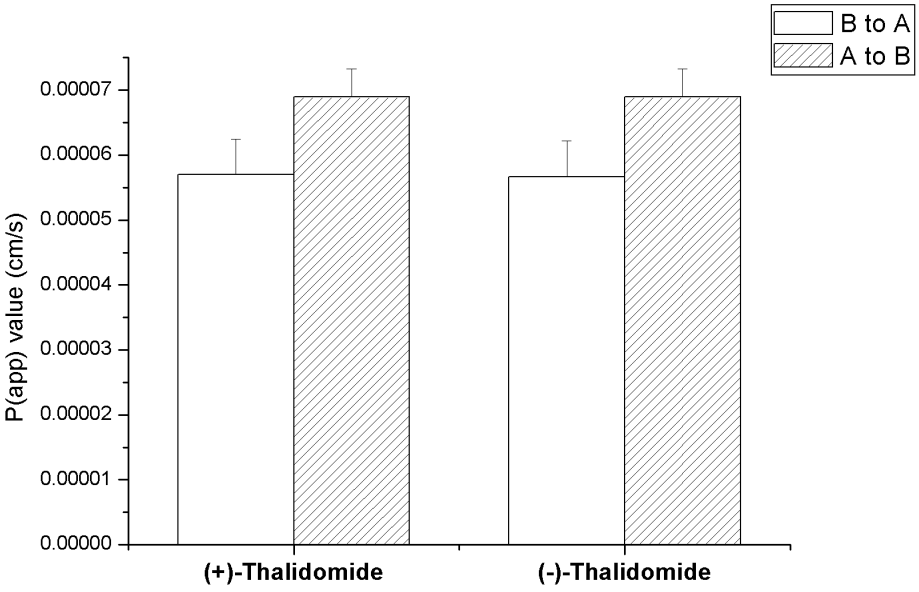


Figure 4.7: Apparent permeability (P_{app}) of (+/-)-thalidomide (100 µM) through Caco-2 cells. The drug was added to the basolateral side (B to A) or to the apical side (A to B) of the monolayers. Values represent mean ± SEM. Differences are not significant ($p>0.05$).

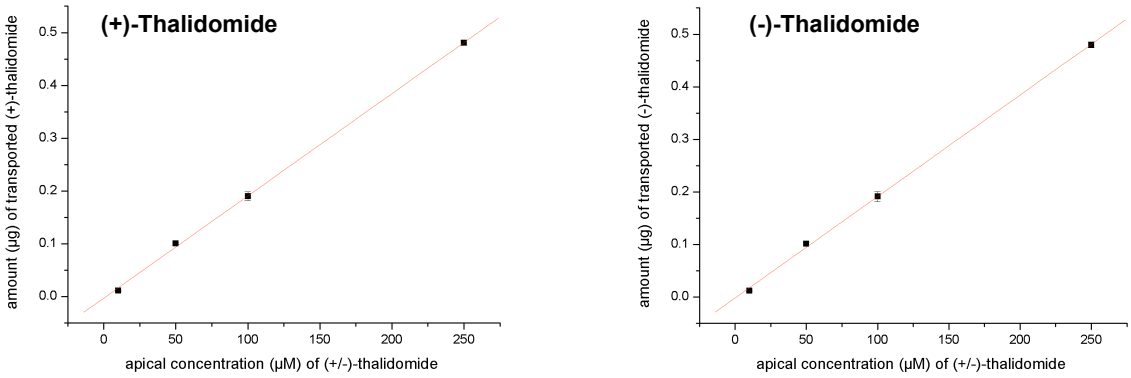


Figure 4.8: Dose-dependent transport of thalidomide through Caco-2 cell monolayers. 10, 50, 100, and 250 µM (+/-)-thalidomide were added to the apical compartment. After 10 minutes the transported amounts (µg) of (+)- and (-)-thalidomide were determined. Values represent means ± SEM.

4.2.5 Discussion

Drug transporters, such as P-glycoprotein, are increasingly recognized as an important determinant of drug disposition (Fromm, 2000). Like cytochrome P450 enzymes, inhibition and induction of P-gp have been reported as the cause of drug-drug interactions (Lin, 2003). Here we showed that the drug thalidomide is not capable of inhibiting P-gp function or inducing its expression. This knowledge may be of importance because thalidomide is a drug that is increasingly used in clinical studies, mainly as an adjuvant therapy. Therefore, we determined thalidomide's potential for interactions with P-gp.

P-gp expression has been shown to be inducible by xenobiotics such as rifampicin (Westphal et al., 2000b). Rifampicin treatment resulted in an increase in intestinal P-gp levels, which correlated with a decrease in oral exposure of both digoxin (Greiner et al., 1999) and talinolol (Westphal et al., 2000b). Further examples for P-gp induction are phenobarbital (Lu et al., 2004), dexamethasone (Fardel et al., 1993), and herbal extracts from St. John's Wort (Pfrunder et al., 2003b; Zhou et al., 2004). Due to enhanced efflux, increased P-gp expression can lead to subtherapeutic concentrations of concomitantly administered substrates. In this study we used the LS180 cell line as an appropriate model to investigate P-gp induction. LS180 cells are derived from a human colon carcinoma cell line and it has been shown that several drugs such as rifampicin or phenobarbital could increase P-gp and CYP3A4 mRNA and protein content in these cells (Schuetz et al., 1996; Pfrunder et al., 2003a). Rifampicin, used as positive control in our experiments, strongly induced MDR1 expression, whereas thalidomide did not change MDR1 mRNA levels.

Several cases of drug-drug interactions caused by P-gp inhibition have been reported. Elevated digoxin plasma concentrations were observed with cardiac drugs that are P-gp inhibitors, such as verapamil or quinidine (Mordel et al., 1993; Verschraagen et al., 1999). Ketoconazole, a potent P-gp inhibitor, caused a marked increase in the CSF concentration of ritonavir and saquinavir (Khaliq et al., 2000). The absorption of talinolol increased with the coadministration of verapamil (Gramatte and Oertel, 1999). By measuring the rhodamine 123 uptake in a cell line over-expressing MDR1, we used a specific assay for the investigation of functional P-gp inhibition. Though this assay is not able to discriminate between substrates and inhibitors, it can be concluded that thalidomide does not interact with P-gp, neither as a substrate nor as an inhibitor. Verapamil, a known inhibitor *in vitro* and *in vivo,* was used as a positive control showing significant accumulation of rhodamine 123 in the cells.

At present, it is unclear whether transporters are involved in the absorption, distribution and elimination of thalidomide. We determined the bi-directional permeability and the dose-dependent

transport of thalidomide enantiomers through Caco-2 cell monolayers. This cell line is generally accepted as *in vitro* model to study intestinal permeation (Artursson et al., 2001). Furthermore, Caco-2 cells are reported to express membrane transporters, such as P-gp and multi-drug associated proteins (MRPs) (Gutmann et al., 1999; Pfrunder et al., 2003a). Therefore, we used this model to assess if thalidomide underwent carrier-mediated transport and whether there is a difference in the permeability between *(+)*- and *(-)*-thalidomide. The ratio P_{app} (B to A) over P_{app} (A to B) is a measure for active, apical directed transport. For paclitaxel, a known P-gp substrate, this ratio was reported to be 41.9 in Caco-2 cells (Hugger et al., 2002). For thalidomide, we observed equal transport for both directions of the monolayer leading to a P_{app} ratio of 0.82. In a previous study, where thalidomide transport through Caco-2 cells was also investigated, similar results were obtained (Zhou et al., 2003). In addition, they showed that verapamil had no effect on thalidomide permeability. A drawback of their study, however, was the lack of chiral separation. The present study is the first one investigating the transepithelial transport of thalidomide including chiral separation of the enantiomers.

Due to the fast chiral inversion at physiological pH, the stereoselective absorption of thalidomide can hardly be determined *in vivo.* In order to avoid racemisation, the samples in our *in vitro* experiments were instantly acidified when drawn from the acceptor chamber (Eriksson and Bjorkman, 1997). Since both enantiomers share the same physical and chemical properties, different absorption rates could indicate an interaction of thalidomide with chiral structures such as proteins. Our results show, however, that there is no difference in the permeability of the enantiomers. Taken together, transport experiments through Caco-2 cell monolayers show linear and no site-directed transport of thalidomide and its enantiomers. This indicates that P-gp is not involved in the absorption process of this drug.

In conclusion, thalidomide exhibited no functional interaction with P-gp in our *in vitro* experiments. Furthermore, thalidomide did not induce P-gp expression. Drug-drug interactions due to these mechanisms are therefore unlikely. Transport studies showed linear permeability that appeared to be neither site-directed nor stereoselective. These results suggest that passive diffusion is involved in thalidomide absorption. Luminal efflux pumps like P-gp seem not to restrict oral thalidomide uptake, which could also explain the high bioavailability found *in vivo*. Moreover, the efficacy of thalidomide in cancer therapy is most likely not limited by P-gp overexpression in tumour tissues. Whether thalidomide has an affinity to other transporters cannot be ruled out by this study and further investigations are required.

4.3 Thalidomide's potential for interactions with multidrug resistance associated protein 2 (MRP2)

4.3.1 Introduction

The family of MRP proteins consists of ATP-dependent transporters that mediate the cellular extrusion of organic anions including conjugated drug metabolites (Konig et al., 1999). In particular, MRP2 mediates the biliary and intestinal secretion of many clinically important anionic drugs, along with the glucuronide- and glutathione-conjugates of many xenobiotics. (Suzuki and Sugiyama, 2000). Thus, beside P-gp and BCRP, MRP2 represents an important efflux pump in the intestine, which can lower the bioavailability of drugs such as furosemide, HIV protease inhibitors, indomethacin, methotrexate, pravastatin, probenecid, and SN38. Regarding the amount of drugs transported by MRP2, drug-drug interactions due to inhibition or induction of this transporter could be of relevance.

So far, there is no information available about thalidomide's affinity to drug transporters. The following assays were used to study the involvement of MRP2 in clinically important drug interactions, as well as in the intestinal absorption of thalidomide.

We used the LS180 cell line as a model to investigate whether thalidomide treatment might increase the expression of MRP2 in the intestine. A previous study showed that in duodenal biopsies obtained from 16 healthy subjects before and after nine days of oral treatment with 600 mg rifampicin per day, MRP2 mRNA and protein was induced by the treatment (Fromm et al., 2000). Therefore, rifampicin was used as a positive control in our experiments.

By measuring the CMFDA accumulation in a cell line over-expressing MRP2, we used a functional assay for MRP2 inhibition. The fluorescent marker CMFDA (5-chloromethylfluorescein diacetate), a precursor of the fluorescent MRP2 substrate MF-SG (methylfluorescein glutathione conjugate), was applied in MDCK cells stably transfected with MRP2. MK-571 was used as a positive control for MRP2 inhibition.

Caco-2 cell monolayers were used to simulate the oral absorption of racemic thalidomide. By using a stereoselectiv HPLC assay we were able to discriminate between both thalidomide enantiomers. The involvement of MRP2 in thalidomide absorption was investigated by co-incubating the cells with known substrates of this transporter (methotrexate, probenecid, pravastatin). Additionally, temperature-dependent transport was determined by performing thalidomide transport at 37°C and at 4°C.

4.3.2 Materials and methods

Induction experiments: materials and methods used for the induction experiments have already been described in chapter 4.2.3.

Sequences of primers and probes and TaqMan analysis were shown in chapter 2.

CMFDA uptake assay

CMFDA was purchased from Molecular Probes (Eugene, OR), whereas MK-571 was from Biomol (Plymouth Meeting, PA, USA). The Madin-Darby canine kidney cell line, stably over-expressing human MRP2 (MDCK/MRP2), was a gift from Dr. R. Evers (The Netherlands Cancer Institute, Amsterdam, The Netherlands). The cells form monolayers and were cultured in DMEM supplemented with 10% FCS. They were cultured under 5% CO_2 / 95% air atmosphere at 37°C in the appropriate medium containing 50 µg/mL gentamycin.

Non-fluorescent and membrane permeable 5-chloromethylfluorescein diacetate (CMFDA) was used as precursor of the MRP2 substrate methylfluorescein glutathion conjugate (MF-SG). The ester is cleaved by cytosolic esterases to the fluorescent, membrane impermeable 5-chloromethylfluorescein (CMF) which can then react with thiols on proteins and peptides (such as glutathione) to form conjugates. MF-SG can be secreted actively by MRP2.

MDCK/MRP2 cells were seeded into 24-well plates and were used when they reached confluence. For loading, they were incubated with 1 mL CMFDA solution (0.5 µM) for 1 hour at 10°C. Cells were washed twice with 0.5 mL HBSS containing (+/-)-thalidomide (10, 100, 300µM), MK-571 (20µM) or pure HBSS at 4°C. Efflux was performed by incubating the cells with 1 mL of the mentioned solutions at 37°C. After 30 minutes, solutions were removed and cells were washed with 1 mL of pure HBSS. For cell lysis Triton X-100 (1%) was applied and 200µL aliquots were analysed (HTS 7000 Plus Bio Assay Reader).

Transport through Caco-2 cell monolayers

Cultivation of Caco-2 cells was described in chapter 4.2.3.

Cells were seeded with a density of 6 x 10^4 cells/cm^2 onto Transwell polycarbonate membrane filters (Costar) with 0.4 µm pore size and 4.7 cm^2 growth area (6-well plate). Cells were grown to confluence and experiments were done 11-16 days after seeding.
Possible inhibition of thalidomide transport was investigated by pre-incubating the cells with the MRP2 substrates pravastatin, probenecid, and methotrexate (Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany) at a concentration of 50 µM for 30 min. Then, thalidomide (100µM) was added on the apical membrane. During the experiments the MRP2 substrates were also present in both compartments. Samples were taken from the basolateral side after 10, 20 and 30 minutes and P_{app} values were calculated (see chapter 4.2.3).
Temperature-dependent transport was performed at 37°C and at 4°C (on ice). Thalidomide (100 µM) was added to the apical side and samples were taken from the basolateral side after 10, 20 and 30 minutes.

4.3.3 Results

MRP2 expression was reduced 2.5-fold with thalidomide (100µM) and rifampicin (10µM) treatment. 10µM thalidomide did not influence MRP2 expression compared to control (Figure 4.8). The expression of the housekeeping gene GAPDH was not altered under any of the treatments (data not shown).
Thalidomide had no effect on the uptake of CMFDA in MDCK/MRP2 cells at concentrations of 10, 100, and 300 µM. MK-571 (20 µM), as a MRP inhibitor, increased CMFDA uptake about two-fold (Figure 4.9).
The apical to basolateral transport of thalidomide was not influenced by the addition of MRP2 substrates (50 µM) such as pravastatin, probenecid, and methotrexate (Figure 4.10). The P_{app} values did not change significantly and no differences between the enantiomers were detectable.
Thalidomide transport appeared to be temperature-dependent (Figure 4.11), as performing the experiment at 4°C resulted in a significant decrease in the P_{app} values ($p<0.0001$). For (+)- and (-)-thalidomide the P_{app} values were reduced to 18.1% and 23.7%, respectively.

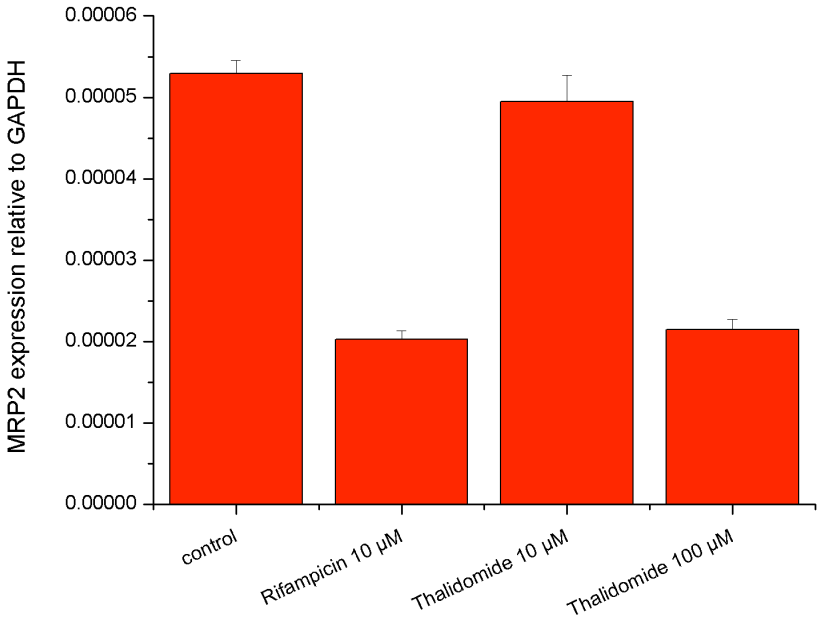


Figure 4.8: Expression of MRP2 in LS180 cells. Cells were incubated for 72h. Values represent mean expression ratios (MRP2 expression / GAPDH expression) ± SEM.

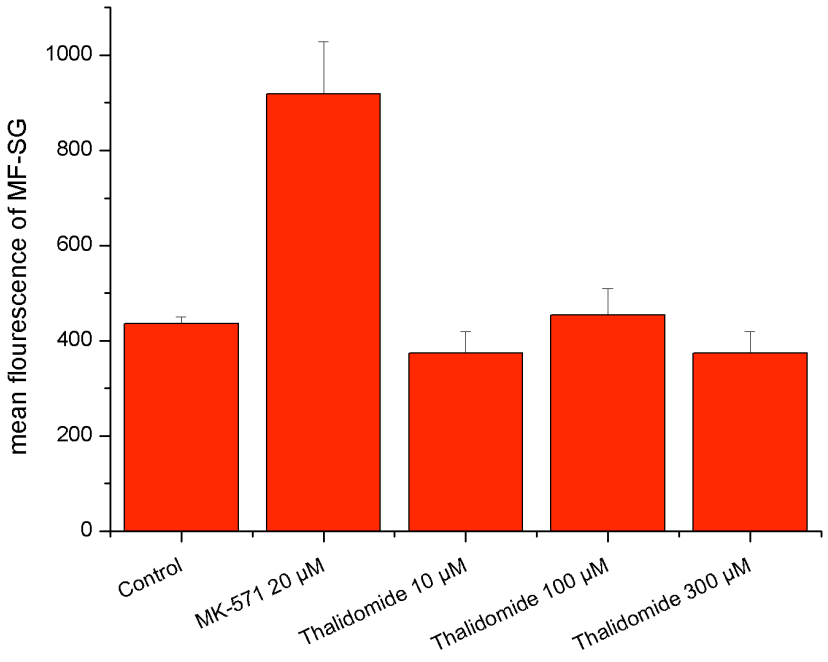


Figure 4.9: Methylfluorescein (MF) accumulation in MDCK/MRP2 cells. MK-571 was applied as a positive control for MRP2 inhibition. Values represent mean intracellular MF fluorescence ± SEM.

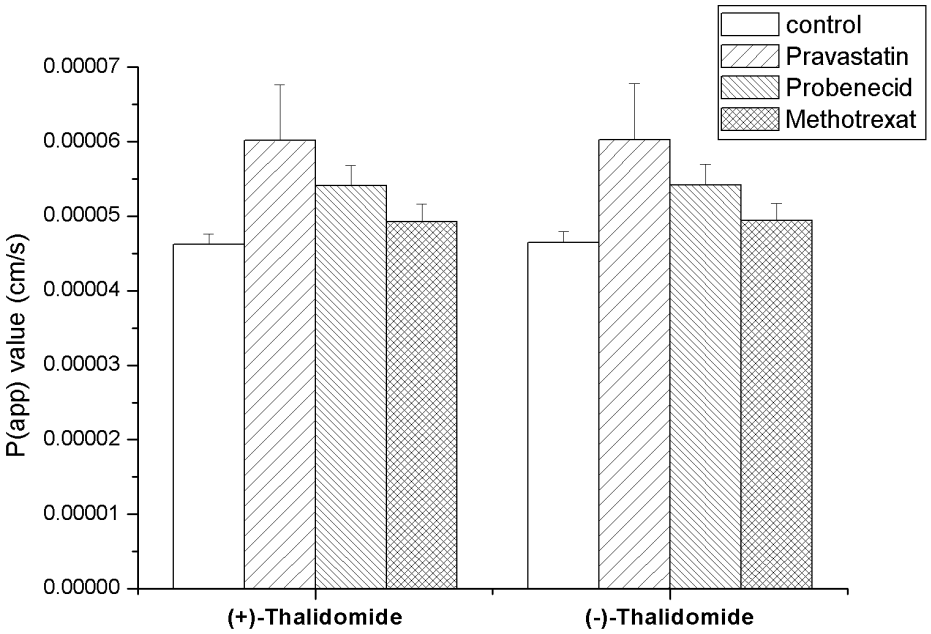


Figure 4.10: Permeability of (+/-)-thalidomide (100 µM) from the apical to the basolateral side of Caco-2 cell monolayers. The MRP2 substrates pravastatin, probenecid and methotrexate (50 µM) were present at both sides during the experiments. Values represent mean±SEM. Differences between the treatments are not significant ($p>0.05$).

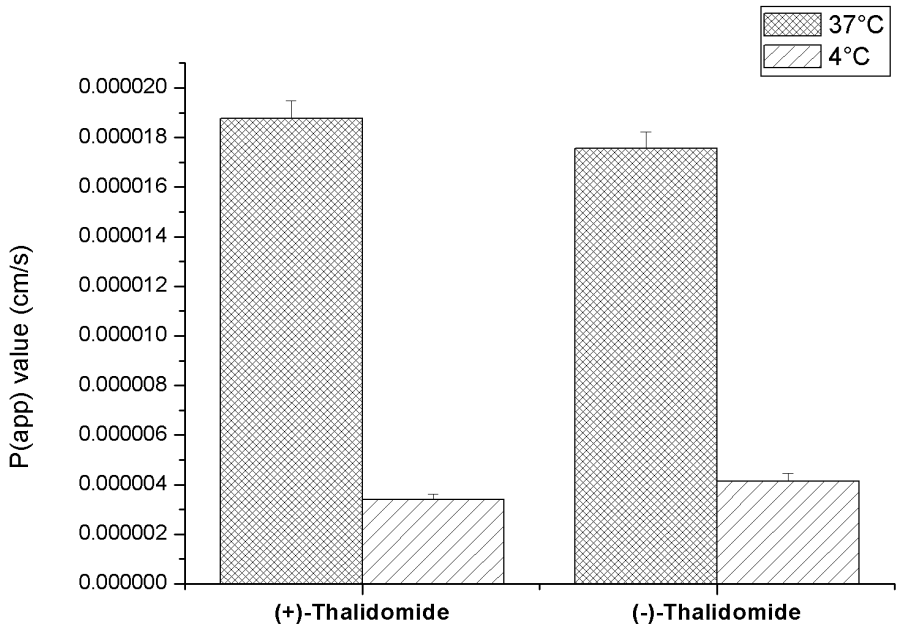


Figure 4.11: Permeability of (+/-)-thalidomide (100 µM) from the apical to the basolateral side of Caco-2 cell monolayers. The experiment was performed at 37°C and at 4°C (on ice). Values represent mean±SEM. Differences between 37°C and 4°C are significant for both enantiomers ($p<0.0001$).

4.3.4 Discussion

Thalidomide treatment did not induce the expression of the luminal efflux transporter MRP2 in LS180 cells. At a concentration of 100 µM thalidomide reduced MRP2 mRNA levels 2.5-fold. The same effect was seen with rifampicin treatment. However, in a study with human subjects treated with rifampicin MRP2 mRNA and protein levels increased in duodenal biopsies (Fromm et al., 2000). Consequently, LS180 cells might be no adequate model for MRP2 induction and an appropriate model has to be found. Concerning MDR1, the LS180 cells perform like human duodenum cells and show an induction under rifampicin treatment (Schuetz et al., 1996; Greiner et al., 1999).

Thalidomide did not influence the uptake of a fluorescent MRP2 substrate in a cell line over-expressing this transporter. Since racemic thalidomide did not show any effect we did not additionally apply the enantiomers. It can be concluded that thalidomide does not interact with MRP2, neither as a substrate nor as an inhibitor.

We investigated the absorption of thalidomide through intestinal cells by using Caco-2 cell monolayers. Caco-2 cells are reported to express membrane transporters, such as P-gp and MRPs, and are generally accepted as in vitro model to study intestinal permeation (Artursson et al., 2001). Therefore we used this model to assess if thalidomide underwent transporter-mediated active transport.

Carrier-mediated transport can be energy dependent or independent (e.g. facilitated). Energy dependent active transport mechanisms are not functional at 4°C and can be abolished by performing the experiment on ice. As we observed a pronounced reduction of the apical to basolateral permeability at 4°C, a basolaterally located active efflux carrier (e.g. MRP1 or MRP3) could transport thalidomide. But the absences of an inhibition with probenecid or methotrexate, both are substrates of MRP1 and MRP3, does not support this hypothesis. However, a further, not yet identified active transporter could transport thalidomide to the basolateral side. Alternatively, the reduced permeability might be attributed to a higher rigidity of the cell membrane at low temperatures leading to a lower diffusive transport. A more likely explanation is a reduced solubility of thalidomide at low temperatures leading to decreased concentrations in the apical compartment.

In conclusion, MRP2-mediated interactions of thalidomide with concomitantly administered drugs are not to be expected. Furthermore, the absorption of thalidomide in the gastrointestinal tract after oral intake will probably not be restricted by the expression of MRP2. The involvement of other transporters cannot be ruled out by these experiments and further studies are required.

5. Development and validation of a HPLC method for the determination of thalidomide enantiomers in whole blood in clinical trials

5.1 Abstract

This assay describes a method for the determination of racemic thalidomide in human blood. The separation of the enantiomers was performed by HPLC using a Daicel Chiracel OJ column. The isocratical eluent was ethanol/hexane (65:35, v/v). The flow rate was 0.85 mL/min at 40°C with detection at 220 nm. After acidification with citrate buffer (pH 1.5) the blood samples were extracted with dichloromethane:hexane (1:1, v/v) and redissolved in 200 µL methanol. The extraction efficiency was about 57% for (+)- and (-)-thalidomide and about 73% for the internal standard anthracene. (+)-Thalidomide, (-)-thalidomide, anthracene and caffeine (which occurred in all blood samples) were well separated (α = 1.48, 1.26, 1.30; R = 3.0, 2.7, 2.1). There was no interaction with caffeine. Calibration curves were performed with 6 standards of racemic thalidomide (n=2). For each enantiomer the range was 0.02 - 2.0 µg/mL (with a limit of quantitation of 20 ng/mL). The concentration of the internal standard was 2.0 µg/mL. The calibration curves were linear with correlation coefficients of 0.9957 for (+)-thalidomide and 0.9945 for (-)-thalidomide. Precision was performed at three concentrations (n=5). The coefficients of variation were well below the 15% limit. Accuracy was also below the 15% limit. We checked the stability of stock solutions of the pure enantiomers after 3 months at –20°C. No racemisation or degradation was observed. Acidified blood samples spiked with the pure enantiomers were also stable after 3 freeze / thaw cycles.

5.2 Introduction: Use of thalidomide in IBD

The inflammatory process in inflammatory bowel disease (IBD) is characterised by increased and continuous production of proinflammatory cytokines by intestinal lamina propria mononuclear cells and peripheral blood monocytes (Mahida et al., 1989; Reinecker et al., 1993). Increased TNF-α levels have been isolated in the serum, in the intestinal mucosa, and in the stools of patients with Crohn`s disease (CD) or ulcerative colitis (UC) (Murch et al., 1991; Braegger et al., 1992; Breese et al., 1994). Therefore TNF-α might play a central role in the inflammatory cascades and represents a therapeutic target in diseases with raised tissue concentrations of this cytokine. This assumption is supported by clinical studies with infliximab, a humanised chimeric monoclonal antibody against TNF-α. Infliximab was shown to be effective in at least two thirds of patients with steroid dependent chronic active CD (van Dullemen et al., 1995).

Thalidomide, an agent with TNF-α suppressive properties, was introduced in 1997 into the therapy of CD (Wettstein and Meagher, 1997). Up to now, there are 4 published clinical trials (with 10-22 patients) that investigated the effect of thalidomide administration (50-400 mg/d) in patients with CD and UC for 12 weeks (Ehrenpreis et al., 1999; Vasiliauskas et al., 1999; Bariol et al., 2002; Bauditz et al., 2002). The data suggest that thalidomide is an effective short-term treatment for symptomatic IBD. One study with long-term use (19-24 months) showed also that thalidomide is effective and safe in refractory CD (Facchini et al., 2001). In another study including 15 patients with refractory disease, thalidomide was effectively used to maintain response to infliximab over a few months (Sabate et al., 2002).

These observational studies of thalidomide in inflammatory bowel disease are promising, but the patient numbers are small. The authors of these studies conclude that the results support the need for placebo-controlled trials, where the efficacy and safety of thalidomide in IBD can be further investigated. For this purpose, a validated HPLC-method for the enantioselective determination of thalidomide enantiomers in blood is described in this thesis.

5.3 Materials and methods

Chemicals

(-)-(S)-Thalidomide, (+)-(R)-thalidomide and (+/-)-(R/S)-thalidomide were obtained from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany), purity > 98%. Anthracene Oekanal® was purchased from Riedel-de Haen Laborchemikalien GmbH&Co.KG (Seelze, Germany), purity: 99.3 %. Ethanol, n-hexane, dichloromethane and methanol were all of HPLC quality (LiChrosolv®) and were purchased from Merck (Switzerland). Citric acid 1-hydrate (p.a.) was obtained from Merck (Switzerland).

Solutions

Citrate buffer (pH 1.5, 0,025 M)

Extraction medium (dichloromethane: n-hexane, 1 : 1 (V/V))

Whole blood for laboratory use (Blutspendezentrum SRK beider Basel).

Stock solutions of racemic thalidomide and anthracene :

Stock 1	(8 mg /100 mL)	8.00 mg (+/-)-thalidomide in 100.0 mL methanol
Stock 2	(2 mg /100 mL)	25.0 mL of stock 1 *ad* 100.0 mL methanol
Stock 3	(0.08 mg /100 mL)	4.00 mL of stock 2 *ad* 100.0 mL methanol
Anthracene	(8 mg / 100 mL)	8.00 mg anthracene in 100.0 mL methanol

Apparatus

The HPLC system (LaChrom) and the software (D-7000 HPLC system manager) were from Merck Hitachi. The following components were integrated into the HPLC system: Interface D-7000, DA Detector L-7455, Column oven L-7300, Autosampler L-7200, Pump L-7100. The Degasser DG-4 was obtained from Henggeler. For the separation of the thalidomide enantiomers a chiral column (Chiralcel OJ, 250 x 46 mm, Daicel Chemical Industries) was used throughout. The column was protected by a LiChrospher C-18 guard column (4 x 4 mm, Merck).

HPLC conditions

The temperature of the column was 40 °C. The mobile phase consisted of 65% ethanol and 35% hexane. The flow rate of the mobile phase was 0,85 mL/min. The injection volume of the samples was 80 µL. UV-detection of the substances was performed at a wavelength of 220 nm. The run-time was 20 minutes.

Sample preparation

2.0 mL blood was given in a glass extraction tube. The samples were acidified by addition of 2.0 mL citrate buffer (pH 1.5, 0.025M). 50 µL anthracene stock solution (internal standard) was added to provide an anthracene concentration of 2 µg/mL. Thalidomide stock solutions were added as necessary. After addition of 5.0 mL of extraction medium (dichloromethane:hexane 1:1(V/V)) the samples were shaken for 20 min. The organic layers were separated by centrifugation (3000 n min^{-1}, 0°C, 10 min.) and transferred to another glass tube. The solvent was evaporated under a N_2 stream at 40°C for 12 min. The residue was redissolved in 200 µL of methanol by means of vortexing and ultra sonic. The solution was then filtered through a 0.2 µm filter and 80 µL were injected into the HPLC system.

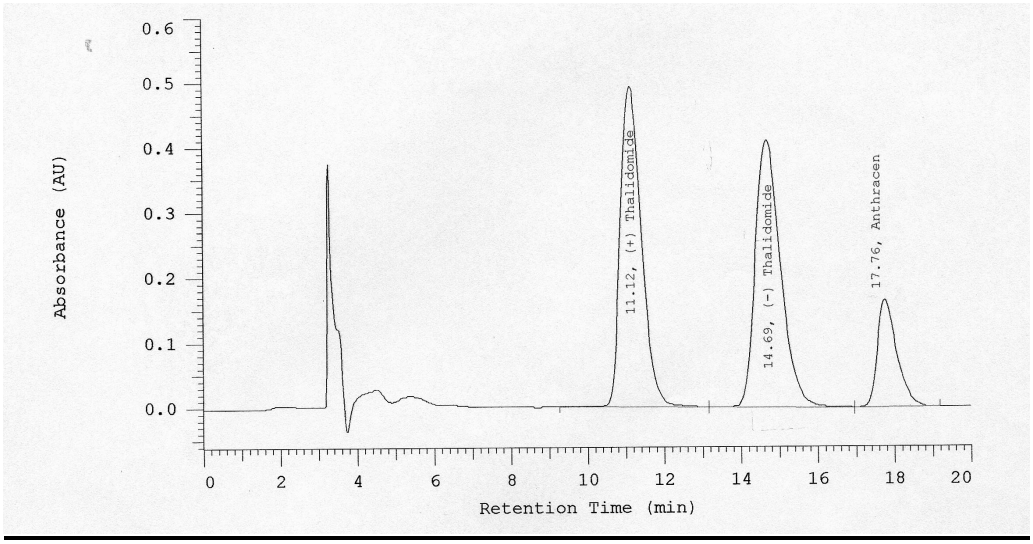
5.4 Results

Chromatography results

(+)-Thalidomide had a retention time of 11.15 min and (-)-thalidomide of 14.75 min. The internal standard anthracene appeared after 17.79 min and the retention time of the unretained solute (t_0) was 3.6 min (Figure 5.1).

The capacity factors ($k = t\text{-}t_0/t_0$) were 2.1 for (+)-thalidomide, 3.1 for (-)-thalidomide and 3.9 for anthracene. The selectivity of the separation ($\alpha = k_2 / k_1$) was 1.48 for the thalidomide enantiomers and 1.26 for (-)-thalidomide and anthracene. The resolution ($R = 2\ (t_2\text{-}t_1) / (w_1+w_2)$) was 3.0 for the enantiomers and 2.7 for (-)-thalidomide and anthracene (where w is the baseline width of a peak measured by extrapolating the relatively straight sides to the baseline). Caffein and (+)-thalidomide were also well separated ($\alpha = 1.3$, $R = 2.1$).

Chromatogram A



Chromatogram B

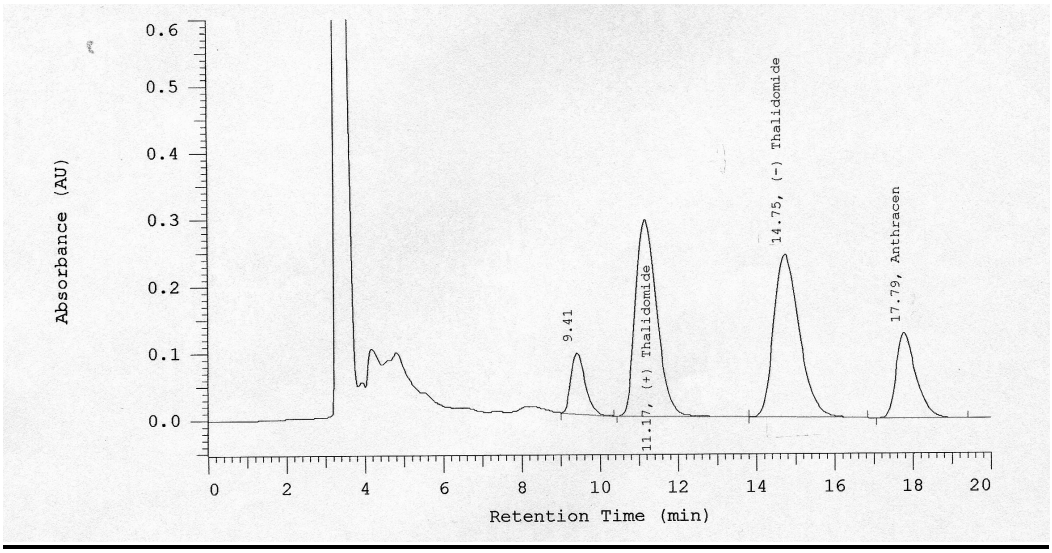


Figure 5.1: The chromatograms above show the separation of racemic thalidomide and the internal standard anthracene. The concentrations were 4 µg/mL for racemic thalidomide (2 µg/mL for each enantiomer) and 2 µg/mL for anthracene. In chromatogram A pure drugs were assayed (racemic thalidomide and anthracene solved in methanol). Chromatogram B was obtained after extracting the drugs from spiked blood samples. The peak appearing after 9.41min is caffeine, which will appear in almost all blood samples.

Calibration curve

Six standards were prepared in duplicate to create the calibration curves. For each enantiomer the range was from 0.02 µg/mL to 2.0 µg/mL. The lower limit of quantification (LLOQ) was 20 ng/mL with a signal to noise ratio (S/N) > 10. The concentration of the internal standard was 2 µg/mL throughout the analyses. The trend line was forced through origin. All response values were based on the ratio of the peak height of the thalidomide enantiomers to that of anthracene.

Standard	(+/-) Thalidomide	(-) Thalidomide	(+) Thalidomide	Stock solution	Anthracene
Standard 1	0.04 µg/mL	0.02 µg/mL	0.02 µg/mL	100 µL stock 3	50 µL (2 µg/mL)
Standard 2	0.2 µg/mL	0.1 µg/mL	0.1 µg/mL	20 µL stock 2	50 µL (2 µg/mL)
Standard 3	0.5 µg/mL	0.25 µg/mL	0.25 µg/mL	50 µL stock 2	50 µL (2 µg/mL)
Standard 4	1.0 µg/mL	0.5 µg/mL	0.5 µg/mL	100 µL stock 2	50 µL (2 µg/mL)
Standard 5	2.0 µg/mL	1.0 µg/mL	1.0 µg/mL	50 µL stock 1	50 µL (2 µg/mL)
Standard 6	4.0 µg/mL	2.0 µg/mL	2.0 µg/mL	100 µL stock 1	50 µL (2 µg/mL)

Table 5.1: Concentrations of thalidomide, its enantiomers and anthracene within the standard curves.

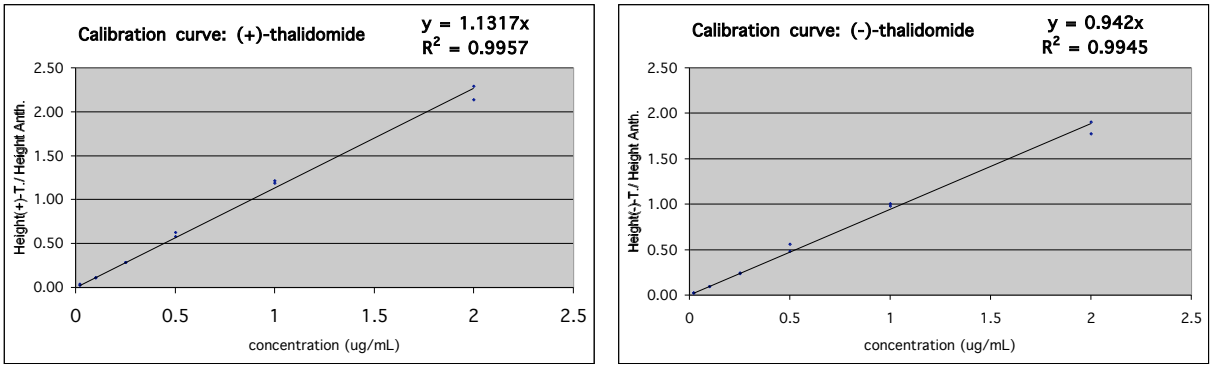


Figure 5.2: Six-point standard curves for (+)- and (-)-thalidomide ranged from 0.02 µg/mL to 2.0 µg/mL. The y-axis is expressed as height of thalidomide peak divided through height of anthracene peak (internal standard). Standard curves were linear with r^2 = 0.9957 and 0.9945 for (+)-thalidomide and (-)-thalidomide, respectively.

Selectivity

For the determination of selectivity blood samples from six drug-free volunteers were analysed by the method. There appeared a small endogenous peak at 11.0 min, which was seen in all blood samples (data not shown). This could be a possible interference with (+)-thalidomide. However, its height was only one third of the height of (+)-thalidomide at the LLOQ (lower limit of quantification). No other peaks occurred which could disturb the chromatographic determination.

Precision

Precision was measured by calculating the coefficient of variation (CV) of 5 determinations at three concentrations (0.02, 0.5, 2.0 µg/mL). For each concentration the CV should not exceed 15%, except for the LLOQ where the CV should not exceed 20%. We used peak height for further calculations, as more accurate results were obtained by this approach. In that case, for all concentrations the coefficient of variation was well below the 15% limit.

0.02 µg mL^{-1} (LLOQ)	**(+)-Th./Anthracene**		**(-)-Th./Anthracene**	
	Area	Height	Area	Height
Vial 1	2.22E-02	2.21E-02	3.17E-02	1.98E-02
Vial 2	3.43E-02	3.04E-02	3.52E-02	2.22E-02
Vial 3	2.21E-02	2.33E-02	2.55E-02	2.39E-02
Vial 4	2.43E-02	2.52E-02	3.29E-02	2.05E-02
Vial 5	2.62E-02	2.67E-02	2.97E-02	1.96E-02
mean	2.58E-02	2.55E-02	3.10E-02	2.12E-02
standard deviation	5.05E-03	3.25E-03	3.69E-03	1.82E-03
coefficient of variation	**19.6%**	**12.7%**	**11.9%**	**8.6%**

0.5 µg mL^{-1}	**(+)-Th./Anthracene**		**(-)-Th./Anthracene**	
	Area	Height	Area	Height
Vial 1	5.76E-01	5.62E-01	5.99E-01	4.64E-01
Vial 2	6.28E-01	5.79E-01	6.58E-01	4.90E-01
Vial 3	6.12E-01	5.64E-01	6.14E-01	4.63E-01
Vial 4	6.40E-01	5.91E-01	6.68E-01	4.97E-01
Vial 5	6.53E-01	5.97E-01	6.58E-01	4.96E-01
mean	6.22E-01	5.79E-01	6.39E-01	4.82E-01
standard deviation	2.97E-02	1.58E-02	3.08E-02	1.70E-02
coefficient of variation	**4.8%**	**2.7%**	**4.8%**	**3.5%**

2.0 µg mL^{-1}	**(+)-Th./Anthracene**		**(-)-Th./Anthracene**	
	Area	Height	Area	Height
Vial 1	2.45E+00	2.24E+00	2.46E+00	1.86E+00
Vial 2	2.45E+00	2.24E+00	2.46E+00	1.86E+00
Vial 3	2.56E+00	2.33E+00	2.57E+00	1.94E+00
Vial 4	2.10E+00	1.92E+00	2.10E+00	1.60E+00
Vial 5	2.72E+00	2.55E+00	2.72E+00	2.10E+00
mean	2.45E+00	2.26E+00	2.46E+00	1.87E+00
standard deviation	2.26E-01	2.25E-01	2.28E-01	1.82E-01
coefficient of variation	**9.2%**	**10.0%**	**9.3%**	**9.7%**

Table 5.2: Results of the determination of precision. At three concentrations (low, middle, high) the coefficient of variation (CV) was calculated for 5 determinations.

Accuracy

The accuracy is a measure of the proximity of mean test results obtained by the method to the true value obtained by the calibration curve. Accuracy was measured using 5 determinations at three concentrations. The mean value should be within 15% of the true value except for the lower limit of quantification where it should be within 20% of the true value. For both enantiomers the deviations of the mean from the true value were all below 15%.

(+)-Thalidomide

Concentration	Calibration curve	mean	Deviation
	(concentration x 1.1317)	(5 determinations)	(accuracy)
0.02 ug/mL	0.022634	0.0255	***11.24%***
0.5 ug/mL	0.56585	0.579	***2.27%***
2.0 ug/mL	2.2634	2.26	***-0.15%***

(-)-Thalidomide

Concentration	Calibration curve	mean	Deviation
	(concentration x 0.942)	(5 determinations)	(accuracy)
0.02 ug/mL	0.01884	0.0212	**11.13%**
0.5 ug/mL	0.471	0.482	**2.28%**
2.0 ug/mL	1.884	1.87	**-0.75%**

Table 5.3: Results of the determination of accuracy. At three concentrations (low, middle, high) the deviation of the theoretical value obtained with the standard curve from the mean of five determinations was calculated.

Extraction efficiency

For the determination of the extraction efficiency the peak height of extracted thalidomide from blood was compared with not-extracted thalidomide. Therefore we added the same amount of (+/-) thalidomide stock solution both to blood and to extraction medium. The solutions were processed according to the extraction method described before (5.3). The experiments were performed at three concentrations (0.1, 0.5, 2.0 µg/mL). The amount of anthracene was 2.0 µg/mL throughout. (+)- and (-)-Thalidomide showed an extraction efficiency of about 57% and anthracene of about 73%.

	(+)-thalidomide recovery	(-)-thalidomide recovery	Anthracene recovery
0.1 µg/mL	55.7%	55.0%	69.1%
0.5 µg/mL	57.9%	57.7%	73.3%
2.0 µg/mL	59.5%	59.4%	76.7%

Table 5.4: Results of the determination of extraction efficiency. Values represent the percentage of thalidomide and anthracene that can be extracted from blood samples using the described extraction procedure.

Stability of stock solutions and blood samples

Stock solutions

In order to determine the stability of thalidomide in stock solutions, solutions of each enantiomer in methanol (0.5 mg/100 mL) were prepared. Aliquots were stored either at room temperature (RT) or at -20°C. Directly after preparing and after 3 months the solutions were assayed to determine degradation (decrease of peak area) and racemisation (percentage of the main enantiomer).

	(+)-thalidomide solution	(-)-thalidomide solution
After preparing		
Racemisation (% main enantiomer)	96.4%	95.3%
Area	2331388	2162740
After 3 months (-20°C)		
Racemisation (% main enantiomer)	96.1%	94.9%
Area	2379842	2258674
After 3 months (RT)		
Racemisation (% main enantiomer)	52.7%	51.0%
Area	344275	397404

Table 5.5: Stability of (+)-thalidomide and (-)-thalidomide stock solutions. Results represent percentage of the main enantiomer and peak area. Stock solutions were assayed directly after preparing, after 3 months at –20°C and after 3 months at room temperature (RT).

The freshly prepared stock solutions showed a slight racemisation of (+)-thalidomide and (-)-thalidomide (96.4% and 95.3% main enantiomer, respectively). After 3 months at -20°C racemisation increased only little (0.3% and 0.4%, respectively). Degradation was not observed, as the peak areas were consistent. After 3 months at room temperature the solutions showed total racemisation (about 50% of both enantiomers). The peak areas decreased strongly (85 % degradation). In order to stabilise stock solutions of thalidomide, they should be kept in methanol at -20°C. This will avoid any racemisation or degradation of the drug for at least 3 months.

Blood samples

The stereospecific stability of thalidomide in blood samples was determined by performing freeze/thaw cycles prior to the extraction procedure. First of all, the stock solutions of the pure enantiomers were assayed in order to specify their purity. Then blood samples were spiked with these stock solutions, acidified and run through 3 freeze/thaw cycles. After the extraction the change in the optical purity (decrease of the percentage of the main enantiomer) was measured.

	(+)-thalidomide	(-)-thalidomide
Stock solution	96.3%	94.9%
Blood samples	96.2%	94.6%

Table 5.6: Determination of thalidomide racemisation in blood samples. Stock solutions of the pure enantiomers and spiked blood samples after 3 freeze/thaw cycles were analysed. Results represent percentage of the main enantiomer.

The stock solutions of (+)-thalidomide and (-)-thalidomide showed a purity of 96.3% and 94.9%, respectively. The blood samples after 3 freeze/thaw cycles, showed only a slight increase in racemisation (purity of 96.2% and 94.6%, respectively). Blood samples have to be acidified instantly. Then there will be almost no loss of the former optical purity.

6. Vasospastic persons with increased endothelin-1 levels exhibit differential expression of ABC-transport proteins

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6.1 Abstract

PURPOSE: To quantify the gene expression levels of the ABC-transporters MDR1 (P-glycoprotein) and MRP (multidrug resistance-associated protein) isoforms in isolated mononuclear cells of vasospastic persons with increased Endothelin-1 plasma levels. METHODS: Quantitative real-time RT-PCR was performed to determine the expression levels of the MDR1 (P-glycoprotein) gene and MRP1 to MRP5 genes as well as the expression of the ETA and ETB receptor in mononuclear cells derived from 11 vasospastic subjects compared to 10 healthy controls. RESULTS: Mononuclear cells of vasospastic subjects showed a significant decrease in the expression of MDR1 (P-glycoprotein) gene ($p=0.029$), MRP2 gene ($p=0.003$), and MRP5 gene ($p=0.013$) when compared to healthy controls. These effects were poorly correlated with ET-1 plasma levels. No significant ETA and ETB receptor expression was observed in both groups. CONCLUSIONS: Vasospastic persons differ in their expression pattern of MDR proteins from healthy controls. This might be an indirect effect of elevated ET-1 levels.

6.2 Introduction

ATP binding cassette (ABC) transporter proteins belong to a large superfamily of transport proteins that are highly conserved across evolution (Higgins, 1992). These transport proteins mediate the translocation of different structurally unrelated molecules across various membranes and are expressed in different tissues. They are located in the plasma membrane or in the membrane of different cellular organelles (Gottesman and Pastan, 1993). Therefore, they control the distribution of endogenous metabolic products and exogenous xenobiotics on a subcellular level as well as in the organism as a whole. Some of these proteins form specific membrane channels (Enkvetchakul et al., 2001). Others facilitate the transport of inorganic ions, or pump various organic compounds (Hipfner et al., 1999). For this transport activity, ABC proteins utilize the energy of ATP hydrolysis (Senior et al., 1995).

Numerous clinical data, mainly derived from cancer research, have revealed that the multidrug resistance phenotype is often associated with the over-expression of certain ABC transporters, termed multidrug resistance (MDR) proteins. P-glycoprotein (P-gp, MDR1, ABCB1) mediated multidrug resistance was the first discovered (Juliano and Ling, 1976; Debenham et al., 1982; Kartner et al., 1983; Ling et al., 1984) and probably still is the most widely observed mechanism in clinical multidrug resistance (Gottesman et al., 2002).

Beside P-gp, other efflux-pumps belonging to the group of multidrug resistance-associated proteins with 9 homologues (MRP1-MRP9) were characterized. Over-expression of some of these

transport proteins lead to MDR phenotype (Kool et al., 1997; Bakos et al., 2000). MDR proteins possess a broad substrate specificity (Gottesman and Pastan, 1993). Therefore, acute inhibition or decreased expression of such MDR proteins may result in an enhanced uptake and systemic accumulation of drugs, which may lead to an increased sensitivity or toxicity.

Self-reported observations of vasospastic subjects revealed an enhanced sensitivity to different drugs such as beta-blockers and calcium channel blockers (many of them are substrates of MDR transport proteins). All these subjects showed characteristic symptoms for the vasospastic syndrome like an inborn tendency towards cold hands and sometimes cold feet, a low body mass index, and low blood pressure that fluctuates markedly (Flammer et al., 2001). They also often show a slower sleep onset (Pache et al., 2001), significantly less feelings of thirst coupled with less daily fluid intake (unpublished data), and a higher plasma level of endothelin (Flammer et al., 2001).

Recently it was shown that Endothelin-1 (ET-1) in subnanomolar to nanomolar concentrations was able to rapidly reduce the activity of MRP2 mediated drug transport in shark rectal gland (Miller et al., 2002). This effect of MRP2 function could also be confirmed in killifish renal proximal tubules (Masereeuw et al., 2000) and a similar inhibitory effect was seen for P-glycoprotein. Both effects could be abolished when an ETB receptor antagonist was given but not when an ETA receptor antagonist was given. This prompted us to investigate the expression levels of P-glycoprotein and MRP1 to MRP5 in subjects with vasospastic syndrome and elevated ET-1 plasma levels, and compare it with the expression of these transport proteins in healthy controls.

6.3 Materials and methods

Blood samples

Blood samples were collected from 11 vasospastic subjects and 10 healthy controls. Vasospastic subjects and controls were recruited from the study “Pathophysiology of vascular dysregulation” (Swiss national protocol number 65/00; this study was performed at the University Eye Clinic of Basel, Switzerland). All participants gave written informed consent for all procedures before inclusion in the study. The protocol was approved by the Ethical Committee of the Department of internal medicine, University Hospital Basel Switzerland, and adhered to the guidelines laid down in the Declaration of Helsinki. A detailed medical history excluded individuals with a history of alcohol or drug abuse, systemic diseases (e.g., diabetes, high concentration of blood lipids, major arterial hypertension or other systemic circulatory diseases other than vasospasm) or who had

been taking any medication at least 4 weeks prior to the study. Subjects were included in the study after an ophthalmological examination without pathological findings and a screening for indicators of vasospasm. After local cooling of the fingers, vasospastic subjects exhibited a stop in blood flow for more than 20 s, which was detected by nailfold capillaromicroscopy (Gasser and Flammer, 1991). In addition, ET-1 plasma levels were determined by a specific radioimmunoassay, as described by Goerre et al. (Goerre et al., 1995). Therefore, blood samples were taken by venopuncture after 30 min of a rest in a supine position. All vasospastic subjects tested here exhibited an increased plasma level of ET-1, ranging from 2.13 to 4.13 pg/ml (reference value for females: 1.42±0.28 pg/ml; for males: 1.67±0.34 pg/ml) (Leu and Huang, 1995). Examination of healthy controls showed no vasospastic response and low ET-1 plasma levels. Individual ET-1 plasma concentrations are shown in Figure 6.1.

Processing of mononuclear cells

Mononuclear cells were isolated from 10 ml heparinized whole blood by density centrifugation as described by Maurer and colleagues (Maurer et al., 1977) using a lymphocyte separating medium (Lymphodex; Innotrain, Kronberg, FRG). After extensive washing (three times) with phosphate buffered saline (PBS) cells were centrifuged at 440x g for 5 min and the supernatant was aspirated. Dry pellets were frozen immediately and stored at -75 °C until use.

Total RNA was isolated using the RNeasy mini kit (Qiagen GmbH, Hilden, Germany) following the instructions provided by the manufacturer. RNA was quantified with a GeneQuant photometer (Pharmacia, Uppsala, Sweden). After DNase I digestion (Gibco, Life Technologies, Basel Switzerland) 2 µg of total RNA was reverse-transcribed by Superscript (Gibco, Life Technologies, Basel, Switzerland) according to the manufacturer's protocol using random hexamers as primers.

Quantitative real-time PCR was described in chapter 2.

Statistical Analysis

Gene expression was compared for each gene between the control group and the vasospastic patients by the two-sided non-parametric Mann-Whitney U-test. The level of significance was to $p<0.05$. Correlation analysis was performed using the non-parametric Spearman's rank correlation.

6.4 Results

The individual pattern of expression of MDR1 and MRP isoforms was qualitatively different between healthy controls and vasospastic patients. Whereas in healthy controls no systematic pattern of gene expression could be observed, each of the vasospastic patients showed a qualitatively similar expression pattern of MDR1 and MRP genes (Figure 6.2).

On average, vasospastic patients expressed about half as much of the MDR1 gene than controls and they showed a smaller inter-individual range of MDR1 expression than controls (Figure 6.3). This was significantly ($p=0.029$) smaller than in the control group. Expression of the MRP1 gene was slightly but not significantly ($p=0.085$) higher in the vasospastic patients and, on average, almost doubled. Expression of the MRP2 and MRP5 genes decreased significantly ($p=0.003$ and $p=0.013$, respectively) in the vasospastic patients. No significant changes in gene expression was observed for the MRP3 and MRP4 genes, although a trend to lower expression could be stated.

Non-parametric rank correlation (Spearman's p) showed, with the exception of MRP1, a weak negative correlation to ET-1 levels ($p= -0.31$ to -0.59). Although correlation was significant for MDR1, MRP2, and MRP5, the values of p indicate only poor to moderate correlation.

The expression of the endothelin receptors ETA and ETB in mononuclear cells of control and vasospastic subjects was not detectable by TaqMan anaylsis. A borderline expression was obtained for both groups with mean threshold cycle (Ct) values between 38.9 and 39.6 (Table 6.1). The Ct value is defined as PCR cycle number, where the PCR product (represented by a corresponding fluorescence) reaches a predefined threshold value. Earlier cycle number corresponds to higher amounts of cDNA of the gene of interest in the sample. Ct values above 38 cycles were judged to represent no or only trace amount of gene expression.

As a positive control, prostate tissue was used, where Ct values of 25.15 and 27.24 for ETA and ETB receptors were obtained, respectively. Since in every PCR cycle the DNA amount is approximately doubled, a 14,000-fold and a 5,000-fold difference for ETA and ETB receptor expression was observed, respectively. Therefore, mononuclear cells seem only to express trace amounts of these receptors.

Sample	ET-A-Receptor	ET-B-Receptor
Controls	39.14	39.33
Vasospastic	38.90	39.60
Prostate	25.15	27.24

Table 6.1: Mean threshold cycle (Ct) values in mononuclear cells of vasospastic and control subjects and in human prostate tissue. Ct value and cDNA concentration of the gene of interest are inversely correlated. A decrease by one in Ct values corresponds approximately to a 2-fold increase of cDNA concentration of the gene of interest. Ct values above 38 cycles are judged to represent no or only trace amount of gene expression.

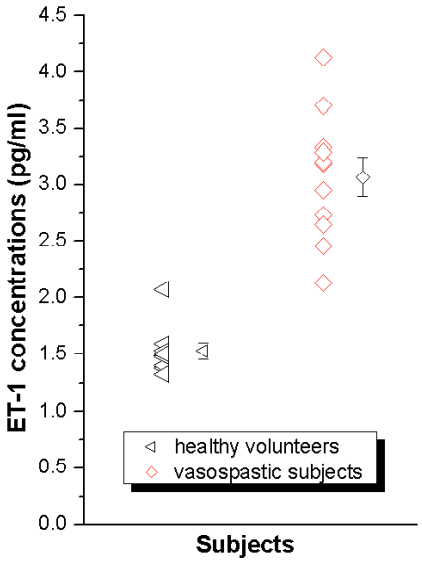

Figure 6.1: ET-1 plasma concentrations. Individual ET-1 concentrations as well as means and standard errors of the means (SEM) in healthy and vasospastic subjects.

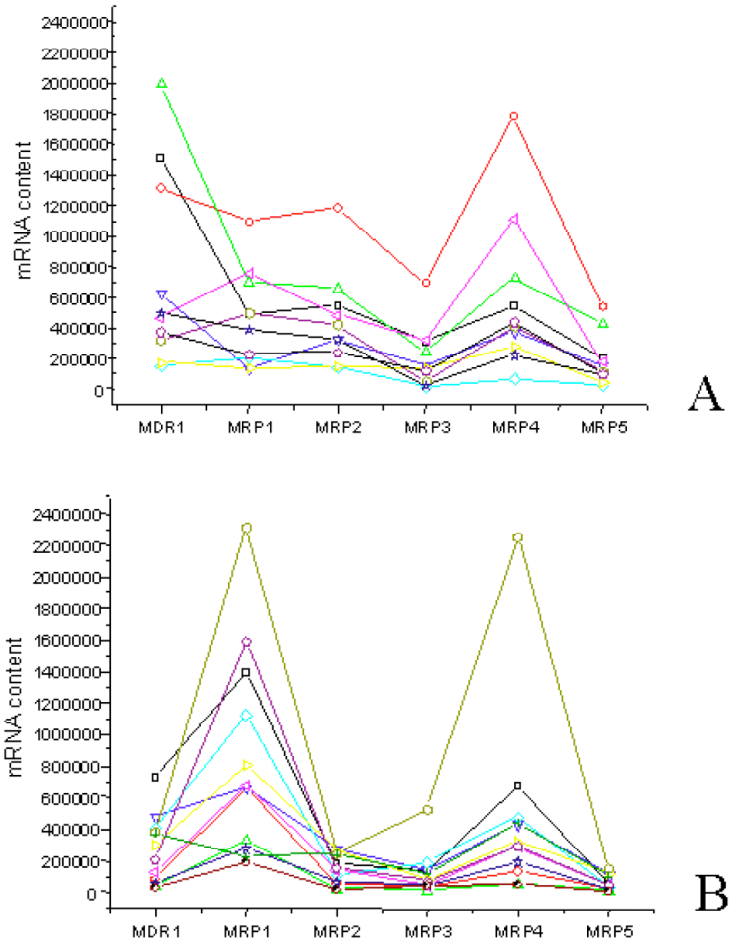

Figure 6.2: Individual gene expression of MDR1 (P-glycoprotein) and MRP isoforms (MRP1 to MRP5) genes: healthy controls (A), vasospastic persons (B).

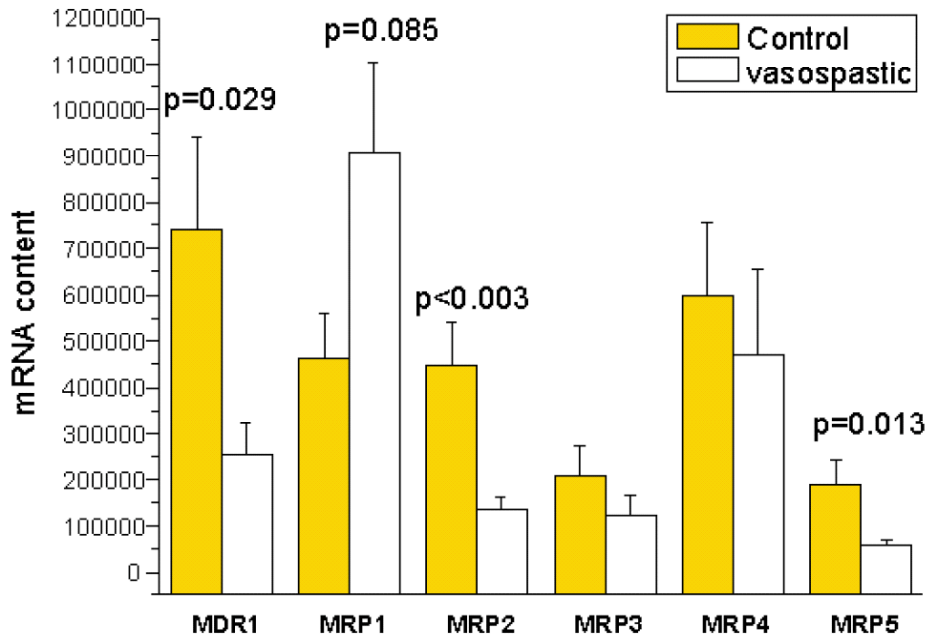

Figure 6.3: Mean gene expression of MDR1 and MRP isoform (MRP1 to MRP5) genes in healthy controls and vasospastic persons. Error bars represent the standard error of the mean.

6.5 Discussion

In addition to their contribution to the protection of the body against xenobiotics and to multidrug resistance in cancer, MDR proteins play an important but not yet fully understood physiological role.

The role of MDR proteins in the protection against toxic agents is supported by the wide substrate specificity of these transporters (Gottesman et al., 2002), the fact that MRP isoforms also mediate the transport of partially detoxified compounds, such as glutathione and glucuronide conjugates, and also by their tissue distribution. These transporters are present in important pharmacological barriers, such as in the blood-retina barrier by the retinal pigment epithelium (Kennedy and Mangini, 2002) as well as the endothelial cells of the brain capillaries (van Kalken et al., 1992) and in the epithelial cells in the choroid plexus (Rao et al., 1999), both contributing to the blood-brain barrier. They could be also identified in the brush border membrane of intestinal cells (Leu and Huang, 1995), the biliary canalicular membrane of hepatocytes (Accatino et al., 1996; Donner and Keppler, 2001), and the luminal membrane in proximal tubules of the kidney (Gutmann et al., 2000). So they are expressed as a consequence of differentiation triggers and in response to environmental challenges. Numerous studies revealed that MDR gene expression is not only

influenced by harmful chemicals and metabolites, but also by stress-evoking stimuli. This stress response can either occur as an increase in MDR mRNA due to heat shock (Kim et al., 1997), UV-, X-, and gamma-irradiation (Hill et al., 1990; Ohga et al., 1996; Harvie et al., 1997) or genotoxic stress (Kennedy and Mangini, 2002). On the other hand, induction of inflammatory response in experimental models of inflammation in rats and mice has been demonstrated to decrease the expression of P-gp at the levels of mRNA (Piquette-Miller et al., 1998). Thereby, P-gp expression is under the control of IL-6 (Sukhai et al., 2001).

Recently, acute inhibition of MDR1 (P-glycoprotein) and MRP2 function by the vasoactive hormone ET-1 (Masereeuw et al., 2000; Miller et al., 2002) was demonstrated in sharks and killifish. In the present study we demonstrate changes in the expression of MDR1 (P-glycoprotein) and MRP isoform genes in leucocytes of vasospastic subjects manifested in a significant down-regulation of the mRNA levels of MDR1 (P-glycoprotein), MRP2 and MRP5 in vasospastic patients with elevated plasma concentrations of ET-1.

Endothelin is one of the most potent vasoconstrictors and was first discovered by Yanagisawa and co-workers in 1988 (Yanagisawa et al., 1988). There exist three isoforms (ET-1, ET-2, and ET-3), each with 21 amino acids. ET-1 is present in many mammalian species, including humans. Although vascular endothelial cells are the major source of endothelin, it is also produced by a wide variety of cell types including renal tubular endothelium, glomerular mesangium, cardiac myocytes, glial cells, the pituitary, macrophages and mast cells (Inoue et al., 1989). Endothelins appear to act mainly as local paracrine/autocrine peptides, but circulating levels of endothelins also have great biological significance, especially in pathological states of increased serum concentration (Inoue et al., 1989; Sakurai et al., 1992).

Two receptors for endothelins have been characterized in humans, designated ETA and ETB receptor (Arai et al., 1990; Sakurai et al., 1990). The order of affinity of endothelins for ETA receptor is endothelin-1>endothelin-2>endothelin-3. ETB receptors show the same affinity for all 3 endothelins (Inoue et al., 1989; Arai et al., 1990; Sakurai et al., 1990; Sakurai et al., 1992). Both receptors are expressed in a wide variety of tissue types (Gandhi et al., 1992; Hayzer et al., 1992; Moreland et al., 1992; Prayer-Galetti et al., 1997).

Concerning leukocytes, opposed results are available: in the human monocytic cell line THP-1 the presence of ETB receptor mRNA was detected whereas another monocytic cell line (U937) lacked in its expression of the transcript (King et al., 1997).

The mechanisms of the changes in the expression of MDR1 (P-glycoprotein) and MRP isoforms in leucocytes of vasospastic persons are yet not understood. Although little is known about the signaling cascades that regulate MRPs, several pathways of their gene regulation appear to occur

through stimulation of enviromental factors. While stress signals increase levels of MRP1 mRNA (Harvie et al., 1997; Oosthuizen et al., 2000), MRP2 gene expression is down-regulated due to inflammatory cytokine release (Sukhai and Piquette-Miller, 2000). As mentioned above, in an animal model MDR1 (P-glycoprotein) and MRP2 mediated transport is under the control of ET-1, which acts here via protein kinase C (Masereeuw et al., 2000; Miller et al., 2002).

There are indications in our experiments that the effect of elevated ET-1 levels on the expression of MDR proteins might be indirect. Although various studies provide evidence of the ET receptors in monocytic cell lines by using binding assays or receptor inhibition experiments (McMillen et al., 1995; King et al., 1997), we could demonstrate that only trace amounts of mRNA transcripts of ETA and ETB receptor could be detected in isolated mononuclear cells of healthy controls and vasospastic persons. Compared to prostate tissue as positive control where both receptors are expressed, mononuclear cells showed a 14 and 5 thousand-fold less expression, respectively.

This view of an indirect mechanism is also corroborated by the poor intraindividual correlation between MDR protein expression and ET-1 plasma levels. The responsible mediating factors for MDR protein regulation are however not yet identified.

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7. Isolated project:

In vitro release of mitomycin C from collagen implants

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7.1 Abstract

PURPOSE: To study mitomycin C loaded collagen implant (CI) pharmacokinetics behaviour *in vitro*. METHODS: The CI were incubated for 15 minutes in different MMC loading solutions with the following concentrations: 0.03 mg/mL (n=9), 0.3 mg/mL (n=10) and 3.0 mg/mL (n=10). The loaded CI were transferred in 100 µL of 0.9% NaCl. Aqueous flow of 5 µL/min was simulated. The MMC concentrations of the samples were determined by high performance liquid chromatography (HPLC). Dissolution kinetics were evaluated by a first-order process. The half-life of dissolution and the time of 95% dissolution were determined. RESULTS: The CI absorbed on average a MMC dose of 0.054, 0.530, and 6.090 µg when incubated in the different MMC loading solutions containing 0.03 mg/mL, 0.3 mg/mL, and 3.0 mg/mL of MMC, respectively. In the release experiments, the mean total dose delivered by CI was 0.0493, 0.585, and 5.291 µg, respectively. A linear correlation between loading concentration and the estimated total dose released was demonstrated. The kinetic parameters showed a fast MMC dissolution. The half-life of the 3 series was 8.8, 10.1, and 10.5 min. CONCLUSIONS: Commercially available collagen implant can be loaded with MMC, and could provide relatively slower release than sponge delivery of MMC. Clinical implications of these results warrant further studies.

7.2 Introduction

Collagen implants (Aquaflow, STAAR AG, Nidau, Switzerland) are currently used to enhance success rates in deep sclerectomy[1-5]. They are manufactured from purified porcine collagen, and are slowly degraded postoperatively during 6-9 months, as has been shown by ultrasonic biomicroscopic studies[6,7]. Mitomycin C has been used to modulate tissue response to surgical trauma in glaucoma surgery[8].

This study was undertaken to examine the pharmacokinetics behaviour of mitomycin C loaded collagen implant (CI*) in vitro*.

7.3 Materials and Methods

Glaucoma collagen implant

The cylindrical collagen implant is manufactured by Staar Surgical AG, Nidau, Switzerland. It measures 2.5 mm in length and 1 mm in diameter. The collagen implant is processed from lyophilized American porcine scleral collagen, which is sterilised by a radiation procedure. This cross-linked, collagen-based biocompatible material does not induce a systematic immunologic reaction[3]. The water content of the hydrated device is 99%. Chiou et al[7,9] reported ultrasonic biomicroscopy (UBM) findings consistent with IOP lowering by aqueous filtration through the thin remaining TDM to an area under the scleral flap, which was hypothetically maintained open by the presence of the collagen implant.

Other available implants are the reticulated hyaluronic acid implant[10] and the hydrophilic acrylic non-absorbable implant (Dr Elie Dahan, personal communication).

Release experiment

Mitomycin C ampoules containing 2 mg were used (Roche Pharma AG, Reinach, Switzerland). Before each experiment, MMC was reconstituted by adding 666.7 µL of water for injection to make up a stock solution containing 3 mg/mL. The collagen implants were incubated for 15 minutes at ambient temperature in 100 µL of different MMC loading solutions with the following concentrations: 0.03 mg/mL (n=9), 0.3 mg/mL (n=10) and 3.0 mg/mL (n=10). The difference between dry and wet weight served as an estimate of total drug loading and was determined using a balance AT201 (Mettler Toledo, Greifensee, Switzerland). The loaded implants were transferred in 100 µL of 0.9% NaCl and the MMC release experiment was performed at 37°C using a Thermomixer 5436 (Eppendorf, Hamburg, Germany). Humour flow of 5 µL/min was simulated by removing a 37.5 µL sample after 7.5 minutes and after 15 minutes and then a 75 µL sample after every 15 minutes until no more MMC release was detectable. The removed volumes were immediately substituted with 0.9% NaCl.

HPLC assay

The MMC concentrations of the samples were determined by high performance liquid chromatography (HPLC) with UV detection at 365 nm. The HPLC system (LaChrom) and the software (D-7000 HPLC system manager) were from Merck Hitachi (Switzerland). A reversed phase column (Symmetry C8, 3.9x150 mm, Waters, Milford, Massachusetts) was used. The mobile phase consisted of 80% phosphate buffer (10 mM, pH 7.0) and 20% ethanol (v/v) with a flow rate of 1 mL/min at 30°C. External calibration was performed with standard curves ranging from 7.5 ng/mL to 30 µg/mL, with a lower limit of quantitation of 7.5 ng/mL.

Evaluation of kinetic parameters

Dissolution kinetics was evaluated by a first-order process represented by the following formula: Dose(released) = Dose(est)*(1-exp(-k*t)), where Dose(est) denotes the estimated total dose released and k denotes the first-order dissolution rate constant. From these estimates, the half-life of dissolution (T1/2 = ln(2)/k) and the time of 95% dissolution (T95% = -ln(0.05)/k) were determined. Parameters were estimated by non-linear regression analysis using Origin Software (version 7, OriginLab Corp., Northampton MA, USA). Dose linearity was assessed by linear regression analysis. In case of significance, analysis of variance was followed by Scheffe`s multicomparison test for pair wise comparisons. Level of significance was p=0.05. Comparisons were performed by SPSS for windows, version 11.0.

7.4 Results

Using 3 different MMC loading solutions containing 0.03 mg/mL, 0.3 mg/mL, and 3.0 mg/mL, we obtained the following results (Table 7.1). The implants absorbed a mean MMC dose of 0.054, 0.530, and 6.090 µg, respectively, which was determined by taking the difference between dry and wet weight (mean dry weight: 0.30, 0.309, and 0.338 µg, mean wet weight: 2.09, 2.077 and 2.348 µg, respectively). In the release experiments, the mean total dose delivered by the implants was 0.0493, 0.585, and 5.291 µg, respectively. A linear correlation between loading concentration and the estimated total dose released was demonstrated (Figure 7.1 and 7.2). The kinetic parameters showed a fast MMC dissolution. The half-life of dissolution (T1/2) of the 3 series was 8.8, 10.1, and 10.5 min, respectively. The time of 95% release (T95%) was 37.8, 43.6, and 45.5 min, respectively. After 90, 120 and 150 min there was no detectable MMC release.

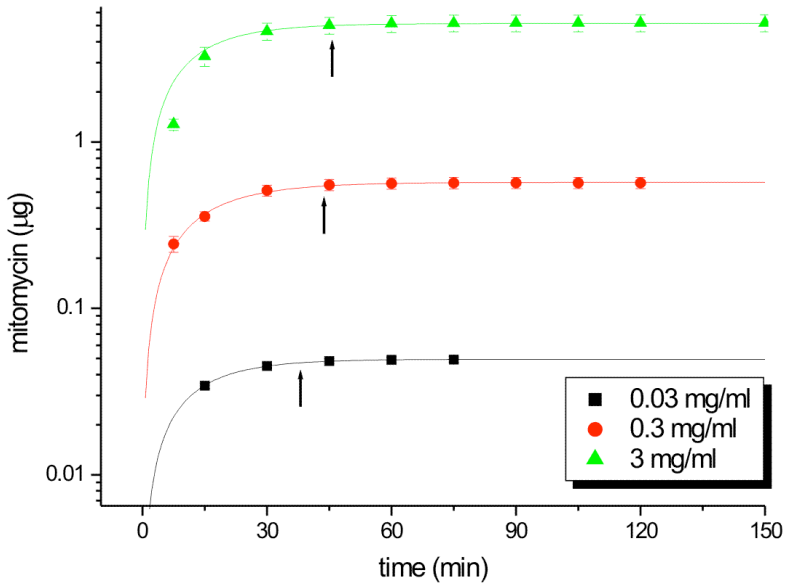

Figure 7.1: Mean (± SEM) cumulative mitomycin C release (µg) over time. Arrows denote times of 95% drug release (T95%).

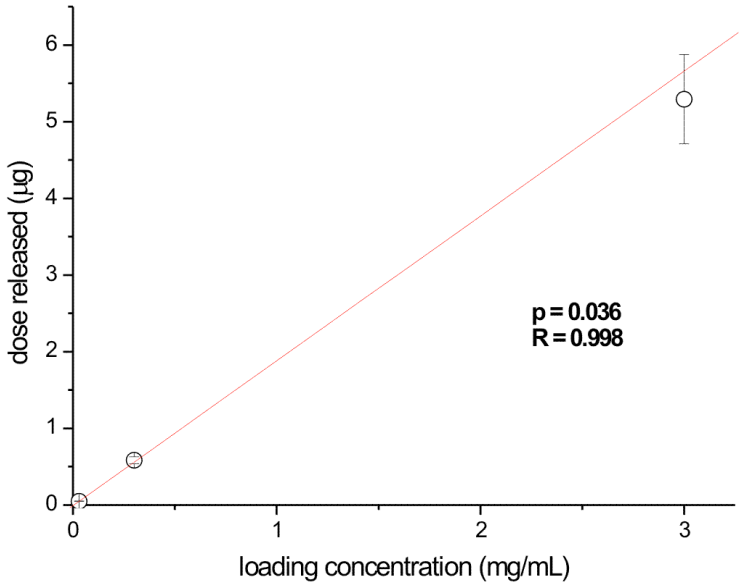

Figure 7.2: Dose-linearity of loading concentration versus estimated totally released drug.

	A) 0.03 mg/mL (n=9)	B) 0.3 mg/mL (n=10)	C) 3 mg/mL (n=10)
$Dose_{weighted}$ (μg)	**0.054 ± 0.003**	**0.530 ± 0.026**	**6.09 ± 0.36** **(A, B vs C: p<0.001)**
$Dose_{est}$ (μg)	**0.049 ± 0.002**	**0.585 ± 0.046**	**5.29 ± 0.58** **(A, B vs C: p<0.001)**
k (min^{-1})	**0.079 ± 0.001**	**0.070 ± 0.002** **(p=0.014)**	**0.067 ± 0.003** **(p=0.002)**
T1/2 (min)	**8.8 ± 0.1**	**10.1 ± 0.3** **(p=0.025)**	**10.5 ± 0.4** **(p=0.002)**
T95% (min)	**37.8 ± 0.5**	**43.6 ± 1.4** **(p=0.025)**	**45.5 ± 1.7** **(p=0.002)**

Table 7.1: Dose (weighted): MMC dose absorbed by the implants, which was estimated as follows: difference between wet and dry weight multiplied with the MMC concentration. Dose (est): Total MMC dose released from the implants, which was estimated by non-linear regression analysis assuming a first-order release process. K: first-order dissolution rate constant. T1/2: half-life of dissolution. T95%: time of 95% dissolution. All p-values correspond to comparisons with treatment A (0.03 mg/mL), unless stated otherwise. All other comparisons were not significantly different.

7.5 Discussion

The use of MMC in deep sclerectomy has been already reported. Kozobolis and coworkers[11], compared deep sclerectomy with MMC to deep sclerectomy without MMC. They reported lower mean IOP achieved, and significantly higher qualified success with the MMC group. Shaarawy and co-workers[12] compared deep sclerectomy with and without the use of collagen implants in a randomised controlled study. They reported similar mean IOP levels between the two groups, but statistically significant higher complete success with the use of the collagen implant. Hypothetically, the results of the procedure could be superior if it was augmented with both an implant and MMC. Operation time could also be relatively shortened if the implant was loaded with MMC. Prior to clinical experimentation the pharmacokinetics of the MMC loaded implant is reported in our article.

Factors affecting MMC efficacy include concentration and volume of MMC used, duration of application, and the use of irrigation after application. Vass and co-workers[13] reported on the scleral concentration of MMC with the use of different concentrations and different volumes of MMC. They reported that scleral concentration increases linearly with increasing concentration,

but not linearly with increasing volume. Never the less both volume and concentration significantly affected scleral concentrations and the authors suggested that both factors should be well taken in consideration.

Regarding the effect of post application irrigation on scleral concentrations of MMC, the same group[14] reported that irrigation reduced MMC concentration only down to half the scleral thickness, leaving the deep intrascleral concentrations unchanged. Which posed the interesting question of if we can use lower unirrigated concentrations, or are higher concentrations needed for clinical effect.

The time of MMC application also significantly affects scleral concentrations[15]. Significant differences have been reported comparing 0.5 to 1, and 1 with 5 min application durations. It is worth mentioning that 64% of the MMC was delivered to the sclera within the first min., which directly pertains to rapid release from the used MMC-soaked application sponge.

In our study we have chosen three different concentrations. The first is frequently used in clinical settings (0.3 mg/mL), and we have chosen to compare it with 10 times less and ten times more the concentration. A flow rate of 5 µL/min was chosen to simulate the estimated flow rate of a trabeculo-Descemet`s membrane following deep sclerectomy.

In a potential clinical setting, the loaded implant could be sutured to the remnants of the sclera after deep sclerectomy, where the flow of aqueous from the anterior chamber via the trabeculo-Descemet`s membrane would carry MMC released to a subconjunctival bleb. MMC could also directly diffuse to the underlying sclera and to the ciliary body and choriod. Whether that would constitute a safety hazard, or would a very low concentration released, be as effective and yet safe is beyond the scope of this study and should be addressed in subsequent studies of safety and efficacy.

Multiple studies have reported on the use of different delivering material of MMC, including a regular surgical sponge, a scleral shield, a presoaked soft contact lens and a reversible thermosetting gel [16,17]. In our study we could clearly show that the commercially available collagen implant could be loaded with MMC, and could subsequently release it. The pharmacokinetics of this relationship is determined *in vitro*.

In experienced hands deep sclerectomy with collagen implant and adjunctive MMC usually takes about 25 minutes (personal communication, André Mermoud, Lausanne, Switzerland). Uploading collagen implant with MMC thus would potentially reduce operation time by 12% (considering MMC application time of 3 mins), by specifically saving on application time.

Collagen implant seems to releases MMC in a considerably slower fashion compared to published reports in the literature regarding release from a regular sponge. The impact of the use of the MMC collagen implant on efficacy and safety warrants further clinical studies.

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8. Conclusions and outlook

Little is known about the etiology and the development of therapy resistance in inflammatory bowel disease. Transporter proteins, in particular P-glycoprotein, seem to be involved in the pathogenesis of IBD (Panwala et al., 1998; Schwab et al., 2003b; Langmann et al., 2004). On the other hand, drug transporters might also be implicated in therapy resistance in these patients (Farrell et al., 2000). Apparently, it still requires further research in the field of IBD and transporters.

In this thesis, the general expression of several transporters in different parts of the intestine of healthy volunteers was investigated. We have shown, for the first time, systematic site-specific expression of MDR1, MRPs, BCRP, and ASBT in humans. The expression pattern of each transporter showed significant alterations along the intestinal tract with low interindividual variability. This knowledge is of importance for further studies, where samples of IBD patients are analyzed. It is essential to provide data from control biopsies that derive from the same anatomic localization in order to obtain reliable results. Furthermore, this knowledge can be useful to develop drug-delivery strategies, which circumvent absorption sites with high drug transporter expression.

The induction of drug transporters might be one reason for the therapy resistance that is frequently observed in patients with IBD. Budesonide, an often-used glucocorticoid, induced the expression of MDR1 in an *in vitro* model of the human intestine. But these findings have to be confirmed in therapy-resistant IBD patients. If this holds true, studies using specific P-gp inhibitors would make sense in order to overcome therapy resistance.

Thalidomide is increasingly used in conditions of uncontrolled inflammation including IBD. Although it is a relative old drug, there is only little information about its affinity to transporters. Our investigations revealed that thalidomide is neither a substrate nor does it induce the expression of P-gp and MRP2. Consequently, thalidomide has no potential for interactions with these transporters. This is of importance since thalidomide is mainly used as an adjuvant therapy. Whether thalidomide has an affinity to other transporters than P-gp and MRP2 has to be determined in further experiments.

Transport studies through Caco-2 cells suggest that thalidomide is absorbed by passive diffusion. Both enatiomers showed the same permeability. Transport proteins in the intestine seem not to influence the absorption process. Nevertheless, additional experiments using a wider concentration range, testing ATP-depletion, and using other inhibitors would be of interest.

The described HPLC assay for the determination of thalidomide enantiomers is a sensitive method to analyze this drug in blood samples. It can be applied in prospective clinical trials. Further studies in IBD patients are necessary because it still has to be evaluated whether they have a substantial benefit from thalidomide treatment.

Vasospastic patients showed an altered expression of drug transporters in isolated mononuclear cells compared to control subjects. The significant down-regulation of MDR1, MRP2, and MRP5 could explain the enhanced drug-sensitivity that was reported by these patients. However, only the measurement of drug plasma concentrations can provide evidence for this assumption. The underlying mechanism for this regulation is still unclear. The elevated Endothelin-1 levels in vasospastic patients did not correlate with transporter expression. Moreover, mononuclear cells showed hardly any expression of endothelin receptors. Therefore, a direct Endothelin-1 effect on transporter expression can be ruled out and further investigations are required.

The transporter expressions determined in this thesis were mainly analyzed on mRNA level, but this does not necessarily correlate with protein expression or function. Additional studies regarding expression on protein levels are therefore desirable. But concerning P-gp, it was shown recently that MDR1 transcript number is at least as valid as MDR1 protein abundance as a predictor of P-gp efflux activity in Caco-2 cells (Taipalensuu et al., 2004)

In this thesis we provided data about the general transporter expressions in biopsies of the human intestine. A further step would imply a separation of the different cell subtypes that comprise a biopsy. It would be of interest whether enterocytes or cells of the immune system show alterations of their transporter expression during drug treatment. Furthermore, the influence of the inflammatory process on transporter expression in these cells should be investigated. Since both enterocytes and immune cells are supposed targets of pharmacotherapy in IBD, the basis of therapy resistance has to be explored in well-characterised cell subtypes.

9. References

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