The Caco-2 cell line in studies of drug metabolism and efflux

Sanna Siissalo

ACADEMIC DISSERTATION

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Abstract

Preclinical prediction of intestinal drug absorption is a continuous challenge in drug development. The absorption of a drug from the gastrointestinal tract is a complicated process, involving passive membrane permeability parameters as well as many active transport and metabolism components. Therefore, well characterised and reliable in vitro methods for studying drug absorption are constantly devised and under refinement.

In this work, the Caco-2 cell line, a widely used model for intestinal drug absorption, was assessed as a platform to study the interplay of phase II metabolism and MRP (multidrug resistance associated protein) -mediated efflux. Expression and function of several metabolic enzymes and efflux proteins have been observed in the intestine and many drug conjugates produced by UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs) are substrates for the apical MRP2 and/or the basolateral MRPs. The kinetics of these interactions is complex, but the human origin and intestinal-like differentiation under appropriate culture conditions appoint Caco-2 cells as a potential platform for these studies.

The Caco-2 cell line studied was thoroughly characterised with regards to different efflux proteins and UGT enzymes. The expression and functionality of MDR1 (multidrug resistance protein 1, P-glycoprotein) and several MRP proteins as well as UGT enzymes were observed in the studied cells, while the expression of SULTs and GSTs (glutathione-S-transferases) have been previously reported by other groups working with Caco-2. In fully differentiated Caco-2 monolayers the expression of most MRPs and UGTs was significantly higher compared to less differentiated cells grown for shorter periods or in flasks, an important observation with direct implications for the sensitivity and specificity of higher throughput cell-based screening assays. Other factors such as the passage number of the cells and the use of inducers also affected the mRNA expression levels.

Based on the observed efflux and phase II metabolism activities, a Caco-2 based screening method was developed for compounds interacting with MRP2, either directly or via their phase II metabolites. The kinetics of these interactions were investigated more closely in permeability experiments, where conjugation of model compounds and the efflux of their metabolites (indomethacin glucuronide, paracetamol sulfate and naphthol glucuronide) were detected. Substrate or product inhibition of the UGT enzyme(s) was evident at higher 1-naphthol concentrations, whereas the complementary role of basolateral efflux proteins was observed at the highest indomethacin concentration as the apical efflux was saturated. Pharmacokinetic modelling could be utilized as a tool for further interpretation of the results.

The combined results of these studies go a long way in improving our understanding of the Caco-2 cell line and its suitability as a model system for drug absorption and metabolism in the intestine.
Acknowledgements

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List of original publications

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II Siissalo, S., Zhang, H., Stilgenbauer, E., Kaukonen, A.M., Hirvonen, J., Finel, M., 2008. The expression of most UDP-glucuronosyltransferases (UGTs) is increased significantly during Caco-2 cell differentiation, while UGT1A6 is highly expressed also in undifferentiated cells. Drug Metabolism and Disposition 36: 2331-2336.


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Abbreviations

A Apical, the “luminal” side of e.g. a Caco-2 cell monolayer
ABC ATP-binding cassette; transporter superfamily utilizing the energy from ATP
ABCB1 Official gene name for the protein MDR1
ABCC1-13 Official gene names for the MRPs
ABCG2 Official gene name for BCRP
ACN Acetonitrile
ASBT Apical sodium-dependent bile acid transporter; influx transporter
ATP Adenosine triphosphate; transports chemical energy within living cells
AZT Azidothymidine, zidovudine (generic name); an anti-HIV drug
B Basolateral, the “circulation” side of e.g. a Caco-2 cell monolayer
BCA Bicinchoninic acid
BCRP ABCG2, Breast cancer resistance protein; apically localized efflux transporter
Caco-2 Human intestinal cell line derived from colonic adenocarcinoma
Caco-2VBL Caco-2 cell line treated with vinblastine during the culturing period
CDCF 5(6)-carboxy-2’,7’-dichlorofluorescein; fluorescent MRP model compound
CDCFDA Diacetate ester of CDCF
cDNA Complementary DNA; DNA synthesized from an mRNA template
CFTR Cystic fibrosis transmembrane conductance regulator
cMOAT Canalicular multispecific organic anion transporter; synonym for MRP2
CYP Cytochrome P450; diverse superfamily of phase I metabolizing enzymes
D “Fully differentiated” Caco-2 cells grown as monolayers on filters for at least 21 days
DMEM Dulbecco’s modified Eagle medium
DMSO Dimethyl sulfoxide
DNA Deoxyribonucleic acid; storage of genetic information
dNTP Deoxyribonucleotide triphosphate mixture
EMEA European Medicines Agency
ESI Electrospray ionisation
FAM Fluorescein-based label for e.g. qRT-PCR probes
FDA U.S. Food and Drug Administration
Fg Fraction of the absorbed dose to escape intestinal first-pass metabolism
GST Glutathione S-transferase; phase II metabolic enzymes
HBSS Hank’s buffered salt solution
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; organic buffering agent
HIFBS Heat inactivated fetal bovine serum
HIV Human immunodeficiency virus
HPLC High performance liquid chromatography
HTS High throughput screening
IU International unit
Jmax Maximum capacity (transport rate) of an efflux protein; [pmol/min/mg transporter]
Km Michaelis constant; substrate concentration at which half of the rate or capacity of a saturable process is achieved; rough measure of affinity [M]
LC-MS/MS Liquid chromatography- tandem mass spectrometry
LogD Distribution coefficient; the ratio of total concentrations of a compound in equal volumes of buffer solution and organic solvent at equilibrium (pH dependent)
LogP  Partition coefficient; the ratio of concentrations of the unionized compound in equal volumes of aqueous and organic solvent at equilibrium
MCT  Monocarboxylate transporter; influx transporter
MDCKII  Madin-Darby canine kidney cell line #II used for e.g. permeability studies
MDR  Multidrug resistance; resistance of e.g. tumours to a broad spectrum of structurally unrelated drugs following the exposure to a single drug
MDR1  ABCB1, Multidrug resistance protein 1; apically localized efflux transporter; P-glycoprotein
MK571  Non-selective MRP inhibitor and leukotriene D4 antagonist
M-MuLV  Moloney murine leukemia virus; a reverse transcriptase for cDNA synthesis
mRNA  Messenger RNA; RNA molecule encoding for a protein product
MRM  Multiple reaction monitoring
MRP  Multidrug resistance associated protein; efflux transporters; nine isoforms are known (MRP1-9; gene names: ABCC1-6, ABCC10-12)
Mw  Molecular weight
NEAA  Non-essential amino acids
NT  Nucleoside transporter; influx transporter
OATP  Organic anion transporting polypeptide; influx transporter/exchanger
OCT  Organic cation transporter; influx transporter
OST  Organic solute transporter; basolateral efflux transporter
P  Passage number of (Caco-2) cells; the number of times the cells have been harvested and reseeded
Papp  Apparent permeability coefficient; [cm/s]
PBS  Phosphate buffered saline
PCR  Polymerase chain reaction; rapid replication of a DNA segment
PDA  Photo-diode-array
pd(N)6  Random hexamer primers
Peff  Passive permeability coefficient across e.g. a lipid bilayer; [cm/s]
PEPT1  Peptide transporter; influx transporter
P-gp  P-glycoprotein; synonym for MDR1
pH  Measure of the acidity or basicity of a solution
pKa  Acid dissociation constant; a measure of the strength of acids and bases in solution
PLE  Porcine liver esterase
PSA  Polar surface area; the surface sum over all polar atoms in a molecule (usually oxygen, nitrogen, attached hydrogens)
qRT-PCR  Quantitative real-time polymerase chain reaction; mRNA expression studies
RNA  Ribonucleic acid; transcribed from DNA
RNAse  Ribonuclease; enzyme catalyzing the degradation of RNA
SNP  Single nucleotide polymorphism; single base-pair error in a DNA sequence
SULT  Sulfotransferase; phase II metabolic enzymes
TAP  Transporter of the ABC subfamily B (MDR/TAP subfamily)
TEER  Transepithelial electrical resistance; a measure of cell monolayer integrity
U  “Undifferentiated” Caco-2 cells grown in culturing flasks to near confluence
UDP  Uridine diphosphate
UGT  UDP-glucuronosyltransferase; phase II metabolic enzymes
Vmax  Maximum capacity of e.g. an efflux protein or maximum reaction rate; [pmol/min/mg transporter (or enzyme)] or [pmol/min/cm²]
1 Introduction

The ultimate goal in drug research is to develop safe, efficient drugs with favourable pharmacokinetic characteristics. Since the gastrointestinal tract is considered to be the most convenient route of drug delivery, knowledge of all the factors influencing drug absorption following peroral administration is of major importance.

The first physical and biochemical barrier to limit drug absorption is the epithelium of the intestinal mucosa. Intestinal absorption of a drug is, of course, one of the elements of interest in clinical bioavailability studies. Due to the importance of the issue, preclinical tools are also needed to predict the absorption of a drug candidate from the intestine as accurately and as early in the course of drug development process as possible.

Since \textit{in vivo} absorption studies in animals are complex, time-consuming, ethically challenging and expensive, there has been a recognized need to develop alternative \textit{in vitro} methods (EMEA, 1997). Cell culture models are used as intermediately complex systems between whole animal studies and isolated enzymes, membrane fractions or artificial lipid bilayers (Quaroni and Hochman, 1996). Their major advantages are the relative ease of maintenance and the fact that experimental conditions, such as pH, temperature and composition of the medium, can be easily controlled or modulated. Thorough characterisation of cell cultures is also much more straightforward than that of whole animals. On the other hand, cell monolayers can be used to study several active and passive permeability properties simultaneously, while more simplified \textit{in vitro} methods only provide information on the specific factors they are designed to model.

Caco-2 is a widely used, well differentiated cell line derived from human colorectal adenocarcinoma. Permeability across Caco-2 cell monolayers is considered to model intestinal absorption, since the cells represent many of the characteristics and functions of the epithelium of the small intestine (Hilgers et al., 1990). The human origin dispels the issue of interspecies differences, but the alleged differentiation of colonic cancer cells to resemble the normal human small intestine cannot be taken for granted. However, Caco-2 cell line has been well characterized during the decades of its use and is currently acknowledged as the “golden standard” of intestinal cell models (Hubatsch et al., 2007). Along with the intestinal-like passive permeability characteristics, several active influx and efflux transporters and metabolic enzymes have been observed in fully differentiated Caco-2 cell monolayers (Sun et al., 2002, Seithel et al., 2006, Meinl et al., 2008).

In addition to predictions of total intestinal absorption, Caco-2 cells can be used to investigate the active and passive processes occurring during the absorption. The information gained on e.g. active influx or efflux of the drug in Caco-2 model can be further applied in predictions of pharmacokinetics of the absorption or even distribution in the whole body (Bourdet et al., 2006). For this purpose, characterisation of the expression and function of the involved transporter(s) in Caco-2 cells and their distribution in human organs is crucial. With this information, pharmacokinetic modelling can be used as a tool to obtain more valid predictions of the kinetic profile of the drug candidate at an early stage in drug development.
The purpose of this dissertation was to examine the interplay of phase II (conjugation) metabolism with MRP (multidrug resistance associated protein) mediated efflux of the metabolites in Caco-2 cell monolayers. This has been assessed from two perspectives: the deliberate use of Caco-2 cell line as a platform for such studies and, on the other hand, elucidation of the active processes occurring during routine Caco-2 experiments.
2 Review of the literature

2.1 Intestinal absorption of xenobiotics

Peroral administration is the preferred route for drug delivery. This is attributable to several factors, above all the long-standing experience in the development of peroral dosage forms and good patient compliance due to the convenience and non-invasiveness of swallowing a tablet or a capsule.

The epithelium of the intestinal mucosa is the barrier to drug absorption from the gastrointestinal tract (Hayton, 1980, Pitman and Blumberg, 2000). This barrier has an important role as a systemic defence mechanism against potentially harmful substances, but it can also limit the bioavailability of perorally administered drugs. In addition to passive permeation properties, active “uptake” and efflux systems, as well as intestinal metabolic enzymes, have been recognized to significantly affect the extent of intestinal absorption (Back and Rogers, 1987, Ayrton and Morgan, 2001, Takano et al., 2006).

The passive route is considered to be dominant in the absorption through the intestinal wall, even though the rising awareness of the involvement of active processes has lead to great efforts to elucidate their clinical significance (Stenberg et al., 2000, Lennernäs, 2003, Kim, 2006). Passive effective permeability (P_{eff}) of a xenobiotic compound is affected by the physico-chemical properties of the molecule, physiological state of the intestinal epithelium and chemical or pharmacokinetic interactions taking place in the lumen (Table 1). There are also differences in the permeability between the different regions along the intestine (Lennernäs, 2007). In order to be absorbed, a drug molecule must be released from the formulation, dissolved in the intestinal fluids and either have favourable passive (para- and/or transcellular) permeability properties, or be a substrate of an “influx” transporter. The paracellular route is mainly important for small, hydrophilic solutes, while transcellular permeation across the lipid bilayers of the epithelial cell membranes dominates for the majority of molecules, due to the extensive surface area of the cells compared to the intercellular spaces (Artursson et al., 1996, Daugherty and Mrsny, 1999, Martinez and Amidon, 2002).

The physiological function of the active transport from the lumen to the circulation is to facilitate the absorption of nutrients and natural compounds. For example, the absorption of proteins occurs via digestion into amino acids or peptides in the stomach followed by active uptake (Matthews and Laster, 1965). However, several drugs have also been observed to utilize these carriers in their absorption (Tsujii and Tamai, 1996). Lately, the increased knowledge of active transport systems has led to applications in drug design (structural mimicking of transported compounds to improve absorption) and in silico modelling of “uptake” transporters and their substrates (Zhang et al., 2002, Chang and Swaan, 2006).
### Table 1. Summary of the most important factors affecting intestinal drug absorption.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Parameter</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Molecular properties</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Size of the molecule</td>
<td>$M_w$ (molecular weight)</td>
<td>Faster diffusion of smaller molecules across membranes; paracellular route for small, hydrophilic compounds</td>
<td>Artursson et al., 1993</td>
</tr>
<tr>
<td>Degree of ionisation at intestinal pH</td>
<td>$pK_a$ (acid dissociation constant)</td>
<td>Ionisation improves solubility but reduces passive transcellular permeability ($pH$ partition theory)</td>
<td>Shore et al., 1957</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Levine et al., 1970</td>
</tr>
<tr>
<td>Lipophilicity</td>
<td>$LogP$ (partition coefficient; aqueous/organic solvent), $LogD$ (distribution coefficient; buffer/organic solvent)</td>
<td>Hydropobicity improves passive permeability (except in extreme cases) but generally reduces aqueous solubility</td>
<td>Winiwarter et al., 1998, Krämer et al., 1999, Goodwin et al., 2001</td>
</tr>
<tr>
<td>Polarity</td>
<td>$PSA$ (polar surface area; linked to hydrogen bonding capacity)</td>
<td>Polarity reduces permeability across lipophilic membranes but improves aqueous solubility</td>
<td>Palm et al., 1997</td>
</tr>
<tr>
<td>Solubility</td>
<td>$Cs$ (saturated concentration; pH-dependent for acids and bases)</td>
<td>Low solubility prolongs the contact with absorptive surface but limits the rate of absorption; bile salts may improve the solubility of lipophilic compounds by micelle formation</td>
<td>Yu et al., 2002</td>
</tr>
<tr>
<td><strong>Physiological factors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal motility</td>
<td></td>
<td>Changes in gut motility affect the duration of the contact with the absorptive surface</td>
<td>Levine et al., 1970</td>
</tr>
<tr>
<td>Unstirred water layer</td>
<td></td>
<td>Slows the absorption of highly permeable compounds</td>
<td>Winne, 1978, Korjamo et al., 2008</td>
</tr>
<tr>
<td>Mucosal permeability</td>
<td></td>
<td>Diseases or e.g. surface active agents can increase the passive permeability of the intestinal wall</td>
<td>Levine et al., 1970</td>
</tr>
<tr>
<td><strong>Kinetic properties</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Affinity to transporters</td>
<td>$K_m$ (Michaelis constant)</td>
<td>Higher affinity (low $K_m$) to apically localized influx transporter or lower affinity to apically localized efflux transporter improves absorption</td>
<td>Kunta &amp;Sinko, 2004</td>
</tr>
<tr>
<td>Transporter capacity</td>
<td>$V_{max}$ or $J_{max}$ (maximum rate of transport)</td>
<td>Higher capacity of influx transporter or lower capacity of efflux transporter improves absorption</td>
<td>Kunta &amp;Sinko, 2004</td>
</tr>
<tr>
<td>Intestinal metabolism</td>
<td>$F_d$ (fraction of the absorbed dose to escape intestinal metabolism)</td>
<td>Extrahepatic first-pass metabolism reduces the bioavailability during absorption</td>
<td>Yang et al., 2007</td>
</tr>
<tr>
<td><strong>Interactions</strong></td>
<td></td>
<td>Various: food, drug or other substances present in the lumen can improve or reduce absorption by affecting some of the above mentioned factors</td>
<td>Fleisher et al., 1999</td>
</tr>
</tbody>
</table>
There is an abundance of active transporters in the intestine, but in most cases they bear no clinical significance for the absorption of xenobiotics (Kunta and Sinko, 2004, Terada and Inui, 2007, TP-search, 2007). If the passive permeability of an actively transported compound is high, the relative contribution of the active component to the absorption is respectively minor. On the other hand, for compounds with low passive permeability, the affinity to a transporter and its capacity are significant factors affecting absorption and bioavailability (Table 1). For some drugs, e.g. L-dopa, the importance of active transport for the intestinal absorption has been observed in vivo (Lennernäs et al., 1993). When active influx and/or efflux processes are involved in the permeation, the apparent permeability across the monolayer is a composite of the passive permeability and the respective active transport process (either increasing or decreasing the overall transport).

Co-administered drugs and other compounds (e.g. in the food) present in the intestine can also interfere with drug absorption. These interactions can, of course, occur via effects on some of the passive permeability properties listed in Table 1 (Prescott, 1977). Nevertheless, interactions related to active transport systems during absorption are currently considered much more common (Zhang et al., 2008). Compounds sharing the same influx transporter act as competitive inhibitors against each other, leading to delayed or reduced absorption, especially if their passive permeability is low. Inhibitors of efflux proteins and metabolic enzymes can, in turn, enhance the absorption of substrates. Induction of these proteins by xenobiotics would inflict the opposite effect than inhibition (Xu et al., 2005). One of the best-known, clinically documented examples is grapefruit juice, for which interactions of some components (e.g. naringin and dihydroxybergamottin) with active influx, efflux and metabolism in the intestine have been reported (Mertens-Talcott et al., 2006).

### 2.2 Intestinal efflux proteins

While active influx transporters have a physiological role in the absorption of essential molecules, efflux proteins in turn act as defence mechanisms, actively protecting the cells by transporting endogenous and xenobiotic toxins or toxic metabolites out of the cells.

According to the current understanding, the efflux proteins belong to the ABC (ATP [adenosine triphosphate] -binding cassette) transporter superfamily (Higgins, 1992, Borst and Oude Elferink, 2002, Schinkel and Jonker, 2003, Chan et al., 2004). ABC transporters use the energy of ATP hydrolysis to transport substrates across the membrane against a concentration gradient. They are associated with many important biological processes. ABC proteins are further divided into subfamilies based on their structural homology. Within these subfamilies, the relevant drug efflux proteins are MDR1 in the MDR/TAP subfamily, several MRPs in the MRP/CFTR subfamily and BCRP in the White subfamily (Klein et al., 1999, Chan et al., 2004). The general structure of ABC transporters consists of 12 transmembrane regions and two nucleotide-binding domains, but there are exceptions: for example MRPs 1-3 and 6-7 have five more transmembrane regions and BCRP is a “half-transporter” (Figure 1).
Figure 1  Predicted secondary structures of drug efflux transporters of the ATP-binding cassette family (Schinkel and Jonker, 2003; reprinted with a permission from the publisher). MRP8 resembles MRP4 and MRP5, while MRP6 and MRP7 are structurally similar to MRP1-3 (Chan et al., 2004).

MDR1 (ABCB1, commonly referred to as P-glycoprotein, P-gp), the classic example, is probably the most studied efflux transporter and, although initially discovered in tumor tissues, it is also present in normal human intestinal epithelium, blood brain barrier and hepatic canalicular membranes (Juliano and Ling, 1976, Cordon-Cardo et al., 1990). The name MDR (multidrug resistance) originates from the ability of cells exposed to a single drug to develop cross-resistance to a broad range of structurally and functionally unrelated drugs due to enhanced outward transport of drugs mediated by the P-glycoprotein (Ford and Hait, 1990). This phenomenon is due to increased expression or activity of MDR1 or some of the MRPs (multidrug resistance associated proteins).

In the human genome, nine genes encoding MRP transporters (as well as one non-functional pseudo-gene, ABCC13) have been identified (Dean et al., 2001, Kruh and Belinsky, 2003, Toyoda et al., 2008). MRP1 (ABCC1) was discovered first, in 1992, and it is expressed in the basolateral membrane of several normal human tissues, transporting a variety of hydrophilic drugs (Cole et al., 1992, Schinkel and Jonker, 2003).

MRP2 (ABCC2, cMOAT) also transports a wide range of organic anions (Nies and Keppler, 2007). It was originally found in the canalicular membranes of hepatocytes, excreting especially conjugated compounds into the bile (Jansen et al., 1993). It is also expressed in the apical membranes of the polarized cells in e.g. kidney, intestine, gallbladder, bronchi and placenta (Nies and Keppler, 2007). Due to its high expression and localisation in the apical membrane in the intestine, MRP2 is probably clinically the most...
important MRP with respect to intestinal absorption (Fromm et al., 2000, Berggren et al., 2007). The significance of MRP2 in the small intestine has even been compared to MDR1 (Dietrich et al., 2003). Of the basolateral intestinal MRPs, especially MRP1 (ABCC1) and MRP3 (ABCC3) have a wide overlap with MRP2 in their substrate specificity (Table 2). They thus have a complementary function to MRP2, except that the basolateral MRPs extrude their substrates into the circulation instead of the intestinal lumen.

The physiological role of MRP3 (ABCC3) appears, indeed, to be the function of an alternative detoxification route if MRP1 and MRP2 are not working properly (Kool et al., 1999, Belinsky et al., 2005). On the other hand, the role of MRP4-6 (ABCC4-6) and MRP7-9 (ABCC10-12) in the intestine is still very much under study (Ritter et al., 2005, Kruh et al., 2007, Russel et al., 2008). According to the current knowledge on the expression levels and tissue localization, the clinical significance of the other members of the MRP family than MRP1-3 is minor for the intestinal absorption of xenobiotics (Chan et al., 2004, Deeley et al., 2006, Yu et al., 2007).

Like MDR1 and MRP2, BCRP (ABCG2) is localized in the apical surface of intestinal epithelial cells, suggesting a role in reducing the absorption of xenobiotics (Mao and Unadkat, 2005, Krishnamurthy and Schuetz, 2007). BCRP also transports a wide range of compounds, including conjugated compounds, thus being involved in the removal of metabolites from the cells.

Since the importance of the intestinal efflux proteins has been established, a lot of attention has been directed towards the genetic variation in their expression (Schwab et al., 2003, Sakaeda et al., 2004, Gradhand and Kim, 2008). Clinically, both ethnic and inter-individual differences have been reported (Evans and McLeod, 2003, Cropp et al., 2008). Interindividual genetic variation is often based on SNPs (single-nucleotide polymorphisms), while ethnic differences are for instance due to allele frequencies in populations. Both types of variations may or may not lead to differences in the functionality of the transporter, e.g. due to alterations in expression and/or activity, and, hence, drug absorption (Maeda and Sugiyama, 2008).
Some substrates and inhibitors of the intestinal efflux proteins and metabolizing enzymes. Note that substrates can also act as inhibitors (competitive inhibition by sharing a binding site or modulation of protein activity).

<table>
<thead>
<tr>
<th>Efflux proteins</th>
<th>Substrates</th>
<th>Inhibitors</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR1</td>
<td>Anti-HIV drugs (e.g. ritonavir, saquinavir), cardiac drugs (e.g. digoxin, quinidine, verapamil), anticancer drugs (e.g. doxorubicin, etoposide, methotrexate, vinblastine), antibiotics (erythromycin, grepafloxacin, rifampicin), hormonal drugs (e.g. dexamethasone, estradiol, hydrocortisone); cyclosporin A, iraconazole, loperamide, midazolam, morphine, prednisolone, terfenadine</td>
<td>Atorvastatin, felodipine, clarithromycin, tamoxifen, progesterone, testosterone, ketoconazole, clortimazole, probenecid, PSC833 (valspodar), GF120918 (elacridar)</td>
<td>Schinkel and Jonker, 2003, Pal and Mitra, 2006</td>
</tr>
<tr>
<td>MRP1</td>
<td>Ritonavir, saquinavir, doxorubicin, etoposide, methotrexate, vinblastine; glutathione conjugates (leukotrienes, prostaglandin), glucuronides (e.g. estradiol), sulfates (e.g. estrone)</td>
<td>Cyclosporin A, probenecid, PSC833 (valspodar), MK571</td>
<td>Bakos and Homolya, 2007</td>
</tr>
<tr>
<td>MRP2</td>
<td>Ritonavir, saquinavir, doxorubicin, etoposide, methotrexate, vinblastine; glutathione conjugates (e.g. arsenic), glucuronides (e.g. of bilirubin, estradiol, leukotrienes), sulfates (e.g. estrone)</td>
<td>Cyclosporin A, probenecid, PSC833 (valspodar), MK571</td>
<td>Hoffmann and Kroemer, 2004, Nies and Kappler, 2007</td>
</tr>
<tr>
<td>MRP3</td>
<td>Etoposide, methotrexate, glutathione conjugates, glucuronides (e.g. estradiol), sulfates, monoanionic bile acids</td>
<td>Indomethacin, probenecid, MK571</td>
<td>Belinsky et al., 2005</td>
</tr>
<tr>
<td>BCRP</td>
<td>AZT, doxorubicin, etoposide, methotrexate, imatinib, pantoprazole, glucuronides (e.g. estradiol), sulfates (e.g. estrone)</td>
<td>GF120918 (elacridar), novobiocin</td>
<td>Mao and Unadkat, 2005</td>
</tr>
<tr>
<td>Phase I enzymes</td>
<td>CYPs</td>
<td>Theophylline (1A1), estradiol (1A1), melatonin (1A1, 2C19), warfarin (1A1, 2C9, 2C19, 3A4), testosterone (1A1, 2C9, 2C19, 3A4), ibuprofen (2C9), naproxen (2C9), diazepam (2C9, 2C19), diclofenac (2C9, 2C19, 3A4), dextromethorphan (2C19), ranitidine (2C19), etoposide (3A4), midazolam (3A4), verapamil (3A4), quinidine (3A4), ethynylestradiol (3A4), erythromycin (3A4), cyclosporin A (3A4), codeine (3A4)</td>
<td>Mexiletine (1A1), sulfafenazole (2C9), flunoxapine (2C9, 2C19), PSC833 (valspodar) (3A4), ketoconazole (3A4), itraconazole (3A4)</td>
</tr>
<tr>
<td>Phase II enzymes</td>
<td>GSTs</td>
<td>A very broad range of electrophilic substrates, e.g. PCBs (polychlorinated biphenyls), anticancer agents (e.g. chlorambucil, cisplatin, mitoxantrone)</td>
<td>Glutathione analogs; product inhibition!</td>
</tr>
<tr>
<td></td>
<td>SULTs</td>
<td>Paracetamol (1A1), minoxidil (1A1), estrogens (1A1 and 1E1) dopamine (1A3), tamoxifen (1E1)</td>
<td>Substrate inhibition!</td>
</tr>
<tr>
<td></td>
<td>UGTs</td>
<td>Ethynylestradiol (1A1), amitriptyline (1A4), trifluoperazine (1A4), paracetamol (1A6), serotonin (1A6), naproxen (1A6, 2B7), scopolatin (1A8), propofol (1A9), entacapone (1A7, 1A8, 1A9, 1A10), codeine (2B4, 2B7), morphine (2B7), buprenorphine (2B7), ibuprofen (2B7), AZT (2B7), S-oxazepam (2B15)</td>
<td>Substrate and product inhibition!</td>
</tr>
</tbody>
</table>
2.3 Drug metabolism in the intestinal wall

The purpose of the biotransformation of xenobiotics is to make them more hydrophilic and, thus, more easily excreted. Drug metabolism is traditionally divided into two phases: in phase I the compound is oxidized, reduced or hydrolyzed and in phase II the products of phase I are conjugated (Figure 2, Williams, 1978). However, these terms may be a little outdated as the phases are not necessarily subsequent (Josephy et al., 2005). Although the liver is the main drug-metabolizing organ, the gut wall can also play an important role in the first-pass metabolism of some drugs (Back and Rogers, 1987, Kaminsky and Zhang, 2003, Yang et al., 2007).

Figure 2 Structures of phase II metabolites. Conjugation can occur at a hydroxyl (most common), a carboxylate, or a primary or secondary amino group in the drug molecule.

The most important enzymes to catalyze phase I metabolic reactions in humans are the oxidative cytochrome P450 superfamily (Lewis, 2003). Several CYP enzymes (especially CYP1A1, CYP2Cs and CYP3As) have been detected in the human intestine, but there is considerable qualitative and quantitative interindividual variation in the expression levels of different CYP enzymes (Paine et al., 1997, Ding and Kaminsky, 2003). However, especially CYP3A4 is expressed at levels high enough to bear clinical significance for drug absorption related metabolism (Anzenbacher and Anzenbacherova, 2001).

Whereas most of the phase I metabolism occurs in the liver, conjugation activity in the intestinal wall may in some cases be close to - or even higher than - that in liver (Back and
Rogers, 1987). Phase II metabolic reactions include sulfation, glucuronidation and glutathione-S-transferation, all of which have been observed in the human intestine (Pacifici et al., 1988, Kaminsky and Zhang, 2003, Van de Kerkhof et al., 2007). Many drugs or their phase I metabolites can be either sulfated or glucuronidated; the balance between the two depends on substrate affinity (usually higher to sulfotransferases) and enzyme capacity (higher for glucuronosyltransferases) (Back and Rogers, 1987). However, there is some variation in the observed expression of e.g. UGT-glucuronosyltransferases in the intestine (Paine and Fisher, 2000, Strassburg et al., 2000, Gregory et al., 2004, Nagar and Remmel, 2006, Nakamura et al., 2008). Intraintestinal variation in the expression of metabolic enzymes has also been observed (Paine et al., 1997, Strassburg et al., 2000).

2.4 Interplay of drug efflux and metabolism in the intestine

In addition to phase I and phase II metabolism, detoxification steps of phase 0, phase III and phase IV have been suggested (Ishikawa, 1992, Homolya et al., 2003). These phases however do not refer to actual biotransformation, but rather to the active disposition directly linked to metabolism. Phase 0 is described as the passage from the lumen into the cell, across the apical cell membrane. During this step, efflux may also be involved, taking up the substrate from the intracellular surface of the apical cell membrane and transporting the compound out before even reaching the cytosol. Apical uptake may, of course, also have a role. Transport of the conjugates formed in phase II (usually MRP- or BCRP-related efflux) is defined as phase III (or phase IV in case intracellular transport processes are referred to as phase III). It should be noted here that the ‘Phase 0-IV’ system was originally designed for the liver, where the phases appear more consecutively.

The most discussed example of the efflux-metabolism interplay is the complex co-operation between CYP3A4 and MDR1 in the intestine (Salphati and Benet, 1998, Wandel et al., 1999, Suzuki and Sugiyama, 2000, Benet and Cummins, 2001, Knight et al., 2006, Pal and Mitra, 2006). Not only do they share a wide range of substrates and inhibitors, but their expression also seems to be co-regulated (Christians, 2004, Lamba et al., 2006). In essence, MDR1 circulates the drugs in the apical membrane of the enterocytes (entry by passive permeability followed immediately by efflux) and, hence, the contact with the CYP3A4 along the length of the intestine is prolonged as the absorption is slowed down.

Several active processes may be involved in the absorption of a single drug (Figure 3). An intriguing example of the efflux-metabolism interplay is the co-operation between the conjugating enzymes (UGTs, SULTs, GSTs) and efflux proteins (MRP1, MRP2, MRP3, also BCRP) (Suzuki and Sugiyama, 2000, Jeong et al., 2005, Oswald et al., 2006a). Instead of sharing the same substrates like CYP3A4/MDR1, these systems are concatenated: the intracellularly formed conjugates are effluxed from the enterocytes back to the lumen of the intestine (MRP2, BCRP) or to the circulation (MRP1, MRP3) (Table 2). However, the products of CYP3A4 can also be substrates of MDR1 or another efflux protein or may be further metabolised by phase II enzymes and consequently effluxed
The assumed goal in all these cases is to protect the cells from the accumulation of potentially harmful metabolites. Some of the conjugating enzymes are inhibited by their own products, a fact that emphasizes the critical role of the efflux for the optimal function of the enzyme (Commandeur et al., 1995, Luukkanen et al., 2005).

Figure 3

The most relevant known active transport, efflux and metabolism processes in the enterocytes (Ito et al., 2005, Hilgendorf et al., 2007, Pang et al., 2007, Terada and Inui, 2007). ISBT = Ileal bile salt transporter, OATP-B = Organic anion transporting polypeptide B, HPT1 = Human oligopeptide transporter, OCTN2 = Organic cation/carnitine transporter, PEPT1 = Oligopeptide transporter, MCT = Monocarboxylic acid transporter, OCT1 = Organic cation transporter, MDR1 = Multidrug resistance protein 1, MRP = Multidrug resistance related protein, BCRP = Breast cancer related protein, CYP = Cytochrome P450 enzyme, UGT = UDP-glucuronosyltransferase, SULT = Sulfotransferase, GST = Glutathione-S-transferase.

To date, the interplay of UGT metabolism and MRP efflux in the intestine has been most extensively studied for flavonoids and toxins (Petri et al., 2003, Zhang et al., 2007b, Sergent et al., 2008). Examples of this interplay influencing the absorption of drugs have been reported in treatment of colonic cancer and in MRP2- and MRP3-deficient rats and mice (Cummings et al., 2004, Zelcer et al., 2005, Oswald et al., 2006b). Specific studies of the interplay of SULT metabolism and MRP efflux in the intestine have not been published so far. On the other hand, the interplay of GST metabolism and MRP efflux has
been studied in cell-free systems and the results suggest that similar interplay may also take place in the intestine (Peklak-Scott et al., 2005).

**2.5 Use of Caco-2 cell line in drug absorption studies**

Cell culture models have been widely used for decades to study drug transport *in vitro* (Audus et al., 1990). The Caco-2 cell line (originating from human colorectal carcinoma) is currently the best characterized, most commonly used cell system for drug absorption studies (Hillgren et al., 1995, Bailey et al., 1996, Le Ferrec et al., 2001). It has also been approved and recommended by the regulatory authorities (FDA, 2006, Elsby et al., 2008). Cultured on polycarbonate filters as monolayers, Caco-2 cells exhibit spontaneous structural and functional differentiation and polarization patterns that are characteristic to the epithelial cells in the small intestine (Figure 4, Pinto et al., 1983). Qualitatively, Caco-2 absorption model gives transport results similar to those observed *in vivo* (Hilgers et al., 1990, Artursson and Karlsson, 1991, Gan and Thakker, 1997). Quantitatively, Caco-2 monolayers are most useful in identifying drugs with potential absorption problems and selecting drug candidates based on absorption characteristics (Artursson et al., 1996).

![Figure 4](image)

*Figure 4*  *Feeding of the Caco-2 cells cultured on Transwell® inserts. The cells are grown on semipermeable filter membranes, where they can be accessed from both sides of the monolayer, allowing polarization and differentiation of the monolayer. Photograph by Anne Soikkeli, 2008.*
Several active uptake and efflux proteins as well as metabolic enzymes are expressed in Caco-2 cells (Peters and Roelofs, 1989, Schmiedlin-Ren et al., 1997, Sabolovic et al., 2000, Sun et al., 2002, Seithel et al., 2006, Meinel et al., 2008). Thus, in polarized Caco-2 monolayers the interplay between these systems can be assumed to resemble that in the small intestine (Figure 3). Considerable variation has still been observed in the active and passive barrier properties of Caco-2 cells from different batches and laboratories, having created a need for thorough characterization of the cell line and the use of standardised methods and protocols in the Caco-2 culturing (Sambuy et al., 2005, Hubatsch et al., 2007, Volpe, 2008).

The expression of proteins responsible for active processes in Caco-2 cells can also be induced by xenobiotics. This is probably due to the cancerous origin of Caco-2, since e.g. vinca-alcaloids induce MDR1 in these cells, according to the concept of multidrug resistance (Eneroth et al., 2001, Laska et al., 2002). MRP2, together with UGT1A6, has been observed to be induced by antioxidants – another indicator of the MRP/UGT interplay (Bock et al., 2000). Induction of many other phase I and phase II metabolic enzymes has also been reported (Engman et al., 2001, Lampen et al., 2004, Chen et al., 2008, Müller et al., 2008).

The interplay of phase II metabolism and MRP efflux of metabolites has, thus, been recognized in Caco-2 cells. Efflux of intracellularly produced UGT, SULT and GST conjugates have been reported, but as in other systems, the studies have so far mostly concentrated on flavonoids, since they are extensively glucuronidated and, thus, glucuronidation activity can be observed in vitro even at lower concentrations (Usta et al., 2007, Zhang et al., 2007a, Brand et al., 2008, Dai et al., 2008). Sulfation and glucuronidation of a drug compound in Caco-2 monolayers followed by efflux of the metabolites has been observed only for methyldopa and raloxifene (Chikhale and Borchardt, 1994, Jeong et al., 2004). Recently, pharmacokinetic models of Caco-2 cells, some of them incorporating metabolism and efflux, have been constructed (Sun and Pang, 2008, Sun et al., 2008, Heikkinen et al., 2009).

2.6 Pharmacokinetic considerations

The principles of pharmacokinetics of efflux proteins and metabolic enzymes are quite similar. Substrates and inhibitors bind to specific binding sites in the protein, followed by the action of the protein or inhibition of the action. The kinetics of these interactions are assumed to follow the Michaelis-Menten equation (Eq. 1) and can be described with the concentration of the compounds at the binding site \[ S \], kinetic parameters \( K_m \) and \( V_{max} \), and the number of the transporters/enzymes available (Table 1). Saturation of the efflux or the metabolic reaction can be observed as the reaction rate or efflux capacity asymptotically approaches the maximum rate \( V_{max} \) as the binding sites in all the proteins are occupied. In practice, determination of the substrate concentration at the binding site is difficult, or even impossible, and hence calculations are based on the bulk concentration of the substrate in the solvent system.
\[
V = \frac{V_{\text{max}} [S]}{K_m + [S]}
\]

(V = reaction or efflux rate; \(V_{\text{max}}\) = maximum rate of metabolism/efflux, when binding sites in all the enzymes/efflux transporters are occupied; \([S]\) = concentration of the substrate compound at the binding site; \(K_m\) = reaction constant)

The efflux proteins reside in the cell membranes and one substrate binding site for verapamil and Rhodamine123 of at least MDR1 is located in the transmembrane domains (Loo and Clarke, 2002). Efflux proteins can then pick up their substrates directly from the lipid bilayer, limiting their distribution into the cytosolic compartment. The metabolic enzymes, in contrast, are mostly located in the endoplasmic reticulum, other cell membranes or cytosol, thus picking their substrates from the intracellular space (Meech and Mackenzie, 1998, Kauffmann, 2004, Neve and Ingelman-Sundberg, 2008).

Inhibitors also bind to the efflux proteins and metabolic enzymes, but they do not necessarily get metabolized or effluxed out by themselves. Instead, they may affect the conformation of the protein (allosteric inhibition, noncompetitive) or occupy the binding site and prevent the efflux or metabolism of the substrates (competitive inhibition). At least MDR1 and MRP2 have been reported to have several binding sites; hence, compounds interacting with the same efflux transporter may or may not affect the interactions of each other (Zelcer et al., 2003, Sharom et al., 2005, Xia et al., 2007).

Two substrates competing for the same binding site act as competitive inhibitors against each other, but the inhibitory effect of the substrate with the higher affinity to the protein is easier to observe. Substrate inhibition of the metabolic enzymes can also occur at high concentrations, when two molecules of the same substrate inhibit the enzyme by binding to it simultaneously (Luukkanen et al., 2005). In the case of product inhibition, intracellular accumulation of the metabolic products (for example if the efflux protein exporting the conjugates is saturated) may also inhibit the enzyme.
3 Aims of the study

The overall goal of this work was to evaluate the feasibility of Caco-2 cell line for studies with compounds that undergo intestinal phase II metabolism (mainly glucuronidation) followed by MRP efflux of the metabolites and/or parent compounds.

The more specific aims were:

1) To thoroughly characterize the used Caco-2 cell line in terms of the expression of efflux proteins and UGT isoforms and to evaluate the effects of cell differentiation and passage number on them (I, II).

2) To evaluate the functionality of phase II metabolism and efflux systems in Caco-2 experiments (I-IV).

3) To apply the characterization results by developing a screening method for compounds interacting with MRP2 either directly or following phase II metabolism (III).

4) To demonstrate the interplay of UGT/SULT metabolism of drugs and MRP efflux of the drug conjugates during Caco-2 permeability experiments (IV).
4 Experimental

4.1 Materials

4.1.1 Model compounds (I-IV)

Rhodamine123 (I), vinblastine (I), CDCF (5(6)-carboxy-2’,7’-dichlorofluorescein) (III), CDCFDA (diacetate ester of CDCF) (III), p-aminohippuric acid (III) and indomethacin (III, IV) were purchased from Fluka (Buchs, Switzerland) and verapamil (I, III) from MP Biomedicals (Aurora, OH, USA). Estradiol (II), 1-naphthol (II, IV), probenecid (III), furosemide (III) and diclofenac (III) were from Sigma-Aldrich (St. Louis, MO, USA), MK571 (III) from Cayman Chemicals (Ann Arbor, MI, USA) and paracetamol (acetaminophen) (III, IV) from Hawkins Inc. (Minneapolis, MN, USA). All the other laboratory chemicals were of analytical grade.

4.1.2 Cell lines (I-IV)

The reagents for cell culturing were purchased from Euroclone (Pero, Italy) except for fetal bovine serum and HBSS (Hank’s buffered salt solution) 10x concentrate from Gibco Invitrogen Corporation (Carlsbad, CA, USA) and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) from Sigma (St. Louis, MO, USA). All the plasticware were obtained from Corning B.V. Life Sciences (Schiphol-Rijk, Netherlands).

Human colon adenocarcinoma Caco-2 (wild type) cells were obtained from American Type Culture Collection (Rockville, MD, USA) at P18. The cells were maintained at +37 °C in an atmosphere containing 5% CO₂ at 95% relative humidity, in a medium consisting of DMEM (Dulbecco’s Modified Eagle Medium, high glucose 4.5 g/l), 10% HIFBS (Heat Inactivated Fetal Bovine Serum, inactivation at +56 °C for 30 min), 1% NEAA (Non Essential Amino Acids), 1% L-glutamine, penicillin (100 IU/ml) and streptomycin (100 µg/ml). The medium was changed three times a week during cell growth and differentiation.

The cells were grown in 75 cm² plastic flasks and harvested weekly with 0.25% trypsin. The cells were seeded at 6.8 x 10⁴ cells/cm² onto polycarbonate filter membranes (pore size 0.4 µm, filter area 1.1 cm²) in 6- or 12-well Transwell® insert plates (Corning® Cat. No. #3412 and #3401, respectively), and grown for 21-28 days for differentiation (I, II) and transport experiments (I, II, IV) (Figure 4), or for efflux experiments in cell culture treated clear, black or clear-bottomed black 96-well plates for 7-15 days (III). For expression studies, undifferentiated cells were grown in flasks to near confluency (I, II). Caco-2VBL (vinblastine treated) cells were handled according to similar procedures as wild type Caco-2 cells, except that 10 nM vinblastine (sterile-filtrated, aliquoted frozen-stock) was added to the culturing medium (I). Since the initiation of vinblastine treatment
substantially disturbed cell growth and attachment, Caco-2VBL cells were used for experiments only after their behaviour in culture had normalized, which took at least 4-5 passaging cycles (recuperation was determined by visual examination during the growth period).

The studied Caco-2 cells were within the passage range between P31 and P53. For Caco-2VBL cells, the passage range was P33/9 to P49/20, expressed as overall passage number of the respective wild type Caco-2 / passage under VBL treatment.

### 4.2 Expression studies: qRT-PCR (I, II)

Materials and reagents for qRT-PCR were purchased from Applied Biosystems (Foster City, CA, USA), except for probes and primers for MDR1, MRP2, BCRP, CYP3A4 and β-actin, which were kindly provided by the University of Kuopio (Korjamo et al., 2005). Reagents for qRT-PCR sample preparation were from Sigma, Ambion (Austin, TX, USA), Molecular Probes (Eugene, OR, USA), MBI Fermentas (Vilnius, Lithuania), Pharmacia Biotech (Amersham Biosciences AB, Uppsala, Sweden) and Promega (Madison, WI, USA). All the other chemicals were of analytical grade and RNase free.

The cells were studied at two levels of differentiation: undifferentiated (cultured to near confluency in flasks) and fully differentiated (cultured on polycarbonate filter membranes for at least 21 days). Sample preparation protocol was carried out in an RNase free environment. Control samples for each process step and genomic DNA contamination were prepared simultaneously. Total RNA was extracted from Caco-2 and Caco-2VBL cells using TRI-Reagent® (Sigma) protocol. The samples were DNase treated with Ambion’s DNA-Free kit and total RNA concentrations were determined with Ribogreen RNA® Quantitation Reagent and Kit (Molecular Probes). Total RNA (2 µg/sample) was reverse transcribed to cDNA with 30 IU M-MuLV reverse transcriptase (Fermentas) in a reaction mixture containing 4 µl of 5x First-Strand buffer (Fermentas), 1mM dNTPs (Fermentas), 3 µg pd(N)6 primers (Pharmacia Biotech) and 20 IU RNase inhibitor (Fermentas) in a total volume of 20 µl. The cDNA was diluted to 8 ng/µl for qRT-PCR.

The relative mRNA expression levels of villin, MDR1, MRP1, MRP2, MRP3, MRP4, MRP5, MRP6, BCRP, UGT1A1, UGT1A4, UGT1A5, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17, UGT2B28 and CYP3A4 were determined with AbiPrism7500 system (Applied Biosystems) and normalized to β-actin using the comparative Ct (threshold cycle, the PCR cycle number at which the fluorescence generated within a reaction crosses the defined threshold) method: the Ct values of the amplified genes were normalized to the Ct values of β-actin assuming equal amplification efficiencies. FAM labeled Assay on Demand TaqMan® Gene Expression Assay reaction mixes were used for β-actin (II), villin, MRP1, MRP3-6 and all the UGTs. The primers and probes for MDR1, MRP2, BCRP, CYP3A4 and β-actin (I) were kindly provided by the University of Kuopio (Korjamo et al., 2005). The samples were analyzed in triplicate using TaqMan Universal PCR Master Mix and 40 µg cDNA in each 15 µl reaction mix. Negative controls for PCR reaction mix, cDNA
synthesis of each sample and cDNA synthesis reaction (without RNA) were included in all the qRT-PCR runs. The reaction profile consisted of 10 min incubation at 95 °C followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C, except for MDR1 and MRP2, for which an extra annealing step of 15 s at 55 °C was added to the cycle. Analysis of qRT-PCR data was performed using Qgene Excel macro (Muller et al., 2002).

Statistical analysis of the normalized qRT-PCT data was performed using the two-sample Student’s t-test assuming unequal variances. Differences in the mRNA expression values were considered significant at p < 0.01.

4.3 Functionality studies

4.3.1 Permeability experiments (I, III, IV)

Transport of the model compounds across the Caco-2 monolayers was studied under sink conditions both in apical-to-basolateral (A→B) and basolateral-to-apical (B→A) transport directions. The same pH (7.4 or 6.5 for CDCF (III), 7.4 for all the other studied compounds) was used in both compartments during each experiment. Before the experiments, the cell monolayers were washed twice with preheated HBSS-HEPES (10 mM HEPES) and then equilibrated for 30 min at 37 °C. The integrity of the cell monolayers was verified by measuring transepithelial electrical resistance (TEER) across each monolayer before and after the experiment using Millicell®-ERS device (Bedford, MA, USA). TEER values above 250 Ω·cm² were considered acceptable.

In the experiments with the fluorescent efflux substrates, rhodamine123 (MDR1 substrate; I) or CDCF (MRP substrate, III) in HBSS-HEPES was placed in the apical or basolateral chamber. In the MDR1 inhibition experiments (I), the donor solutions contained 200 µM verapamil in addition to rhodamine123. The initial concentrations (rhodamine123: 50µM apical or 5 µM basolateral; CDCF: 10 µM at pH 6.5 or 50 µM at pH 7.4) were verified by immediately taking a sample from the donor compartment, so that 500 µl (A) or 1.5 ml (B) of the solution was present at the beginning of the experiment. Samples (400 µl apical, 1.5 ml basolateral) were taken from the receiver compartment at 15 min or 5 min (rhodamine123, B→A) intervals over 60 minutes and replaced immediately with buffer. Sampling intervals and initial concentrations were optimised for each direction to ensure sample concentrations above the limit of quantification while maintaining sink conditions.

In the metabolism/transport experiments with 1-naphthol, indomethacin and paracetamol (IV), the parent compound solution (5 µM, 20 µM, 50 µM, 200 µM or 500 µM – except for paracetamol, 5 µM was considered too low, so 1000 µM was used instead) was placed in the apical or basolateral chamber and samples were taken from both apical (400 µl) and basolateral (1 ml) compartments at 30 min, 60 min and 120 min and replaced with the parent solution (apical) or study buffer (basolateral). At the end of the experiments, the filters were washed briefly with buffer, cut off with a scalpel and
transferred into separate microcentrifuge tubes with 250 µl buffer. The cells on the filters were disrupted by freeze-thawing and the tubes were centrifuged at 13,000 rpm (Eppendorf Centrifuge 5415D, Eppendorf AG, Hamburg, Germany) for 5 min, after which 200 µl samples were drawn from each supernatant for the determination of residual model compounds and metabolites in the cells. All the samples were stored at -20 °C until they were sent for LC-MS/MS analysis (4.4.3).

Apparent permeability coefficients (\(P_{\text{app}}\), cm/s) were calculated based on Eq.2.

\[
P_{\text{app}} = \frac{(dQ/dt)}{(A \times C_0 \times 60)}
\]

d\(Q/dt\) = cumulative transport rate (nmol/min), A = surface area of the cell monolayer (1.1 cm\(^2\)) and C\(_0\) = initial concentration in donor compartment (nmol/ml)

Efflux ratios were calculated from the apparent permeability coefficients (Eq. 3).

\[
\text{Efflux \_ratio} = \frac{P_{\text{app},B\rightarrow A}}{P_{\text{app},A\rightarrow B}}
\]

4.3.2 Enzyme activity assays (II)

Differentiated and undifferentiated Caco-2 cells were cultured on 6-well plates and collected at passage 43. The cells were washed and scraped into cold phosphate buffered saline (PBS), collected by centrifugation at 3,200 x g, suspended in cold water, divided to aliquots and stored at -80 °C. Aliquots of both differentiated and undifferentiated cells were thawed on the day of the activity assays and centrifuged at 40,000 x g (Sorvall RC 5B Plus, Thermo Fisher Scientific Inc., Waltham, MA, USA) for 30 min. The cell pellet was suspended in 200 µl cold water and sonicated mildly on ice (Branson sonifier 450, narrow tip, output level 4, 50% duty, 6 pulses with 1 min interval after the first 3 pulses) and the protein concentration was determined using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) using bovine serum albumin as standard. The 1-naphthol glucuronidation was essentially performed as previously described (Kurkela et al., 2003), in the presence of 100 µM substrate and 40 µg protein, incubated for 120 min at 37 °C. The estradiol glucuronidation assay was performed similarly, except that the substrate was 17-\(\beta\)-estradiol at 100 µM. The reactions were terminated with cold perchloric acid and the samples chilled and centrifuged prior to the analysis with HPLC (4.4.2).
4.3.3 De-esterification experiments (III)

Caco-2 cells grown to confluency in a plastic flask (7-8 days, to mimic the cells grown in 96-well plates) or fully differentiated cell monolayers (at least 21 days) on polycarbonate filter membranes were washed 4 times with PBS solution (without Ca\(^{2+}\) and Mg\(^{2+}\)), scraped off and disrupted in Milli-Q water (Millipore, Molsheim, France). The cells were exposed to four freeze-thawing cycles: rapidly frozen in liquid nitrogen and immediately thawed in 37 °C water bath. The disrupted cell suspension (100 µl /well, containing 10 µg of total protein) was pipetted into a black 96-well plate. One hundred microlitres of freshly prepared CDCFDA solution (1 nM to 5 µM) was added into each well and the fluorescence resulting from the de-esterification of CDCFDA into the fluorescent CDCF was monitored with the multilabel counter as described in section 4.4.1. Spontaneous de-esterification in the study buffer was used as a negative control and de-esterification in the presence of porcine liver esterases (PLE) (0.25 µg/ml to 250 µg/ml; 27 IU/mg) from Fluka (Buchs, Switzerland) as a positive control.

4.3.4 Efflux assays in 96-well format (III)

Caco-2 cells grown in clear, black or clear-bottomed black 96-well plates (Corning® Cat. No. #3599, #3916 and #3603, respectively; seeding density 22,500 cells/well) for 7-15 days were used for these experiments. To ensure the viability of the cells over longer growth periods, Corning® CellBind® plates (Corning® Cat. No. #3340) were used to improve the attachment of the cells for growth periods exceeding 9 days. The cells were washed twice with HBSS-HEPES (10 mM HEPES, pH 7.4) and then equilibrated for 15-20 min in the washing buffer solution. The washing solution was then removed and the cells were loaded for 5-30 min with 200 µl of 0.5 to 10 µM CDCFDA (all the solutions were prepared in HBSS-HEPES pH 7.4). The loading solution was removed and the cells were washed briefly with buffer. The solutions containing the studied compounds (probenecid, MK571, verapamil, paracetamol, diclofenac, indomethacin, p-aminohippuric acid or furosemide) were pipetted into the wells. HBSS-HEPES pH 7.4 was used as a reference (100% efflux) in each experiment. All the incubations were conducted at +37 °C. In the experiments where the fluorescence was measured directly from the cell plate, the total volume was 200 µl in each well. Where a separate, fresh black plate was used for the analysis of samples, 200 µl of the total volume of 250 µl was collected from each well. In either approach, the fluorescence was determined as described in section 4.4.1.

4.3.5 Statistical analysis

Where appropriate, statistical analysis of the results from the functionality studies was performed using two-sample Student’s t-test assuming unequal variances. The results are expressed as means ± standard deviation, and the differences were considered statistically significant at p < 0.01.
The statistical quality of a screening method can be assessed with a value called Z factor (Eq. 4). An ideal assay would have a Z factor of 1.0, which can never actually be achieved. Assays with Z factor values > 0.5 (or 0.4 for cell-based assays) are considered excellent, while assays with Z factors < 0 are ‘essentially useless’ (Zhang et al., 1999).

\[
Z \text{- factor} = 1 - \frac{3 \times (\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}
\]

\(\mu = \text{mean}, \sigma = \text{standard deviation}, p = \text{positive controls}, n = \text{negative controls}\)

4.4 Analytical methods

4.4.1 Fluorescence reader (I, III)

Fluorescence of the rhodamine123 and CDCFDA/CDCF samples was analysed in 96-well plates using Wallac Victor® 1420 multilabel counter (Turku, Finland) at 485 nm excitation and 535 nm emission wavelength. The analytical method was calibrated for each analysis over the concentration range 0.001 – 2 µM (R^2 > 0.99).

4.4.2 HPLC (II)

The 1-naphthol glucuronides were detected by HPLC as previously described (Kurkela et al., 2003), except that the column this time was Hypersil BDS-C18, 150 x 4.6 mm, 5 µm (Agilent Technologies, Santa Clara, CA, USA). The estradiol glucuronides were separated using a Shimadzu HPLC and the column was Chromolith Speed ROD RP-18e 50-4.6 mm (Merck, Darmstadt, Germany). The glucuronides were detected by fluorescence, excitation at 216 nm and emission at 316 nm.

4.4.3 LC-MS/MS (IV)

The samples from the metabolism/permeability experiments were analysed at Novamass Ltd (Oulu, Finland). The parent compounds and metabolites were detected with LC-MS/MS system using electrospray ionisation (ESI) and multiple reaction monitoring (MRM). Prior to the analysis, the samples were thawed at room temperature, shaken, centrifuged at 16,100 g (Eppendorf Centrifuge 5415D, Eppendorf AG, Hamburg, Germany) for 10 min and pipetted to Total Recovery vials (Waters Corporation, Milford, Massachusetts, USA).
A Waters Alliance 2695 chromatographic system (Waters Corp., Milford, MA, USA) with autosampler, vacuum degasser and column oven (set to 35 °C) was used. The precolumn was Phenomenex Luna-C18, 4.0 × 2.0 mm, 3.0 µm (Phenomenex, Torrance, California, USA), and the analytical column was Waters XBridge Shield RP18, (2.1 × 50 mm, 3.5 µm). The eluents were 2 mM ammonium acetate (pH 7.0) and acetonitrile (ACN). For 1-naphthol, indomethacin and their conjugates, a linear gradient elution from 10% to 90% ACN in one minute was followed by two minutes of isocratic elution with 90% ACN and column equilibration at the flow rate of 0.4 ml/min. For paracetamol and its conjugates, a linear gradient elution from 5% to 90% ACN in 2.5 minutes was employed, followed by column equilibration at 0.3 ml/min. The flow was split post column to a Waters 2996 photo-diode-array (PDA) detector and a mass spectrometer ion source at the ratio of 3:1, respectively. LC-MS/MS data were acquired with a Micromass Quattro Micro triple quadrupole mass spectrometer (Micromass Ltd., Manchester, England), equipped with a Z-Spray electrospray ion source, using a negative ionisation and a multiple reaction monitoring mode. The monitored MRM transitions were m/z 319 > 143 for 1-naphthol glucuronide (collision energy 20 eV, cone voltage 30V), m/z 356 > 312 for indomethacin (10 eV, 32V), m/z 532 > 356/193 for indomethacin glucuronide (14/30 eV, 22V), m/z 150 > 107 for paracetamol (20 eV, 32V) and m/z 230 > 150 for paracetamol sulfate (18eV, 32V). In addition, 1-naphthol was monitored by UV detection at the wavelength of 295 nm. Nitrogen was used as both desolvation and nebulising gas, while argon was used as a collision gas at 3.8 × 10⁻³ mbar pressure. The mass spectrometer and HPLC system were operated with Micromass MassLynx 4.0 software. For the metabolites, raw data (MS area) was used directly as a marker of the relative amount of the metabolite in the samples.
5 Results and discussion

5.1 Expression of efflux proteins and UGT enzymes in the Caco-2 cell model (I, II)

The mRNA expression level profile of the studied efflux proteins in Caco-2 cells was observed to be qualitatively quite similar to the respective reported profile in human small intestine (Table 3). These results further support the use of Caco-2 as a model for the epithelium of small intestine, even though the cell line originates from the colon.

Table 3. Observed relative mRNA expression profiles of principal efflux transporters and UGT enzymes in fully differentiated Caco-2 cells vs. reported mRNA/protein expression in human small intestine and colon. Note that the expression levels in different systems can not be directly compared to each other, since different methods have been used. Within each system: +++ = very high expression, ++ = high expression, + = observed, - = not observed.

<table>
<thead>
<tr>
<th></th>
<th>Caco-2 (observed)</th>
<th>Small intestine</th>
<th>Colon</th>
<th>Note</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDR1</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>Apical; inducible by vinblastine</td>
<td>A</td>
</tr>
<tr>
<td>MRP1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Not localised to membranes in Caco-2!</td>
<td>A, B</td>
</tr>
<tr>
<td>MRP2</td>
<td>+++</td>
<td>+++</td>
<td>-</td>
<td>Apical</td>
<td>A, B</td>
</tr>
<tr>
<td>MRP3</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>Basolateral</td>
<td>A, B</td>
</tr>
<tr>
<td>MRP4</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>Basolateral</td>
<td>A, B</td>
</tr>
<tr>
<td>MRP5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Basolateral ?</td>
<td>A, B</td>
</tr>
<tr>
<td>MRP6</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>Basolateral ?</td>
<td>A, B</td>
</tr>
<tr>
<td>BCRP</td>
<td>++</td>
<td>+++</td>
<td>++</td>
<td>Apical</td>
<td>A</td>
</tr>
<tr>
<td>UGT1A1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>C, D, E</td>
</tr>
<tr>
<td>UGT1A3</td>
<td>?</td>
<td>+ / -</td>
<td>+</td>
<td>Good quality primers not available</td>
<td>C, D, E</td>
</tr>
<tr>
<td>UGT1A4</td>
<td>+</td>
<td>+ / -</td>
<td>+</td>
<td></td>
<td>C, D, E</td>
</tr>
<tr>
<td>UGT1A5</td>
<td>+</td>
<td>+ / -</td>
<td>+/-</td>
<td></td>
<td>C, E</td>
</tr>
<tr>
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<td>+++</td>
<td>+</td>
<td>+</td>
<td></td>
<td>C, D, E</td>
</tr>
<tr>
<td>UGT1A7</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>Largely extrahepatic</td>
<td>C, D, E</td>
</tr>
<tr>
<td>UGT1A8</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>Largely extrahepatic</td>
<td>C, D, E</td>
</tr>
<tr>
<td>UGT1A9</td>
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<td>+ / -</td>
<td>+</td>
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</tr>
<tr>
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<td>Largely extrahepatic</td>
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</tr>
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<td>UGT2B4</td>
<td>+/-</td>
<td>+/-</td>
<td>-</td>
<td></td>
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</tr>
<tr>
<td>UGT2B7</td>
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<td>+</td>
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<td>C, D, E</td>
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</tr>
<tr>
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<td>+</td>
<td></td>
<td>C, D, E</td>
</tr>
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<td>++</td>
<td>-</td>
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<td></td>
<td>C, E</td>
</tr>
<tr>
<td>UGT2B28</td>
<td>+/-</td>
<td>+</td>
<td>?</td>
<td></td>
<td>D, E</td>
</tr>
</tbody>
</table>

References within Table 3: A) Hilgendorf et al., 2007, B) Prime-Chapman et al., 2004, C) Tukey and Strassburg, 2001, D) Gregory et al., 2004, E) Nakamura et al., 2008.
Of the presently studied efflux proteins, the mRNA expression of MDR1 has been found to correlate with protein abundance and efflux activity (Taipalensuu et al., 2004). On the other hand, MRP1 has been found in Caco-2 cells at protein level, but was localized in cytosolic compartments instead of the basolateral membrane (as in vivo), suggesting that the mRNA expression may not be indicative of MRP1 activity (Prime-Chapman et al., 2004). However, the same study also showed localization of the MRP2-4 in Caco-2 monolayers reflective of the in vivo situation with a corresponding activity of at least MRP2 and MRP3.

In contrast to the efflux protein expression pattern, the expression profile of UGT enzymes in Caco-2 cells somewhat differs from the UGT expression profile in the small intestine (Table 3). However, there are some conflicting reports on the expression of different UGTs in the intestine (Gregory et al., 2004, Nakamura et al., 2008). Variable results have also been obtained with the previous studies in Caco-2 (Münzel et al., 1999, Ishida et al., 2007, Nakamura et al., 2008). Several UGT isoforms appear to be readily inducible with e.g. flavonoids in Caco-2 cells and in the intestine, thus accentuating the need to thoroughly characterize the cell batch in use (Galijatovic et al., 2000, Van de Kerkhof et al., 2008).

5.1.1 Effect of cell differentiation

For the efflux protein (I) and UGT (II) mRNA expression studies, Caco-2 cells of each studied passage were cultured either as monolayers on polycarbonate filters for at least 21 days to achieve full differentiation (differentiated, “D”), or in culture flasks to near confluence (undifferentiated “U”). The polarization of Caco-2 monolayers grown on filters was clearly demonstrated by the increased expression of the protein villin in comparison to its level in the respective undifferentiated samples of the same passage number (Figure 5). Villin is a good marker for the differentiation, as it is expressed in the microvilli of the apical brush border of the enterocytes (Hodin et al., 1997).

The expression of the studied efflux proteins was observed to be higher in the differentiated cells (with the exception of MRP4), which may have implications for higher throughput cell-based screening assays (Figure 6). Transport studies are usually conducted with fully differentiated cells grown on inserts for at least 21 days, while most of the current screening methods (e.g. Calcein AM test) are based on shorter growth protocols, where the cells are grown for no more than 7-8 days (Eneroth et al., 2001, Yamashita et al., 2002, Shirasaka et al., 2006). The expression profile of different efflux proteins may then resemble that of undifferentiated rather than differentiated cells. This may, in turn, affect the sensitivity and specificity of efflux screening methods.
Figure 5  The effect of cell differentiation on villin expression. The mRNA expression of villin, normalized with respect to β-actin expression, in Caco-2 samples. Statistically significant differences (p < 0.01) between the undifferentiated and differentiated samples of the same passage are indicated with an asterisk above the respective bars.

Figure 6  The expression ratios of different efflux proteins in differentiated vs. undifferentiated Caco-2 cells. 1 = same mRNA expression in differentiated and undifferentiated cells.

Cell differentiation also increased the expression of most of the UGTs. A good example of this effect is UGT1A8: relatively low expression was observed in undifferentiated cells, while in the differentiated cells the mRNA levels were 5-10 fold higher (Figure 7A). For some of the UGT isoforms, the effect of differentiation was even more pronounced: for example, the differences in mRNA levels of UGT2B17 between the undifferentiated and differentiated cells were approximately 50-fold (Figure 7B). Some UGTs with this kind of an expression profile (e.g. UGT2B10) were barely detected in the undifferentiated samples.
Figure 7  Examples of UGT mRNA expression patterns in Caco-2 cells: The effect of differentiation and passage number on the relative expression levels of A) UGT1A8, B) UGT2B17 and C) UGT1A6, normalized with respect to β-actin expression. Statistically significant differences (p < 0.01) between the undifferentiated and differentiated samples of the same passage are indicated with an asterisk above the respective bars.
Of all the studied UGTs, UGT1A6 provided an interesting exception: the mRNA expression was consistently high in all the samples, including the undifferentiated cells (Figure 7C). The expression profiles for all the individual studied UGTs are provided in the supplement S1 of the original publication II.

Similar effect of differentiation on the expression of glutathione-S-transferases and sulfotransferases in Caco-2 cells has also been reported (Peters and Roelofs, 1989, Meinl et al., 2008). Thus, the whole phase II metabolism profile, as well as the MRP2/MRP3/BCRP-mediated efflux of conjugated metabolites, is clearly different in the undifferentiated cells (e.g. cells, with a short growth protocol, used for higher throughput screening methods) than in the fully differentiated cells.

5.1.2 Effects of passage number and vinblastine treatment

The passage number of Caco-2 cells is known to affect many of the features of the cell line, such as growth in culture, viability and efflux protein expression (Briske-Anderson et al., 1997, Anderle et al., 1998). Therefore, a certain, validated passage number range is usually employed in each laboratory routinely using Caco-2 cells.

The mRNA expression levels of the studied efflux proteins were not strongly affected by the passage number (Table 1 in original publication I). On the other hand, the expression of many UGTs was found to increase at higher passage numbers (II) (Figures 7A and 7B). Therefore, in our laboratory, differentiated Caco-2 cells at the passages ~ P40 would be the best choice for studies with drugs that may undergo glucuronidation during first-pass metabolism in the intestine.

As expected, the mRNA expression levels of MDR1 were constantly (approximately 2-3-fold) higher in the vinblastine induced Caco-2 cell line (Caco-2VBL) than in the respective wild type Caco-2 samples (I). This observation was confirmed in the functionality studies (Figure 9). Considerable variation was observed between the different wild type Caco-2 cultivation lots, whereas the expression in Caco-2VBL cells remained more consistent. Vinblastine did not affect the expression levels of any of the other apical or basolateral efflux proteins, CYP3A4 (probed due to the known interplay with MDR1) or the UGTs, except for a slight decrease in BCRP in differentiated Caco-2VBL cells compared to wild type cells.
5.2 Functionality of efflux proteins and UGT enzymes in the Caco-2 cells

Figure 8  Structures of the model compounds used in functionality studies: Rhodamine 123 for MDR1 (I), CDCF for MRP2 (III) and 1-naphthol and estradiol for the UGTs (II).

5.2.1 Functionality of MDR1 in Caco-2VBL and wild type cell monolayers (I)

Transport of Rhodamine123 (substrate for MDR1 and BCRP, Figure 8) was observed to be higher in basolateral-to-apical (B→A) than in apical-to-basolateral (A→B) transport direction (efflux ratios ranging between 2 and 5 at passages <40), indicating some efflux activity in wild type Caco-2 cells (Figure 9). The higher expression of MDR1 in Caco-2VBL cells was featured by more pronounced efflux activity (efflux ratios of 8-24).

The functionality of MDR1 was strongly dependent on the passage number in the wild type cells (Figure 9). The efflux activity was higher at low passage numbers compared to the later passages (efflux ratios P29 > P36 > P44 > P49 > P53). The findings are in full agreement with the mRNA expression results. In a previous study from our laboratory, loss of MDR1 activity was also observed at higher passage number Caco-2 cells of the same origin (Koljonen et al., 2006). In Caco-2VBL cells, the functionality of MDR1 was more consistent across all the passages.
Co-administration of 200µM verapamil, inhibitor of MDR1, MRP1 and MRP3 but not MRP2 or BCRP (Barrand et al., 1993, Honda et al., 2004, Zhang et al., 2005), in the donor chamber reduced the B→A transport of rhodamine123 in Caco-2VBL cells (efflux ratios of ~1), indicating inhibition of MDR1-mediated efflux (Figure 4 in original publication I). In the wild type cells, the presence of verapamil had no significant effect on rhodamine123 transport at P53, demonstrating the absence of functional MDR1 efflux at later passages in the used Caco-2 cell clone.

Due to the higher activity and more constant functionality of MDR1 in the Caco-2VBL cell line compared to the wild type cells, Caco-2VBL cells could be used over a prolonged passage span for the screening of potential MDR1 substrates. Vinblastine and its analogous alcaloid vincristine have also been used by other groups to increase the MDR1 activity in Caco-2 cells (Eneroth et al., 2001, Korjamo et al., 2005, Shirasaka et al., 2008). On the other hand, the lower expression of MDR1 in conjunction with the similar expression profile of other efflux proteins and metabolic enzymes, together with the relative ease of culturing, appoint the wild type Caco-2 cells as the cell line of choice for the screening for other efflux proteins, especially the apically localized MRP2 or BCRP.

### 5.2.2 Functionality of MRP2 (III)

Functionality of MRP2 was assessed in permeability experiments with CDCF (5(6)-carboxy-2',7'-dichlorofluorescein), a fluorescent substrate of the apical MRP2 as well as...
the basolateral MRP3 and MRP5 (Zamek-Gliszczynski et al., 2003, Pratt et al., 2006). The permeation of CDCF across the Caco-2 monolayers was very low at pH 7.4 (lower than that of mannitol, the marker for paracellular permeability and cell monolayer integrity), with both apical to basolateral (A→B) and basolateral to apical (B→A) P<sub>app</sub> values <10<sup>-7</sup> cm/s (Figure 10). The permeation was still quite low at pH 6.5 (on both sides of the monolayer), yet efflux activity was observed (efflux ratio of 8.3). Verapamil, an inhibitor of MDR1, did not affect the permeation. Altogether, these results indicate that MRP2 is the main efflux protein affecting CDCF permeability in Caco-2 cells.

![Figure 10](image.png)

**Figure 10**  Transport of CDCF (P<sub>app</sub> ±SD, n=3) across fully differentiated Caco-2 monolayers at pH 7.4 and at pH 6.5 and with MDR1 inhibitor, verapamil. Statistically significant differences (p < 0.01) are indicated with an asterisk above each pair of experiments.

With the opening of the lactone ring at higher pH, CDCF is a dicarboxylic acid and highly ionized at pH 7.4 (pKa-values of 3.58±0.04 and 4.86±0.02 were obtained for the two carboxylic acid groups by potentiometric titration). According to the pH partitioning theory, it is thus not able to passively permeate cell membranes in efflux activity experiments (Figure 8). CDCF has previously been used as an MRP-efflux marker in hepatocytes, where the ionized CDCF is actively taken up by OATPs (organic anion-transporting polypeptides) (Zamek-Gliszczynski et al., 2003). Some laboratories have reported functional OATP also in Caco-2 cells (Seithel et al., 2006, Maeda et al., 2007), but no evidence of OATP activity was observed in the present study.

### 5.2.3 Functionality of UGT isoforms (II)

According to the results from the expression studies, the main UGTs in our Caco-2 cells are UGT1A6 in the undifferentiated cells and UGT1A6, UGT1A8, UGT2B7 and UGT2B17 in the differentiated cells (many other isoforms are also expressed). These results were supported by the enzyme activity assays with 1-naphthol (good substrate for
UGT1A6) and estradiol (substrate for many UGTs but not UGT1A6) (Kurkela et al., 2003, Lépine et al., 2004, Itäaho et al., 2008). Glucuronidation of 1-naphthol was observed both in undifferentiated and differentiated Caco-2 cells, whereas estradiol was not glucuronidated in the samples obtained from undifferentiated cells (Figure 11). In the differentiated cells, estradiol was glucuronidated both to the 3-OH and 17-OH metabolite, indicating glucuronidation activity of at least two other UGTs besides UGT1A6. Estradiol is glucuronidated at the 3-OH position by UGTs 1A1, 1A3, 1A8, 1A10 and 2B15, while glucuronidation at the 17-OH position is catalyzed by UGTs 1A4, 2B4, 2B7 and 2B17 (Itäaho et al., 2008). Based on the expression results and glucuronidation rates of different UGTs, the glucuronidation of estradiol in Caco-2 cells was probably catalyzed by at least UGT1A8 and/or UGT1A10 (estradiol-3-glucuronide) and UGT2B7 and/or 2B17 (estradiol-17-glucuronide).

Figure 11  Glucuronidation rates of 1-naphtol and estradiol in differentiated (D) and undifferentiated (U) Caco-2 cells.
5.3 Efflux-metabolism interplay in the Caco-2 cell model

Figure 12 Potential patterns of interplay between the most important drug efflux and metabolism processes observed during the Caco-2 permeability studies. Localisation of the efflux proteins are presented as diamonds and drug molecules as circles; parent drugs in yellow and orange, phase I metabolites in blue and phase II metabolites in magenta. BCRP is also localized in the apical membrane of the Caco-2 cells and may contribute to the transport of phase II metabolites.

5.3.1 Efflux assays (III)

As an application for the demonstrated MRP2 functionality and the proposed interplay with phase II metabolism in Caco-2 cells (Figure 12), a higher throughput screening method was developed for substrates and/or inhibitors of MRP2, along with compounds having metabolites formed in Caco-2 cells that interact with MRP2. CDCF was chosen as a marker for MRP2 interactions due to the fluorescence (rapid and easy analytics) and the observed properties as a good, apically specific substrate for MRP2 in Caco-2 cells. The encountered problem with the poor membrane permeability at pH 7.4 was overcome by administering the compound as a diacetate derivative, CDCFDA. The non-fluorescent diacetate form passively diffuses across the cell membrane and is then readily hydrolyzed into the fluorescent CDCF by the intracellular esterases (Breeuwer et al., 1995). Hereby the poor passive permeability can even be utilized, as CDCF has to be effluxed out from the cells and the efflux is interfered by all the compounds interacting with MRP2.

De-esterification kinetics of CDCFDA in Caco-2 cell suspension was compared to the passive hydrolysis in study buffer and, as a reference, to the de-esterification in porcine liver esterase (PLE) solutions (Landowski et al., 2006). Similar de-esterification kinetics were observed in Caco-2 monolayer homogenates and at the highest PLE concentration of 250µg/ml, with more than 100-fold CDCF formation compared to the spontaneous hydrolysis in the study buffer (results in more detail in the original publication III).
Several factors were optimized during the screening method development (Table 4).

### Table 4. Optimisation of the experimental set-up of the MRP2 screening method.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Considered options</th>
<th>Final protocol</th>
<th>Rationale</th>
</tr>
</thead>
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<td>Cell line</td>
<td>• Caco-2</td>
<td>Caco-2</td>
<td>• Human origin and the established expression MRP2 and other transporters and phase II metabolizing enzymes possibly involved in MRP2 interactions.</td>
</tr>
<tr>
<td></td>
<td>• MDCKII-MRP2</td>
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</tr>
<tr>
<td>Culture plate</td>
<td>• 96-well cell culture plates:</td>
<td>Clear 96-well plates for cell culture. Fluorescence measurements of the samples from black plates.</td>
<td>• Clear 96-well plates are the standard for HTS cell culturing: most affordable and allow for visual inspection of the cells.</td>
</tr>
<tr>
<td></td>
<td>- Clear</td>
<td></td>
<td>• Black plates for fluorescence measurements: less background and cross-talk.</td>
</tr>
<tr>
<td></td>
<td>- CellBind&lt;sup&gt;®&lt;/sup&gt;</td>
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<td></td>
</tr>
<tr>
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<td>- Black</td>
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<td></td>
<td>- Black, clear bottom</td>
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</tr>
<tr>
<td>Assay method</td>
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<td>Sampling into a separate plate followed by immediate analysis.</td>
<td>• Greatly improved signal to noise ratio at measurements justifies an additional protocol step.</td>
</tr>
<tr>
<td></td>
<td>• Sampling into a separate plate for analysis</td>
<td></td>
<td>• Fluorescence reading immediately post-sampling to reduce the possible effect of spontaneous hydrolysis of residual CDCFDA.</td>
</tr>
<tr>
<td>Cell growth period</td>
<td>• 8 days</td>
<td>8 days</td>
<td>• Cells grown in 96-well plates (also CellBind&lt;sup&gt;®&lt;/sup&gt;) for more than 8 days were more fragile during washing steps.</td>
</tr>
<tr>
<td></td>
<td>• 15 days</td>
<td></td>
<td>• Complete differentiation would require 21 days on a filter.</td>
</tr>
<tr>
<td>CDCFDA loading time</td>
<td>• 10 min</td>
<td>10 min</td>
<td>• Sufficiently long equilibration with CDCFDA solution to allow intracellular hydrolysis and accumulation of CDCF in the cells.</td>
</tr>
<tr>
<td></td>
<td>• 30 min</td>
<td></td>
<td>• Initially selected 30 min was later shortened to 10 min; reproducibility of the results was not affected.</td>
</tr>
<tr>
<td>CDCFDA loading concentration</td>
<td>• 0.5 µM</td>
<td>5 µM</td>
<td>• Lowest concentration providing sufficient responses with good enough reproducibility.</td>
</tr>
<tr>
<td></td>
<td>• 2 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• 5 µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling time</td>
<td>• 20 min</td>
<td>30 min</td>
<td>• Best responses with lowest variation.</td>
</tr>
<tr>
<td></td>
<td>• 30 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• 40 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• 60 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO concentration as a co-solvent</td>
<td>• 0.5%</td>
<td>max. 1%</td>
<td>• 2% DMSO did not affect the results, either; 1% was chosen as a limit just to be on the safe side.</td>
</tr>
<tr>
<td></td>
<td>• 1%</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>• 2%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

In the final experimental set-up, Caco-2 cells are seeded in 96-well plates and cultured according to the laboratory standard protocols for 8 days. The cell monolayers are washed twice, loaded with CDCFDA solution (5 µM, 200 µl/well) for 10 minutes and briefly washed again. The plate is then ready for the experimental and reference (100% efflux, HBSS-HEPES pH 7.4) solutions (30 min incubation, 250 µl/well). During the incubation, intracellularly produced CDCF is effluxed out of the cells by MRP2 and the flux is
potentially inhibited if the test compound also interacts with MRP2. At the end of the incubation period, samples (200 µl) are withdrawn into a separate plate for immediate analysis with a fluorescence plate reader. The results are assessed by comparing the effluxed fluorescence values to the reference wells in the same plate (efflux into the study buffer) and to results obtained for known MRP2 substrates, inhibitors and non-substrates. The final protocol was tested with known efflux substrates, inhibitors (MK571 and probenecid) and a non-substrate (furosemide) (Figure 13). In most cases, the method succeeded in pointing out the substrates or inhibitors from the non-substrates. However, there are some limitations and challenges. A known issue is related to the false negative responses of hydrophilic compounds in cell-based screening methods due to the insufficient distribution into the cell membrane (Wortelboer et al., 2003). Another concern is related to the incomplete differentiation of Caco-2 cells cultured for screening purposes: in the absence of fully polarized monolayers, the expression and activity of some uptake and efflux transporters or metabolic enzymes may be compromised.

As the method is intended to act as a preliminary screen of any interaction, it will not separate the substrates from the inhibitors; neither the interactions with the parent compound from interactions with intracellularly formed metabolites. The involvement of basolateral MRPs can not be completely ruled out, since the growing of the cells on a flat plastic surface is likely to affect the morphology of the basolateral membrane and, consequently, the localisation of the basolateral efflux proteins.

![Figure 13](image-url)  
**Figure 13**  
Testing of the MRP2 screening method with known substrates, inhibitor and non-substrate: Inhibition of efflux of the fluorescent CDCF compared to the fluorescence observed in the reference solution (HBSS-Hepes pH 7.4; 100%) at the conditions selected for the final protocol (Table 4). Statistical significance of the difference compared to the reference, i.e. 100% (p < 0.01) is indicated above each bar.
The quality of the developed screening assay was studied by determining the Z factor (Zhang et al., 1999). An assay with Z factor > 0.5 (or even 0.4 for cell-based assays) is considered excellent, while +/- type assays typically have Z factors ~0. When the response caused by 1 mM diclofenac was compared to the negative control (study buffer), a Z factor of 0.47 was obtained with the final protocol. However, +/- type responses sufficient for initial screening could also be obtained for other compounds studied, also at lower concentrations.

5.3.2 Permeability experiments (IV)

Since the functionality of both phase II metabolism and MRP efflux had been established in the studied Caco-2 cell line, permeability experiments were conducted according to validated routine protocols, with substrates known to undergo glucuronidation and/or sulfation. Due to the extensive observed glucuronidation, 1-naphthol was chosen as a model compound from the UGT activity experiments. Formation of 1-naphthol glucuronide was, indeed, substantial also in the permeation studies: at the lowest concentration studied (5 µM) the concentrations of parent 1-naphthol were below the limit of quantification in the receiver compartments, whereas glucuronides were detected in both compartments at all time points.

Both apical and basolateral efflux of naphthol glucuronides was observed at all the studied concentrations (Figure 14). In view of the results from the expression studies, this is probably due to glucuronidation by at least UGT1A6 followed by the apical efflux by MRP2 and/or BCRP and the basolateral efflux by MRP3 and/or the other basolateral MRPs. The efflux of 1-naphthol glucuronide was higher to the basolateral than to the apical side of Caco-2 monolayers, as was also previously reported by others (Mizuma et al., 2004). This could be due to several factors: higher affinity of 1-naphthol glucuronide to basolateral efflux transporters, higher capacity of basolateral efflux systems or the inhibition of apical efflux by the glucuronide conjugates.
Figure 14  The relative amounts of 1-naphthol glucuronide observed in apical and basolateral Transwell® compartments at 120 min during 1-naphthol Caco-2 permeability studies at different initial parent donor (A. apical and B. basolateral) concentrations (n = 3).

Lower amount of 1-naphtol was conjugated to glucuronides at higher parent 1-naphthol concentrations, indicating substrate or product inhibition of the metabolizing enzyme(s) (Figure 14). This observation was further supported by the higher apparent permeability of 1-naphthol at higher donor concentrations (Table 5).

Table 5.  Transport of the parent compounds across Caco-2 monolayers in the metabolism/permeability studies ($P_{app}$ ±SD, n=3).

<table>
<thead>
<tr>
<th>Conc (µM)</th>
<th>1-Naphthol</th>
<th>Indomethacin</th>
<th>Paracetamol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A ‡ B (x 10^6 cm/s)</td>
<td>B ‡ A (x 10^6 cm/s)</td>
<td>Efflux ratio</td>
</tr>
<tr>
<td>5</td>
<td>n.d.</td>
<td>n.d.</td>
<td>-</td>
</tr>
<tr>
<td>20</td>
<td>4.2 ± 0.6</td>
<td>1.9 ± 0.6</td>
<td>0.4 ± 1.1</td>
</tr>
<tr>
<td>50</td>
<td>12.0 ± 1.4</td>
<td>7.6 ± 1.6</td>
<td>0.6 ± 0.5</td>
</tr>
<tr>
<td>200</td>
<td>55.5 ± 2.9</td>
<td>42.0 ± 3.0</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>500</td>
<td>55.2 ± 0.9</td>
<td>47.8 ± 4.5</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>1000</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

In contrast to 1-naphthol, the permeability of parent indomethacin across Caco-2 monolayers was quite consistent in both directions over the whole studied concentration range (Table 5). Transport of indomethacin has, indeed, previously been proven to be passive in Caco-2 cells (Neuhoff et al., 2005). Indomethacin glucuronides were formed in
all the studies, but at the lowest parent concentrations the metabolite concentrations were below the limit of quantification (Figure 15).

![Graph](image)

**Figure 15** The relative amounts of indomethacin glucuronide observed in apical and basolateral Transwell® compartments at 120 min after indomethacin Caco-2 permeability studies at different initial parent donor concentrations (n = 3).

Indomethacin glucuronidation did not demonstrate similar distinct inhibition pattern than the one observed for 1-naphthol in this study. On the other hand, the increase in the formation of indomethacin glucuronide was not linear with the increase in the parent concentration (Figure 15). Since the linearity in the analytics of the glucuronide was not properly established, definitive quantitative conclusions for indomethacin glucuronidation in Caco-2 monolayers can not yet be drawn. Qualitatively, the UGTs involved in the glucuronidation of indomethacin in the differentiated Caco-2 cells may include UGT1A1, UGT1A3, UGT1A9 and UGT2B7 (Sabolovic et al., 2000, Mano et al., 2007). It has, however, been reported that at higher indomethacin concentrations, the glucuronidation process and, thus, the formation of the glucuronides is decreased (Kuehl et al., 2005). Indomethacin itself is not a substrate for MRP2, so the reported concentration-dependent competitive inhibition of MRP2 may well be due to the efflux of glucuronides (Kouzuki et al., 2000, El-Sheikh et al., 2007). Indomethacin also stimulates the MRP2-mediated efflux of glucuronides, which in turn may account for the profoundly apical efflux of indomethacin glucuronide at the lower concentrations (Zelcer et al., 2003). At the presumably highest intracellular glucuronide concentrations, the observed basolateral efflux provides a possible concrete example of the complementary role of the basolateral efflux proteins in case of saturation of the apical efflux (Figure 15).

The permeability of the parent paracetamol was also passive and consistent in both directions over the studied concentration range (Table 5). Paracetamol glucuronidation was not observed in these studies; however, paracetamol sulfate was observed in both
chambers at all the parent donor concentrations in both directions (Figure 16). This is due to the fact that the SULTs often have much higher affinity to the substrates they share with the UGTs (Van den Hurk et al., 2002). Hence, the UGTs only contribute to the overall metabolism at high substrate concentrations, which may well not be the case during Caco-2 permeability experiments. In these studies, several SULTs may have been involved in the production of the observed metabolites (Meinl et al., 2008).

**Figure 16** The relative amounts of paracetamol sulfate observed in apical and basolateral Transwell® compartments at 120 min during paracetamol Caco-2 permeability studies at different initial parent donor (A. apical and B. basolateral) concentrations (n = 3).
5.3.3 Pharmacokinetic modelling

Complex kinetics was observed in the Caco-2 permeability experiments involving both phase II metabolism and efflux of metabolites. There were occasions in which the observations could be explained best with a combination of active processes. Under these circumstances, pharmacokinetic modelling can provide a helpful tool. The interacting processes can be separated and the kinetic parameters can be modified individually. This way, the most plausible explanation could be applied to the observed phenomena.

Modelling of metabolism and efflux simultaneously has already been attempted in Caco-2 cell experiments (Sun and Pang, 2008). However, the observations published so far have been quite theoretical, without supporting practical data. This is quite understandable, since the studies included in this dissertation have clearly demonstrated the intricacy of the subject.

The pharmacokinetic modelling based on the data presented in this dissertation is ongoing (Figure 17). There are still some challenges to overcome (e.g. the absolute quantification of the metabolites formed during Caco-2 experiments), but along with the rapid progress in this field, the generalisation of these observations will certainly continue to contribute to the overall knowledge of the feasibility of Caco-2 cell line for studies of drug metabolism and efflux.

Figure 17  Schematic representation of the interplay of UGT/SULT conjugation and MRP efflux in the enterocytes and a simplified diagram of the pharmacokinetics involved. Aki Heikkinen (University of Kuopio) and Jussi Malkki (Orion Pharma) are acknowledged for their contributions in the preparation of this figure.
6 Conclusions

Based on all the results obtained during this study, the Caco-2 cell line can be applied as a platform for studies, where phase II metabolism and MRP efflux are observed simultaneously.

The following conclusions can be drawn from the results:

1. Many efflux proteins and UGT enzymes are expressed in Caco-2 cells. With few exceptions (MRP4 and UGT1A6), the expression is higher in fully differentiated cells, compromising the activity of these systems when using incompletely differentiated cells.

2. Several factors influence the expression and functionality of efflux proteins and phase II metabolic enzymes in Caco-2 cells. These factors include passage number, level of differentiation and growth protocols (e.g. the use of inducers). Hence, the Caco-2 cell batch used in efflux/metabolism studies should always be characterized at the relevant passage window and level of differentiation. Furthermore, the functionality of relevant enzymes and efflux proteins must be confirmed.

3. The developed Caco-2 based screening method, utilizing the probe pair CDCFDA/CDCF, provides a promising screening tool for compounds interacting with MRP2. The method is also suitable for the screening of indirect interactions, i.e. compounds, the glucuronides (and/or other phase II conjugates) of which are MRP2 substrates or inhibitors.

4. The interplay of phase II metabolism and MRP efflux is functional during Caco-2 permeability experiments. The kinetics of this interplay is quite complex, demonstrating e.g. saturation of the efflux and substrate/product inhibition of the conjugative enzymes.
References


Kurkela, M., Garcia-Horsman, J.A., Luukkanen, L., Morsky, S., Taskinen, J., Baumann, M., Kostiainen, R., Hirvonen, J., Finel, M., 2003. Expression and characterization of recombinant human UDP-glucuronosyltransferases (UGTs). UGT1A9 is more resistant to detergent inhibition than other UGTs and was purified as an active dimeric enzyme, J Biol Chem 278: 3536–3544.


Meinl, W., Ebert, B., Glatt, H., Lampen, A., 2008. Sulfotransferase forms expressed in human intestinal Caco-2 and TC7 cells at varying stages of differentiation and role in benzo[a]pyrene metabolism, Drug Metab Dispos 36:276-283.


12,000 gene sequences tags and correlation with permeability of 26 drugs, Pharm Res 19: 1400-1416.


