Chapter 1

Introduction
General introduction

The plasma membrane forms an impermeable barrier for hydrophilic compounds that constitute many of the nutrients, essential factors and drugs that must enter the cell, and conversely, for the toxic and signaling molecules that must be released from the cell into the extracellular media. To overcome this obstacle, cells have evolved various transport systems to ensure the selective permeability of their membranes. At the heart of these systems are transmembrane transport proteins that selectively recognize their substrates and move them across the membrane at the expense of energy. Transporters use different sources of energy to facilitate this transport process. Equilibrative transporters (e.g. the equilibrative nucleotide transporters, ENTs) transport their substrate downstream its concentration gradient until equilibrium is obtained. Concentrative transporters (e.g. the Apical Sodium-dependent Bile acid Transporter, ASBT) couple the transmembrane-concentration gradient of sodium (or other factors) to the transport of their substrate, thereby highly concentrating it within the cell. Another family of transporters (e.g. members of the Organic Anion Transporting Polypeptide family, OATPs) are thought to work by exchanging an intracellular organic anion for an extracellular substrate resulting in the accumulation of the latter. A universal source of energy in diverse cellular processes is ATP and not surprisingly, many transporters have chosen this as their source of energy. These ATPases couple the energy released by the hydrolysis of ATP into inorganic phosphate and ADP to the transport of a substrate against its concentration gradient.

Among the ATPases, members of the ATP Binding Cassette (ABC) transporters form a distinct family that is based on shared, highlyconserved signature motifs. ABC transporters form a large gene superfamily that is evolutionarily preserved and they have been identified in all the organisms studied to date, from bacteria to man. These transporters are remarkable in that their substrates include many different and dissimilar substrates that span the diverse array of endo- and xenobiotica known to man. In humans, 48 ABC transporters have been identified and only for a small subset have the physiological role and transport properties been elucidated. Based on phylogenetic analysis the human ABC transporters have been further subdivided into 7 subfamilies. Among these, the ABC-C branch contains 12 members thus forming the largest subfamily of human ABC transporters.

The ABCC subfamily contains the Multidrug Resistance Proteins 1-9 (MRP1-9) along with SUR1, SUR2 and CFTR. Whereas MRP1-8 are bona fide transporters for which transport of organic anions has been demonstrated, this is not the case for SUR1, SUR2 and CFTR. SUR1 and SUR2 are regulators of inwardly rectifying potassium channels and CFTR functions as a chloride channel. Whether MRP9 is also an organic anion transporter remains to be determined. Interest in the multidrug resistance proteins was sparked by their possible involvement in clinical
resistance of tumors to chemotherapeutic agents. The first member of this family to be cloned, MRP1, confers resistance to a broad spectrum of anticancer drugs when overproduced in cells. Rather early, however, it became apparent that MRPs transport a wide variety of organic anions and compounds that are conjugated with sulfate, glucuronate, glutamate and with glutathione (GSH), placing them in a position to participate in many different physiological processes. The importance of MRPs is further emphasized by the fact that mutations in two members of this family, MRP2 and MRP6, are the cause of two inherited human diseases, the Dubin-Johnson syndrome and Pseudoxanthoma elasticum, respectively.

The substrate specificity of MRPs, as mentioned above, is broad, and to a certain extent overlaps. Several substrates such as the steroid conjugate estradiol-17-β-D-glucuronide (E₂₁₇βG) and the anticancer drug methotrexate (MTX) are transported by multiple MRPs. Despite their partially overlapping substrate specificity, MRPs can be distinguished based on their size and predicted membrane topology (figure 1), localization to different membrane compartments in polarized cells (apical vs. lateral membrane) and on their tissue distribution (table 1). This emphasizes the need to study the different MRP isoforms in detail in order to obtain a better understanding of their physiological roles and their possible contribution to multidrug resistance of tumors.

This thesis describes the characterization of the transport properties of MRP3 and MRP4, with an emphasis on their substrate specificity, physiological role and their possible contribution to clinical multidrug resistance of tumors. Whereas these lines of research may seem unlinked, they are in fact interwoven. From studying drug resistance one can infer possible substrates and physiological roles and vice versa. Another intriguing question addressed in this thesis is the mechanism by which MRPs transport their substrates across membranes, which was studied in the context of MRP2 but is likely to be relevant for the other MRP members as well. Beyond the basic mechanistic aspect, this has also implications for drug-drug interactions and for the possibility of modulating the MRP-mediated transport process by developing transport inhibitors or stimulators.

How do MRPs recognize and transport their substrates? (Chapter 2)

How MRPs transport their substrates is not known in detail and is a question that received much attention in recent years. ABC transporters are large transmembrane proteins and their structural analysis has proven difficult. High-resolution structures of several bacterial transporters have been recently reported, while for the drug transporting MRP1 and MDR1 P-glycoprotein only low-resolution structures have been obtained. In the absence of a high-resolution atomic
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P-glycoprotein  TAP  ABCG2

MRP1  MRP5

Figure 1. Predicted topology of the major classes of mammalian ABC transporters. This simplified scheme shows the intracellular nucleotide-binding domains (NBDs) and the transmembrane segments and indicates the N and C termini of the protein. Note that the predicted topology is often based on minimal data, as in the case of ABCG2 (BCRP1/MXR/ABCP) and MRP5 (ABCC5). TAP, the transporter associated with antigen presentation, probably has more than six transmembrane segments. The half-size transporter TAP functions as a heterodimer of TAP1 and TAP2, and ABCG2 functions as a homodimer. Taken from Borst et al. 7

structure, the mechanism of transport has been inferred from a combination of transport, binding and mutational studies.

For MDR1 P-glycoprotein, these experiments led several groups to propose that MDR1 contains 3-4 substrate-binding sites that show positive heterotopic cooperativity. 38-41. It still is a debate whether all sites are transport capable, or whether some are only modulatory sites. 42 Moreover, whereas one may envision these sites as separated domains within the transport molecule, it is not unconceivable that these sites may exist within one large binding pocket that contains distinct functional regions. 43 Recently, structural support for this type of model was obtained in the bacterial multidrug transporter AcrB, admittedly not an ABC transporter, where it was unequivocally demonstrated that multiple ligands bind within a large cavity that is formed by the transmembrane domains of this transporter. 44

The anionic nature of MRP substrates makes them very unpractical for studying the substrate specificity and transport mechanism in intact cells. However, this very physico-chemical
Table 1: Summary of tissue distribution, polarized localization and substrate specificity of MRP1-9.

<table>
<thead>
<tr>
<th>Size</th>
<th>Tissue distribution</th>
<th>Polarized localization</th>
<th>Substrate specificity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRP1</td>
<td>Long Ubiquitous</td>
<td>Basolateral</td>
<td>X-GlcA, X-Sulf, X-SG, GSH, X+GSH, MTX</td>
</tr>
<tr>
<td>MRP2</td>
<td>Long Liver, Gut, Kidney</td>
<td>Apical</td>
<td>X-GlcA, X-Sulf, X-SG, GSH, X+GSH, MTX</td>
</tr>
<tr>
<td>MRP4</td>
<td>Short Ubiquitous, high in kidney and prostate</td>
<td>Basolateral/Apical</td>
<td>Cyclic nucleotides, MPNs, X-GlcA, X-Sulf, GSH, X+GSH, bile acids, MTX</td>
</tr>
<tr>
<td>MRP5</td>
<td>Short Ubiquitous</td>
<td>Basolateral</td>
<td>Cyclic nucleotides, MPNs, X-SG</td>
</tr>
<tr>
<td>MRP6</td>
<td>Long Kidney, liver, low in other tissues</td>
<td>Basolateral</td>
<td>X-SG, cyclic peptides</td>
</tr>
<tr>
<td>MRP7</td>
<td>Long Ubiquitous</td>
<td>?</td>
<td>X-GlcA</td>
</tr>
<tr>
<td>MRP8</td>
<td>Short Ubiquitous</td>
<td>?</td>
<td>Cyclic nucleotides, MPNs</td>
</tr>
<tr>
<td>MRP9</td>
<td>Short Ubiquitous</td>
<td>?</td>
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* X-GlcA, Glucuronate conjugate; X-Sulf, Sulfate conjugate; X-SG, Glutathione conjugate; MPNs, monophosphorylated nucleotides

property makes them excellent substrates for use in vesicular transport assays. These assays have been the major experimental procedure employed to delineate the substrate specificity and transport mechanism of MRPs. In these assays, inside-out vesicles are prepared from cells that have been engineered to overproduce the transporter of interest so that transport of a (radiolabeled) substrate in the presence of ATP will be directed into the lumen of the vesicle (figure 2). Subsequently, the uptake of radiolabeled tracer can be measured and serve as an indication for the transport activity. Applying this approach to MRP1 revealed that it transports negatively charged compounds, or compounds conjugated with GSH, sulfate, glutamate or glucuronate. In these assays, the highest affinity substrate identified for MRP1 is leukotriene C4 (LTC4) which has an apparent K_m value of ~100 nM. The importance of this finding was later demonstrated in mice deficient for Mrp1 that display an impaired inflammatory response as a result
of the loss of LTC₄ transport capacity. Using this same approach, the substrate specificities of MRP1-8 have been also defined over the last couple of years.

![Diagram of vesicular transport assay](image)

**Figure 2:** Schematic outline of vesicular transport assays. Inside-out membrane vesicles are isolated from cells that overproduce the transporter of interest. The reaction mix contains the substrate that is radioactively labeled. Adding ATP starts the reaction. The assay is terminated by filtering the reaction mix through a filter followed by several wash steps. Accumulated radioactivity associated with the vesicles is measured by liquid scintillation from which the ATP-dependent contribution of the transporter can be determined.

Most of our knowledge on the mechanism by which MRPs transport their substrate comes from extensive studies with MRP1. The picture that initially emerged from these assays is that MRPs transport negatively charged molecules in a process that seems to follow simple Michaelis-Menten enzyme kinetics. However, this simple model was challenged early on by the observations that MRP1 and MRP2 can transport and confer resistance to the anticancer drugs etoposide, doxorubicin and the vinca alkaloids vincristine (VCR) and vinblastine. These drugs are not negatively charged and are not known to form charged metabolites. Several lines of evidence suggest that the drug resistance phenotype of MRP1 cells to these drugs is dependent on the presence of GSH. Firstly, depleting these cells of GSH by treatment with
buthioninesulfoximine (BSO) can reverse the resistance phenotype to these drugs. Secondly, mouse embryonic stem cells lacking Mrp1 have a higher intracellular GSH concentration, presumably due to the fact that GSH secretion by Mrp1 is absent. Exposing stem cells that contain Mrp1 but not those that lack it to etoposide leads to a decrease in the intracellular GSH concentration, concomitant with elevated GSH secretion into the medium. Finally, a series of elegant experiments by the Cole and Deeleey lab (summarized in Leslie et al.) demonstrated that indeed, transport of VCR into MRP1-containing vesicles is stimulated by GSH, and conversely, that VCR stimulates GSH uptake.

Evers et al. extended these studies in polarized kidney cells that overproduce MRP2. In these cells MRP2 localizes to the apical membrane and mediates GSH transport into the apical compartment that is further stimulated by the presence of vinblastine. These findings led to the proposal that MRP2 co-transport GSH together with drug as depicted in figure 3. To model these observations Evers et al. suggested that MRP contains a complex substrate binding site in which drugs are transported from the D site and GSH from the G site. Simplistically, a GSH conjugate would then fit with its GSH moiety directed toward the G site whereas the D site would bind the rest of the molecule. In these experiments, however, the ratio between GSH and the co-transported substrate differed from the expected 1:1 ratio expected of obligatory co-transport, but this was largely attributed to technical issues.

Further challenge to this model comes from recent experiments where it was demonstrated that GSH also stimulates transport of organic anions by MRP1 and not only of neutral drugs, and that unexpectedly, in some cases transport of an anionic substrate by MRP1 is even dependent on GSH. Furthermore, in the case of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol-glucuronide and estrone-sulfate transport by MRP1 no reciprocal stimulation of GSH transport could be detected, suggesting that for these substrates, GSH acts as a positive heterotrophic stimulator and not as a co-substrate. In line with this, GSH transport by MRP1 can be stimulated by verapamil and several verapamil analogs as well as by flavonoids in the absence of detectable transport of these compounds. Stimulation is caused by a strong decrease of the $K_m$ for GSH from > 1 mM to 100-200 µM. Stimulation of GSH transport by these compounds is therefore not consistent with co-transport together with GSH.

The above-mentioned findings cannot be reconciled with co-transport as the model to explain MRP-mediated transport. Complex heterotropic interactions, like those described above for MRP1, have been reported for MRP2 and MRP3 as well. Uniquely, we have also recently demonstrated positive homotrophic interaction for transport of E$_2$17βG by MRP2, i.e. E$_2$17βG stimulates its own transport, an observation independently made by Bodo et al. Positive homotropic interactions have not been previously observed for MRP1. Compounds that strongly stimulate transport of E$_2$17βG by MRP2 do so by increasing the apparent affinity of the
transporter for this substrate, and interestingly, are not necessarily themselves transported by MRP2. This has led us to propose that MRP2 contains a bi-partite binding site that contains (minimally) two substrate-binding sites; a Modulatory site and a Transport site from which substrate is transported (figure 3) \(^\text{23}\). Whether a substrate X stimulates or inhibits transport of another substrate Y depends on the relative affinity of substrate X for the two distinct sites. A complex binding site as we propose would also allow us to accommodate the results from mutational studies of MRP1-3. These studies demonstrate that a defined mutation can have differential effects on the transport of distinct substrates by the same transporter \(^\text{73-78}\). Whereas transport of one substrate may be abolished, that of another may remain unaffected or even increase. Moreover, a bi-partite binding site located within a large cavity, as seen in AcrB \(^\text{44}\), would be able to explain why mutations spread throughout different transmembrane domains of MDR1 and MRP1 can have dramatic effects on their transport properties, as was previously suggested \(^\text{43}\).

At present, we see no compelling evidence for the alternative that the M site can function as a transport site, but given the postulated presence of at least 2 transport sites in P-glycoprotein, this alternative remains open for the MRPs.

In view of the complex positive heterotropic interactions seen for MRP1 and MRP2 it is necessary to reconsider the results interpreted as co-transport of drug and GSH reported previously. If GSH can bind both the M site \(^\text{60-65}\) and the T site \(^\text{25,59,61,66,87}\) in our model (figure 3), apparent co-transport could be due to cross-stimulation, in which GSH at the M site stimulates transport of drug in the S site and vice versa. A consequence of this reasoning would be that transport of GSH by MRP1 and MRP2 should also show positive homotropic cooperativity. However, the low transport rates obtained with GSH make this hard to test.

The question remains as to why should multidrug transporters contain multiple drug binding sites. The answer to this may be trivial. The M and T sites share similarities and both recognize E\(_{2}\)17\(\beta\)G \(^\text{23,72}\), GSH \(^\text{59,62-84}\) and sulfinpyrazone \(^\text{23,55}\). This may simply reflect symmetry that was preserved during the shift from a half transporter to a full transporter (figure 1), presumably via gene duplication. The binding of ligands that are not transported and the cooperativity between sites is less easily explained. Atkins et al. \(^\text{79}\) have recently proposed that the positive homotropic cooperativity found in some P450 drug metabolizing enzymes, might be explained by assuming a threshold effect of drug toxicity: If both substrate and product are toxic, it is better to have a mix of substrate and product. This way, none reach their toxic threshold concentration. Assuming this for MRP2 may then offer a plausible explanation for some of the complex homo- and heterotropic transport interactions seen with MRP2 substrates. This may reflect an additional point of regulation that keeps the levels of two interacting substrates under a certain toxic threshold by further regulating their transport. If the two substrates can stimulate each other's transport this will ensure that none will accumulate to toxic levels within the cell.
Figure 3: A schematic representation of the two mechanistic models of MRP-mediated transport that are discussed. The co-transport model (A) assumes that two substrates that stimulate each other’s transport (e.g. GSH and VCR, see text) are transported together in a transport cycle via the transport sites T1 and T2. The two-site model that we propose (B) assumes the existence of a Modulatory site that only modulates transport and a Transport site from which substrates are transported. Reciprocal stimulation as observed for GSH and VCR is then interpreted as cross-stimulation. Whether a substrate stimulates the transport of a second substrate or inhibits it will depend on the relative affinities of the substrates to the M and T sites.

In a similar vein, it is possible that an organic anion transporter able to transport valuable cellular compounds, such as GSH in the case of MRP1 and MRP2, would function best, if transport would be disproportionally low at low substrate concentrations to limit loss of cellular constituents, but rapid when the concentration is high. Similarly, when transport of a substrate results in trans toxicity it would again be advantageous to have disproportionately lower transport at low substrate concentration. This could be the case for transport of $E_2\beta$G by MRP2. The main canicular bile salt transporter BSEP is trans inhibited by $E_2\beta$G and this has been postulated to be the reason for estradiol induced cholestasis. Therefore, the homotropic nature of MRP2-mediated $E_2\beta$G transport is beneficial since it will ensure relatively low $E_2\beta$G transport at a low substrate concentration. Finally, homotropic cooperativity allows instantaneous transporter recruitment, and operates faster than an increase in protein synthesis, phosphorylation or recruitment of additional protein to the membrane as has been described.
The advantage of heterotopic interactions and stimulation of organic anion transport by ligands that appear not to be transported is not clear. The complex results obtained may simply be the consequence of the ability of MRP2 to bind many different ligands. Some of these may optimally bind only with a partner, resulting in heterotopic positive cooperativity, or even co-transport. Others may fit the binding pocket, but lack the properties required for transport. It is also possible that heterotopic cooperativity could have a direct physiological advantage, e.g. the ability of the major bile acids taurocholic and glycocholic acid to stimulate transport of E$_2$$\beta$2G by human MRP2 $^{23,72}$ and that of GS-DNP by rat Mrp2 $^{76}$. Bile acids regulate the activity of MRP2 in multiple ways. They modulate transport by MRP2, influence its expression $^{82}$, and as has been shown by Bodo et al. $^{72}$ are transported by it. This clearly demonstrates that an endogenous substrate may have a substantial effect on the overall activity of MRP2 and raises the intriguing possibility that regulation of transporters by endogenous compounds is a general phenomenon. We have recently observed that steroid conjugates stimulate MRP2- and MRP3-mediated transport at physiologically relevant concentrations lending further support to this idea. The fact that allosteric interactions have been found in plant $^{83}$, rodent $^{76,84}$ and human MRPs stresses the need to ascertain the physiological role of this phenomenon.

The presence of multiple drug binding sites in multidrug transporters has also pharmacological implications for the treatment of human ailments. Many of the drugs that show positive heterotopic cooperativity are commonly used in patients and therefore one must consider the possibility that both adverse and beneficial drug-drug interactions can occur at the transporter level. It is now well established that intestinal MDR1 P-glycoprotein $^{85,86}$, MRP2 $^{87}$ and BCRP $^{88,89}$ can limit the oral availability of substrate drugs in humans, rats and mice. Stimulating the activity of these transporters will lead to a further decrease in the bioavailability of drugs and thus to a lower treatment efficacy. Although speculative, it is possible to envision a condition where one would be interested to stimulate transport i.e. to stimulate the excretion of a toxic metabolite.

MRP3 (Chapters 3-5)

The existence of multiple MRP isoforms other than MRP1 and MRP2 was predicted from screens of expressed sequence tags and from analysis of cDNA from drug selected cell lines $^{90,91}$. Within a short period of time, several groups independently cloned the cDNAs of MRP3-6 and embarked on their analysis $^{28,92-96}$. The cloning of MRP7-9 followed several years later, completing the cloning of the human MRP family $^{97-101}$.

MRP3 (ABCC3) was initially cloned from rat liver as an inducible homolog of MRP1/2 in a screen to identify novel hepatic ABC transporters $^{102}$. Subsequently, the human isoform was cloned by various groups from the intestinal Caco2 cell line $^{103}$, human liver cDNA $^{26,104}$ and from a Cis-
platin resistant cell line. Human MRP3 is a 1527 amino acid membrane spanning glycoprotein with a predicted mass of 170 Kd. The predicted topology of the putative transmembrane domains of MRP3 indicates that it has an overall organization similar to that of MRP1,2,6 and 7 (figure 1). Comparison of the MRP3 sequence with that of the other MRP family members reveals that it is most closely related to MRP1 and MRP2 sharing with these transporters 58% and 47% amino acid identity, respectively.

The tissue distribution of MRP3 was studied at the mRNA and protein level and is narrow when compared to the more ubiquitous expression of MRP1. In the rat, Mrp3 is detected at high levels in the small intestine, colon and at low levels in the kidney and lung. No expression of Mrp3 was detected in normal rat liver. However, in livers of Eisai hyperbilirubinemic rats (EHBR) rats or rats that have undergone bile duct ligation (BDL) Mrp3 is strongly induced. The tissue distribution of human MRP3 was also studied and to a large extent overlaps that found in rats. Expression of MRP3 mRNA is high in the small intestine, colon, adrenal gland and liver and is moderate to very low in lung, kidney, placenta, gallbladder, pancreas, prostate and spleen. This expression pattern was largely confirmed by protein analysis with the exception that MRP3 protein could not be detected in the spleen and lung. Our detailed analysis of the tissue distribution of mouse Mrp3 (see chapter 5 of this thesis) is more or less in line with the tissue distribution of human MRP3 with the exceptions that relatively high levels of MRP3 are found in the lung and spleen and none in the kidney. The undetectable level of Mrp3 in rat liver samples stands in stark contrast to the relatively high levels of MRP3 in the liver of humans and mice. Moreover, in humans there is often a lack of correlation between the mRNA levels and the protein levels detected either by protein analysis or direct tissue staining. Initially, it was proposed that the reason for the relatively high levels of mRNA and protein MRP3 in some of the human liver samples that were analyzed is the result of the specimen originating from a diseased liver. Indeed, there is strong evidence that MRP3 levels are increased under conditions of liver disease (e.g. cholestasis, see below). However, it is now evident that MRP3 can be readily detected in normal human liver samples. A plausible explanation for this discrepancy can therefore be that the hepatic MRP3 level varies substantially between individuals as recently reported by Hitzel et al. The variation in hepatic levels of MRP3 can be thus attributed to the overall liver status as well as to external factors such as medication and dietary components next to species differences. Finally, in most of the tissues studied MRP3 is found in epithelial cells. Despite an early confusing report that claimed apical localization in these cells, it has been now unequivocally shown that in these cells MRP3 localizes to the basolateral membrane (see also chapter 5).

Initial studies on the substrate specificity of MRP3 were motivated by its possible involvement in multidrug resistance of tumor cells as it is most closely related to MRP1 that has been shown to confer resistance toward diverse anticancer drugs. Moreover, reports by Young et
al. \textsuperscript{114,115} suggested that MRP3 expression strongly correlated with the resistance of small and non-small cell lung carcinoma cell lines to doxorubicin, and more moderately with resistance to etoposide and VCR. An additional study also demonstrated a correlation between resistance to doxorubicin and MRP3 expression in bladder carcinoma \textsuperscript{116}. The possible contribution of MRP3 to anticancer drug resistance was studied in cell lines that have been engineered to overproduce MRP3. In these initial experiments, MRP3 mediated only low-level resistance to the two related podophyllotoxin derivatives etoposide and teniposide and to short-term, but high concentration methotrexate exposure (see below) \textsuperscript{91}. One of the studies reported also marginal resistance toward VCR, potentially broadening the range of anticancer drugs recognized by MRP3 \textsuperscript{117}. However, a major complication of these studies was that the resistance phenotype was weak and more importantly, was analyzed in cells that contain many endogenous transporters that also show clonal variation. To overcome these complications, we expressed MRP3 in a fibroblast-like cell line that was generated from the kidneys of mice that have homozygous deletions of \textit{Mdr1a/b} and \textit{Mrp1} (see chapter 3 of this thesis) \textsuperscript{25}. Due to the lack of these major drug transporters the cells are hypersensitive to anticancer drugs \textsuperscript{118} and are a suitable model to study the contribution of an exogenously introduced drug transporter. In these cells, MRP3 mediates resistance only to etoposide and teniposide. No resistance against VCR or a series of other anticancer drugs was observed. The relative resistance factors that we observed against etoposide in our MRP3 overproducing cells are the highest reported. From this we infer that the anticancer substrate specificity of MRP3 is narrow and includes only etoposide, teniposide and MTX and does not include several other drugs that were suggested based upon correlation studies. In view of this, we do not expect MRP3 to be a major determinant in clinical multidrug resistance of tumors.

To further characterize the substrate specificity of MRP3, a second approach was applied. Vesicular transport assays are a powerful technique to delineate the substrate specificity of a transporter and several groups have employed this technique for MRP3, most often using \textit{S. frugiperda} (Sf9) cells as their overexpression system. From the initial reports it was evident that MRP3 has distinct substrate specificity as compared to previously characterized MRPs. Whereas GSH conjugates are very good substrates of MRP1 and MRP2, they are rather poor substrates for MRP3 and unlike the former, MRP3 does not transport GSH \textsuperscript{24,25,84}. In this assay system, the transport activity of MRP3 results only in a 2-3 fold higher transport above control of LTC\textsubscript{4} and GS-DNP, two model GSH conjugated substrates. In contrast, glucuronate and sulfate conjugated substrates are transported at high rates and with high affinity. The spectrum of conjugated substrates that MRP3 transports include conjugates of steroids \textsuperscript{24,25,84}, prostanoinds \textsuperscript{119}, bile acids \textsuperscript{70,120} and those of commonly used drugs (e.g acetaminophen \textsuperscript{121}) and anticancer agents (e.g. etoposide) \textsuperscript{25}. In fact, the highest affinity substrates identified to date for MRP3 are the two glucuronide conjugates of the bile acids hyodeoxycholic and hyocholic acid (K\textsubscript{m} < 0.4 μM) that are
formed during the detoxification of chenodeoxycholic and lithocholic acid, respectively (our unpublished data, in collaboration with Hanns-Ullrich Marschall, Karolinska Insitute, Stockholm).

Another class of MRP3 substrates that have drawn great interest are (anti)-folates. MRP3, as has been also shown for MRP1, MRP2 and MRP4, can mediate resistance to MTX, but this is critically dependent on the exposure schedule \(^{28,29,122}\). In long-term exposure experiments cells overproducing these transporters show no resistance to MTX. However, in short term, high concentration drug exposure these transporters confer very high resistance to MTX. The molecular basis for this observation has now been clarified. MTX enters cells primarily via the reduced folate carrier and once within the cell is further poly-glutamylated by folylpoly-\(\gamma\)-glutamate synthetase (FPGS) to form MTX-Glu\(2,7\) that exert most of the toxic effect of MTX. MRP1-4 have been shown to transport MTX (MTX itself contains one glutamate group) \(^{13,28-30,68}\). Whereas MRP3 can still transport MTX-Glu\(2\) at substantially lower rates as compared to MTX (~5% of the MTX transport rate), the other MRPs mentioned cannot. Higher glutamylated forms of MTX are not transported by any of these transporters. Transport of MTX by MRP1-4 is characterized by low-affinity (\(K_m\) values \(\sim 0.2\)-5 mM) and high rates of transport.

In view of these findings it is now possible to explain the unique schedule-dependent MTX resistance mediated by MRPs. Toxicity of MTX reflects the relative rates of MTX polyglutamylation by FPGS and the rate of extrusion by MRPs. In short term exposure experiments to high MTX concentrations, MTX accumulates in the cell to high levels, resulting in saturation of FPGS and in an increase in the ratio of free MTX:polyglutamylated MTX. MRPs are low affinity/high capacity transporters of MTX and under this condition are expected to extrude MTX from the cell efficiently and limit the subsequent availability of substrate for FPGS. This is not the case, however, in long-term exposure using low MTX concentrations. Under this condition, polyglutamylation of MTX is favored. The low affinity of MRPs for MTX prevents them from substantially impacting the intracellular concentration of this drug and eventually sufficient polyglutamylated MTX species, that cannot be transported by MRPs, will be formed and kill the cell. Interestingly, MRP1-4 transport also the natural folates, folic acid and leucovorin, but again with low affinity. It would make no sense for MRPs to transport these compounds with high affinity as that would lead to depletion of the folate pool.

Whether MRP1-4 are involved in clinical resistance to MTX is still unknown. However, we think that this is doubtful in light of the very low affinity of these transporters for MTX. Most likely the free concentration of MTX is too low for MRPs to recognize them efficiently. However, the contribution of MRPs to MTX resistance is dependent on the competition between transport out of the cell on the one hand and glutamylation by FPGS on the other hand. Therefore, it is possible that in cells where the latter reaction is limiting, the transport of MTX by MRPs can have an impact on toxicity. The availability of Mrp1-4 \(^{-/-}\) mice will allow critical testing of this issue.
A great deal of effort has been invested into defining the physiological role of MRP3. From early on, several groups have suggested that MRP3 may play an important role in the enterohepatic circulation of bile salts. Briefly, bile salts are synthesized in the liver in a multi-step enzymatic process from cholesterol, secreted into bile and reabsorbed predominantly in the terminal ileum into the portal system so that under normal conditions less than 5% of the bile salt pool is lost daily (Figure 4). MRP3 has been suggested to play a role in this cycle in two different anatomical sites. The first site is in the enterocytes of the small intestine where MRP3 could transport reclaimed bile salts into the portal system thus ensuring their proper circulation. A second site is in the liver under pathological conditions of impaired bile flow, e.g., cholestasis. In cholestatic states gene expression is altered so as to minimize hepatic damage. The induction of MRP3 under this pathological state could protect the liver by allowing the secretion of toxic bile salts and organic anions into the circulation, and subsequent elimination via urine.

Several lines of evidence support the role of MRP3 in in vivo bile acid transport. MRP3 is detected in the small intestine and more importantly, in the terminal ileum where absorption of bile acids is thought to occur through uptake by ASBT. In rat small intestinal enterocytes Mrp3 is detected on the basolateral membrane, as is the case with mouse Mrp3 (chapter 5 of this thesis). This at least places MRP3 in a suitable position to participate in the intestinal step of the enterohepatic circulation. In the cholestatic state, the localization of MRP3 in the basolateral membranes of hepatocytes would allow it to transport toxic organic anions into the circulation that would otherwise accumulate within the cell. This is indirectly suggested by the increased expression of Mrp3 in the TR'/EHBR rats that lack Mrp2 in the apical membrane of their hepatocytes or in cholestatic rat models. In a similar vein, liver samples from Dubin-Johnson patients show increased MRP3 levels as well. Furthermore, the transport of taurocholate across the sinusoidal membrane in perfused rat livers is increased in livers from EHBR rats and correlates, at least partially with the expression level of Mrp3 in these livers.

Direct functional support for the participation of MRP3 in physiological transport of bile acids came from studies of its substrate specificity. In vesicular transport assays, rat Mrp3 readily transports glycocholate and taurocholate, the latter with high affinity (K_m of 16 μM). In these assays, Mrp3 transports also the 3-sulfo-conjugates of lithocholic (K_m of 3 μM) and taurochenodeoxycholic acid that are formed at increased levels during cholestasis. However, whereas the rat isofom transports bile acids with high affinity, transport of these compounds by human MRP3 occurs with low affinity. In vesicular transport assays, MRP3 transports glycocholate. However, it does so with low affinity (K_m of 250 μM). Transport of taurocholate in these assays...
HEPATIC SYNTHESIS = FECAL LOSS
E.H.C. = 95% of BILIARY SECRETION

**Figure 4:** The enterohepatic circulation of bile salts. Bile salts are synthesized in the liver from cholesterol and are secreted into bile by the bile salt efflux pump, BSEP (ABCB11). Bile salts reach the intestine where they assist in absorption of dietary fats and lipid soluble vitamins. Bile salts are taken up in the terminal ileum in a process mediated by the apical sodium-dependent bile salt transporter. Subsequently, they are transported across the enterocytes and secreted, by a yet unidentified transporter, into the portal system from which they are taken up back into the liver mainly by action of the sinusoidal sodium-dependent transporting polypeptide, NTCP. Under normal conditions <5% of the bile salt pool is lost in the feces. Values (mmol/day) for humans are presented.

is marginal but in cells that co-express ASBT and MRP3 clear transport of taurocholate by MRP3 can be demonstrated, albeit again at low affinity (chapter 4 of this thesis)\textsuperscript{70}. Only sulfo-conjugated bile acids are transported by MRP3 with high affinity, as is the case for rat Mrp3. From these assays one would conclude that human and rat Mrp3 have different affinities for bile acids, as was shown later for MTX and GS-DNP as well\textsuperscript{132}. A difference in the transport properties of MRP orthologs is not uncommon. In the case of MRP1 it is clear that whereas human MRP1 confers resistance to anthracyclines the mouse isoform does not and this is dependent on a single negatively charged residue at position 1086 that is not present in murine Mrp1 but is found in the
human isoform. However, despite this relatively large difference in substrate specificity, the major function of MRP1 as the high affinity LTC₄ transporter is conserved in humans and rodents. The same would be expected for MRP3 if bile acid transport was a major function of MRP3. The large difference in the transport properties of bile acids by the rodent and human isoforms strongly argues against these being the major endogenous substrates of MRP3. Furthermore, it suggests that in humans MRP3 would be only important under conditions of severely elevated bile acid levels. To critically test this claim we therefore generated mice that have a homozygous disruption of Mrp3.

The Mrp3 (−/−) mice were generated by stem cell technology and they are viable, fertile and show no apparent abnormality (chapter 5 of this thesis). The mice are not hypersensitive to etoposide, the only drug to which MRP3 mediates substantial resistance to, unlike their Mrp1 (−/−) counterparts. This probably indicates that either Mrp3 does not protect crucial organs that are sensitive to etoposide (e.g. bone marrow) or more likely, that overlapping transporters such as Mrp1 provide sufficient protection to these organs. To test whether Mrp3 is involved in transport of bile acids in the intestine, we used an Ussing chamber set up with ex vivo terminal ileum sections known to contain Mrp3. In these assays, we find no difference in the trans-ileal (mucosal to serosal) transport of taurocholate across ileum sections from Mrp3 (−/−) and Mrp3 (+/+ ) mice.

To test the hypothesis that Mrp3 is important for protecting the liver under cholestatic conditions we ligated the common bile duct to simulate an acute form of obstructive cholestasis. In rats, this treatment leads to a strong increase of basolateral Mrp3. Mice however, have high levels of hepatic Mrp3 to begin with and this treatment results only in a marginal further increase in Mrp3 levels. Bile duct ligation (BDL) results in a large increase in serum bile acids level that is observed in the control and mutant mice, but no difference was observed between these two groups. Our results are challenged by the recent report by Bohan et al. who studied the effect of a two-week BDL treatment on mice. This treatment results in a 6-fold increase of the basal hepatic Mrp3 levels concomitant with a strong increase in serum bile acid levels. Induction of hepatic Mrp3 by this treatment proceeds through a TNFα-dependent induction of the nuclear receptor liver receptor homolog-1 (LRH1). Accordingly, in mice lacking the TNF receptor, Tnfr(−/−) mice, induction of Mrp3 by BDL is abrogated and serum levels of bile acids are elevated to a lower extent than in control mice. The authors interpret this as evidence for the role of Mrp3 in the basolateral clearance of bile salts under BDL in mice, different from our observations. A simple reason for this discrepancy can be the duration of BDL. Bohan et al. studied the effect of 14 days of BDL whereas we did not extend our studies beyond 7 days. We do not, however, think that this is a likely explanation. More likely, the differences can be attributed to experimental methodology.

LRH1 regulates many aspects of hepatocyte biology including metabolism and transport processes. Repression of LRH1-mediated transcription in BDL treated Tnfr(−/−) mice is likely to
induce numerous hepatic gene alterations, as this is a key hepatic nuclear receptor. Additionally, we do not find strong induction of Mrp3 after BDL treatment whereas this is reported in that study. This discrepancy may be easier to reconcile. The studies were done in mice from different genetic backgrounds (C57BLJ\textsuperscript{135} and 50%/50% Ola129/FVB in our studies) and this may impact the endogenous hepatic Mrp3 levels. Possibly, our mice may display a constitutively induced Mrp3 phenotype, for reasons unclear to us, preventing it from being further induced. We are currently analyzing the levels of hepatic Mrp3 in mice from different genetic backgrounds to test this hypothesis.

BDL also impairs the MRP2-mediated secretion of bilirubin-(mono/di)glucuronide into bile and this results, as is seen in the TR\textsuperscript{136-138}/EHBR rats, in elevated conjugated bilirubin appearing in their serum. Similarily, we find elevated levels of conjugated bilirubin in the serum of mice that have undergone BDL, but the levels in Mrp3\textsuperscript{\textminus} mice are 50% lower than those of control mice. This suggests that Mrp3 transports bilirubin glucuronide across the sinusoidal membrane and accounts for ± 50% of this capacity. Clearly, other transporters are involved in this process as well. We are currently studying in closer detail the role of Mrp3 in bilirubin glucuronide transport under cholestatic conditions.

Our results with the Mrp3\textsuperscript{\textplus} mice suggest that Mrp3 is not involved in the in vivo transport of bile acids, contrary to the accepted view in the field. This raises the question as to what is the physiological role of MRP3? A plausible hypothesis is that MRP3 is involved in the detoxification of both endo- and xenobiotics, predominantly those that have been enzymatically conjugated to glucuronic acid. Our finding with bilirubin glucuronide in the cholestatic Mrp3\textsuperscript{\textminus} mice can be seen also within this context. Bilirubin enhances its own clearance by regulating the cascade of enzymes and transport systems that affect it via PXR- (pregnane X receptor) and CAR (constitutive androstane receptor)-dependent pathways\textsuperscript{139,140}. These nuclear receptors are known to regulate phase I and II metabolic enzymes, as well as phase III transport systems\textsuperscript{82,141-146}. It is not surprising that both CAR and PXR can regulate Mrp3 in mouse liver\textsuperscript{141,147}, and this further raises the possibility that bilirubin induces not only its glucuronidation by UGT1A1 but also the expression of hepatic Mrp3. We can only speculate that this will occur when biliary secretion of bilirubin is impaired so that it accumulates within the liver to concentrations that activate these nuclear receptors. We have recently noted that bile acids, CAR and PXR ligands induce MR3 expression in human colon carcinoma HCT-8 cells, and more importantly, in human hepatic carcinoma HepG2 cells (our unpublished results). The latter are often used as a model cell line for hepatocytes and our results suggest that the regulation of MR3 expression in hepatic cells is conserved between humans and mice. However additional studies are required to substantiate this metabolic pathway.
In a broader context, it is likely that Mrp3 plays a role in a detoxification axis involving nuclear receptors ⇒ enzymatic oxidation/conjugation ⇒ transport. In this context, transport for example of glucuronidated hyodeoxycholic acid can be seen as a pathway starting from lithocholic acid (LCA) activation of PXR/FXR ⇒ hydroxylation by CYP3A ⇒ induction of UGT2B4/7 ⇒ increased formation of HDCA-GlcA that is subsequently transported by MRP3 \(^{146}\) (figure 5).

Alternatively, a parallel pathway involving a similar cascade that induces sulfotransferases results in the synthesis of 3-sulfo-LCA, again an MRP3 substrate \(^{143-145}\). Whereas examples of endogenous compounds have been given, xenobiotics can trigger similar pathways \(^{146}\). Therefore, it can be expected that Mrp3 will have an impact on hepatic (and possibly intestinal) kinetics of xenobiotics that undergo extensive conjugative metabolism prior to their transport. Since the liver is the major metabolic organ involved in these reactions the effect of Mrp3 on these metabolites will be profound when they are predominantly secreted in the urine. This would then suggest that subsequent to conjugation, the metabolites are transported across the sinusoidal membrane by Mrp3 for further urinary excretion \(^{149}\). Two drugs that display these characteristics that we are currently studying are acetaminophen and morphine. The glucuronide metabolites of both of these drugs are predominantly excreted in the urine, although they are formed in the liver. Morphine is an intriguing compound as it is a rare example where one of its metabolites, morphine-6-glucuronide, is more potent than the aglycone and is recognized specifically by Mrp3 (our unpublished results).

**Figure 5**: Detoxification of LCA in cholestasis. In cholestasis, LCA levels increase and activate the nuclear hormone receptors FXR and PXR. These in turn down-regulate CYP7A1 the rate-limiting enzyme in bile acid synthesis and up-regulate CYP3A4 and UGT2B4/7 resulting in hydroxylation of LCA to HDCA that is subsequently conjugated with glucuronate to form HDCA-GlcA that is transport by MRP3. A parallel pathway (not depicted) involves the induction of sulfotransferase 2A1 by PXR/FXR, which catalyzes the conjugation of LCA with sulfate. The resulting LCA-3-sulfate is also transported by MRP3.
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An additional and highly speculative hypothesis is that MRP3 is involved in the transport of physiologically important conjugated steroids. We have recently found that 5β-pregnane-3α,20α-diol glucuronide is a high-affinity inhibitor of MRP3-mediated E₂17βG transport, whereas to achieve similar inhibition of MRP1 or MRP2 at least 10-fold higher concentrations are required (our unpublished results). We infer that this pregnane conjugate is a substrate of MRP3, but this remains to be tested. A different conjugate, 5-pregnen-3β-ol-20-one sulfate (pregnenolone sulfate) stimulates transport of E₂17βG by MRP3 (~ 3-fold) and MRP2 (~ 20-fold) and several pregnane mono- and di-sulfates that are associated with intrahepatic cholestasis of pregnancy are transported by both transporters (our unpublished results). Combined with the localization of MRP3 in the liver, adrenal gland and human term placenta this could point to an important role of MRP3 in steroid metabolism. The Mrp3(-/-) mice show no overt abnormalities as might have been expected had their steroid metabolism been severely disturbed. Whether MRP3 plays a role in steroid metabolism is still unclear but is an exciting possibility that warrants further attention.

To conclude, in recent years our knowledge of MRP3 has increased. Whereas we know much about the substrate specificity and possible contribution of MRP3 to drug resistance, its physiological role is still unclear and remains a major unanswered question. The current view that MRP3 transports major species of bile acids in vivo is not supported by our experiments with the Mrp3(-/-) mice. Alternative hypotheses that link MRP3 to detoxification pathways, and more speculatively to steroid metabolism are discussed above. Whereas these hypotheses suggest a highly specialized physiological role for MRP3 based on its substrate specificity it cannot be ruled out that MRP3 has no such "specialized" role. The similarity between MRP2 and MRP3 is striking. The substrate specificity of MRP2 and MRP3 overlap with the exception that MRP2 transports GSH whereas MRP3 does not. In tissues where MRP3 is detected in the basolateral domain of epithelial cells, MRP2 is found on the opposing apical membrane. The main difference between these two transporters lies in the affinities with which they recognize and interact with their shared substrates, e.g. MRP3 seems to be a higher affinity glucuronide transporter than MRP2. In contrast, GSH conjugates are good substrates of MRP2 whereas they are poor MRP3 ones. This raises the possibility that MRP2 and MRP3 evolved together and what controls the directional transport of their common substrates are their relative affinities towards these. A direct consequence of this speculation would be that GSH conjugates would be preferably transported by MRP2 and glucuronide conjugates by MRP3. Transport of an inactivated conjugate by MRP2 makes sense, as it would result in their elimination from the body via bile, feces or by direct excretion into urine. However, the physiological advantage of transporting inactivated glucuronide conjugates across the basolateral membrane of polarized cells by MRP3 is not immediately evident. In all the tissues where MRP3 is detected this would result in pumping the conjugate back into the body instead of assisting in its elimination. We can only speculate that this may have an
advantage in circumventing many of the uptake systems present in the gut whose activity can result in a futile enterohepatic cycle for these conjugates. An additional advantage for preventing these conjugates from reaching the intestine may to avoid exposing them to the high activity of bacterial β-glucuronidases that is present in the gut. Glucuronidases will result in deconjugation of the metabolite back to the parent compound and possibly to its enterohepatic circulation and in some case even to enterocyte toxicity. The intestinal toxicity of SN-38 is thought to occur by this mechanism \(^{151,152}\). This hypothesis, although highly speculative, remains to be critically tested.

**MRP4 (Chapters 4-6)**

MRP4, like MRP3 was initially identified by a genomic approach searching for MRP-like cDNAs \(^{90,91}\). The cloning of MRP4 by several groups \(^{93,153,154}\) followed, and revealed that the protein is composed of 1325 amino acids and lacks the extra N-terminal transmembrane domain found in MRP1 (Figure 1). MRP4 is most closely related to MRP5 and to the recently described MRP8 and MRP9. MRP4 is detected in most tissues and cell lines at low to moderate levels, the prostate and the kidney being exceptions where the expression is high \(^{93,122,153}\). The polarized localization of MRP4 is still in dispute. Whereas in the prostate MRP4 localizes to the basolateral and lateral membranes of the acinar cells \(^{122}\) (and our unpublished results) and in hepatocytes to the sinusoidal membrane \(^{155}\), van Aubel et al. \(^{153}\) reported that rat and human MRP4 are detected in the apical membranes of cells of the proximal tubule in the kidney. In MDCK-II/MRP4 cells MRP4 was detected in the basolateral membranes \(^{156}\), however, the MRP4 cDNA construct used in that study contains the V5 and HIS\(_9\) tags in its C' terminal region. This region is important for the sorting and routing of other ABC-C family members \(^{157-160}\). New transfected cell lines with high levels and membrane targeted MRP4 and analysis of additional tissues should assist in settling this issue. At present, however, one cannot rule out the possibility that MRP4 localizes to different membrane domains in a tissue specific manner. An even more intriguing possibility is that differential splicing regulates the routing of MRP4 either to the apical or basolateral membrane. Notably, Lee et al. \(^{93}\) originally cloned two isoforms of MRP4 at equal frequencies. The longer isoform has an open reading frame encoding a protein of 1325 amino acids which was designated MOAT-B (MRP4). This ORF was the one used in their subsequent studies. However, the second variant that has a shorter and altered C'-terminus has not been further characterized. There is also evidence that the expression of MRP4 is regulated by complex splicing events that can result in nonsense-mediated decay of its transcript \(^{161}\). It is possible that in different tissues this process is regulated differently. Different regulation can result in one tissue having predominantly the longer transcript, and another tissue predominantly the shorter variant. Although speculative, if this C'-terminal region contains...
crucial sorting signals its presence or absence may offer an explanation for the current discrepancy. Clearly, this possibility requires further examination.

Interest in the possible role of MRP4 in drug resistance was spurred by the observation that it is amplified in a cell line that shows cross resistance to the nucleoside-based antiviral drugs (9-(2-phosphonylmethoxyethyl)adenine) (PMEA) and azidothymidine (AZT). Resistance toward these drugs was the result of enhanced efflux of PMEA (which is a monophosphorylated compound) and of AZT-monophosphate, respectively. Furthermore, the level of MRP4 amplification in these cells correlated with the resistance toward these drugs and resulted in their lower anti-HIV activity. This was the first example of an ABC transporter that transports nucleotides out of cells. Subsequently, cells transfected with an MRP4 cDNA were used to define the substrate specificity of MRP4. Transfected cells that overproduce MRP4 are resistant to several base and nucleoside analog drugs used in antiviral chemotherapy (e.g. PMEA). Additionally, overexpression of MRP4 in cells results in resistance to the anticancer drugs thioguanine (TG) and 6-mercaptopurine (6-MP). Only low-level resistance was found for cladribine, but not to any of the other nucleoside analogs that are often used in the chemotherapy of cancer. Both MRP4 and MRP5 confer resistance against 6-MP and TG and this was further studied by Wielinga et al. who demonstrated that both transport the thiopurine mononucleotides after they are formed from the base. Whereas MRP4 has a preference for methylated thio-IMP, MRP5 prefers the unmethylated nucleotides. Studies with MRP4 transfected cells also revealed that MRP4 confers resistance against MTX. However, similar to MRP1 and MRP3, cells that contain MRP4 are resistant only to short-term, high-dose MTX exposure as was discussed above. To date, no reports have indicated a correlation between MRP4 and clinical drug resistance and it is still not known whether MRP4 contributes to clinical resistance to nucleobase and nucleoside based antiviral and anticancer therapy. As Chen et al. point out, however, MRP4 transports both MTX and 6-MP. These two drugs are used in the treatment of childhood acute lymphoblastic leukemia and MRP4 may influence both arms of this treatment. Clearly, additional studies are required to investigate this possibility.

The utilization of vesicular uptake studies revealed that the substrate specificity of MRP4 is yet broader than initially thought. MRP4 transports E\textsubscript{2}17βG and is inhibited by several other glucuronide conjugates. However, the finding that MRP4, like its closest homolog MRP5, transports the 3',5'-cyclic nucleotides cAMP and cGMP with high affinity and at physiologically attainable concentrations set MRP4 in the spotlight. Cyclic nucleotides signal primarily as intracellular second messengers, but they are known also to have extracellular receptors and functions. The role of extracellular cyclic nucleotide has been best studied in the slime mold Dictyostelium discoideum where secretion of cAMP by solitary amoebae under low nutrient conditions initiates the aggregation and formation of a multi-cellular organism. The high-affinity
transport of cAMP and cGMP by MRP4 and MRP5, however, is fiercely debated. Our results stand in strong contrast to findings of others. In vesicular transport assays transport of these cyclic nucleotides has characteristics of low-affinity transport and very high concentrations of cAMP/cGMP, far above their reported K_m's for these transporters, only show marginal inhibition of transport of another substrate. Similarly, Chen et al. 30 found only 50% inhibition of MRP4-mediated MTX (20 μM) transport by 300 μM of cGMP or cAMP (a concentration 30- and 6-fold higher than the respective K_m's reported for cGMP and cAMP transport by the same group). At this concentration, MRP4 should be fully saturated by the cyclic nucleotides and inhibition of the transport of a poorer substrate like MTX should be complete, assuming simple transport kinetics.

Further support for low-affinity transport of these nucleotides by MRP4 and MRP5 comes from our studies with intact cells. Cells that overproduce MRP4/5 secrete more cGMP and cAMP into the medium in an ATP-dependent manner, as expected. However, transport from these cells increases linearly as a function of the intracellular concentration over a calculated range of 20-600 μM. Moreover, MRP4 and MRP5 overproduction does not lead to a substantial decrease in intracellular levels of these cyclic nucleotides, even when phosphodiesterases are inhibited, a condition where the maximal effect of these transporters would be expected. Combined, these results suggest that MRP4 and MRP5 are low-affinity transporters of cAMP and cGMP.

The involvement of GSH in the transport of cAMP by MRP4 is also controversial. Whereas one group found that decreasing the cellular GSH levels leads to a concomitant reduction in the MRP4-mediated cAMP transport, our studies with intact cells do not show this. However, a recent report by Rius et al. demonstrated that MRP4 requires GSH to transport taurocholate in vesicular transport assays (see below) and that taurocholate reciprocally stimulates transport of GSH. In our studies with vesicular transport assays, addition of GSH neither stimulated nor inhibited transport of several MRP4 substrates indicating that GSH is not a general requirement for transport by MRP4. It seems therefore likely that neither MRP4 nor MRP5 play a major role in attenuating cyclic nucleotide signaling and in their physiological secretion. At best, they could function as overflow transporters under conditions where intracellular cyclic nucleotides are highly elevated and phosphodiesterase activity is low.

Recently, MRP8 was also reported to extrude cyclic nucleotides from cells, but the kinetic parameters of this process were not reported. High-affinity transport of cyclic nucleotides is known to exist and whether MRP8 represents the transporter responsible for this action remains to be determined.

More recently, we have suggested three other exciting possible functions for MRP4. The first is based on findings in the Fxr (−/−) mice. In livers of Fxr (−/−) mice Mrp4 was strongly upregulated suggesting a pathway in which elevated bile acids induce the expression of Mrp4 that subsequently reduces their toxicity, similar to what has been discussed above for MRP3.
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finding that Mrp4 is upregulated in these mice led us to hypothesize that it transports (conjugated) bile acids (Chapter 5 of this thesis). We find that bile acids, especially sulfated ones that are elevated under cholestatic conditions are potent competitive inhibitors of MRP4-mediated transport. A recent study demonstrated that MRP4 is localized to the sinusoidal membrane of hepatocytes and transports taurocholate in a GSH-dependent manner with high affinity. GSH transport was reciprocally stimulated by taurocholate and at the single concentration tested a ratio close to 1 was observed for GSH and taurocholate transport. This is suggestive of cotransport according to the authors but a study over a range of concentrations is required to elucidate the transport mechanism.

A second possible physiological role of MRP4 may be in steroid metabolism. We had noticed that steroid conjugates, that share structural similarities with bile acids, inhibited MRP4 to varying degrees. Among these, the potent inhibition by dehydroepiandrosterone-sulfate (DHEAS) attracted our attention. DHEAS is the most abundant circulating steroid in humans and its concentration reaches 10 μM in the blood. Whereas its synthesis in the adrenal cortex is well characterized, it is unknown how it is secreted into the circulation. We detect MRP4 in human adrenal samples but with our antibodies were unable to localize it in tissue sections. In transport assays MRP4 transports DHEAS with high rates and with high affinity (Km of 2 μM) suggesting the possibility that MRP4 plays a role in secretion of DHEAS from its site of synthesis. Additionally, DHEAS is thought to act as a steroid precursor reservoir that target tissues can convert to downstream steroids after it is taken up by carrier proteins. Once taken up, an equilibrium between the sulfated and non-sulfated form of DHEAS is obtained depending on the relative activities of sulfatases and sulfotransferases. MRP4 may be a component of this regulatory system as it is able to transport DHEAS.

A third possible physiological function for MRP4 is in the transport of prostanoids (chapter 6 of this thesis). Prostaglandins (PGs) are generated by metabolism of arachidonic acid and are important signaling molecules that exert diverse cellular reactions. PGs interact with extracellular receptors that mediate their actions. Transport of GSH conjugated prostaglandins has been previously shown for MRP1-3, but this is considered to be a step in their biological inactivation. Non-conjugated PGs, e.g. PGE1 and PGE2, are thought to diffuse across the membrane despite their negative charge and the fact that a transporter, PGT, is required to take them up into cells. In vesicular transport assays we find that MRP4 transports PGE1 and PGE2 with high affinity. The affinity of MRP4 for these PGs is similar to that reported for arachidonic acid from which cyclooxygenase (COX) enzymes catalyze their synthesis. Additionally, MRP4 is more sensitive to inhibition by other PG derivatives (e.g. PGF2α, and TXB2) when compared to MRP1-3 and MRP5, which are only marginally or not inhibited by these derivatives at concentrations that fully inhibit MRP4. This suggests further that MRP4 may also be
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Capable of transporting other prostanoids. In cells, overexpression of MRP4 results in reduced accumulation of PGs after transfection of cells with a PGT cDNA construct to allow their entry. Conversely, decreasing endogenous levels of MRP4 by siRNA constructs increases accumulation of PGs in these cells demonstrating that even low levels of MRP4 have profound effects on PG kinetics. Interestingly, non-steroidal antiinflammatory drugs (NSAIDs) inhibit MRP4 much more than MRP1. NSAIDs work predominantly by inhibiting the COX enzymes that participate in the synthesis of PGs. It is possible, however, that part of their effects are also mediated by inhibition of MRP4 and the release of PGs into the extracellular space. It is now clear that MRP4 transports PGs in vesicular transport assays and from cells when these are loaded via PGT. PGs are de novo synthesized in response to various stimuli in a variety of cells and elicit diverse effects \(^{178}\) and are thought to passively diffuse across the plasma membrane in a process driven by the pH and voltage gradients \(^{182}\). Moreover, it has been suggested that de novo synthesized PGs and those taken up by PGT are found in distinct compartments (V.L Schuster, personal communication). It remains therefore to be seen whether MRP4 plays a role in the release of de novo synthesized PGs as well. Several classes of high affinity substrates have been now identified for MRP4. The Mrp4\(^{(-/-)}\) mice will allow a critical examination of the role of Mrp4 in their physiology.

Concluding words

The cloning of the last member of the MRP family of transporters in 2002 rounds off a decade of research that started with the cloning of MRP1 in 1992. With the exception of MRP9 all of the MRPs have been expressed in cells and their substrate specificity has been studied using the different assays described above. It is now clear that MRPs are promiscuous transporters that are capable of transporting several classes of endo- and xenobiotic substrates. The goal of the coming years is to try and place these transporters within a biological context and elucidate their role in different biological processes in vivo. For MRP3 and MRP4, that have been the focus of this thesis, the tools to do so are available.
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