MRP2-4, from drug resistance to physiology

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Conclusions and perspectives

With the cloning of the last MRP member in 2002 a decade of exciting MRP research comes to an end. During these years, three major questions fueled the study of MRPs: (1) How do MRPs transport their substrates across biological membranes? (2) What is the contribution of MRPs to multidrug resistance of cancer? (3) What are the physiological roles of MRPs? The work presented in this thesis is an attempt to address some of these issues for three members of the MRP family, MRP2-4.

How do MRPs transport their substrates?

Our understanding of how MRPs transport their substrates across biological membranes is derived, in the absence of high-resolution structures, from indirect assays, by comparison with related transporters (e.g. MDR1 P-glycoprotein), and by comparison with crystal structures of bacterial ABC transporters that have been recently solved. From the combination of these studies, several general principles can be distilled.

The promiscuous nature of MRPs must imply that they contain a flexible substrate-binding site that accommodates chemicals with divergent structures. Drug efflux transporters are not unique in this, as evolution has chosen this solution for other proteins as well. A flexible and dynamic binding site is found in mammalian nuclear receptors\(^1\) and metabolizing enzymes\(^2\) as well as in bacterial drug-dependent transcription factors\(^3\). In these proteins the substrate binding site is formed by a large, relatively hydrophobic cavity. This allows these proteins to fit different substrates, as is likely to be the case for MRPs as well.

There is strong evidence that MRPs contain more than one ligand binding site\(^4\) (chapter 2 and references therein). This is now recognized for MRP1-4 and is likely to be the case for the remaining MRPs. These sites display complex homo- and heterotropic allosteric interactions, but the importance of this for the function of MRPs is not clear. Some MRPs evolved to use GSH as co-stimulator/substrate for transporting their substrate\(^5,6\) (see below), and it is conceivable that many of the interactions observed with other compounds mimic that of GSH. It is tempting, however, to speculate that other endogenous compounds, such as steroid conjugates (our unpublished results), serve as endogenous modulators of MRPs, like GSH. A similar reasoning can be applied to drugs that display complex interactions with MRPs\(^4,7,8\). The allosteric properties of MRPs must be important for their function as otherwise, over an evolutionary time scale and in the absence of a selective pressure, they would be lost. Moreover, allosteric interactions similar to the ones observed with human MRPs have been reported for rat\(^9,10\) and plant\(^11\) MRP homologs. However, why MRPs display allosteric effects is not clear and some ideas on this were put forward
in chapter 1 of this thesis. The in vivo relevance of these observations is even less clear. The allosteric properties of MRPs have been demonstrated in vesicular transport and cellular assays. It is now imperative to demonstrate that they take place in intact animals and can affect pharmacokinetics, oral availability and possibly toxicity. We expect this to be the case.

An additional question addressed in this thesis derives from the reciprocal stimulation of substrate transport seen in MRPs and best studied for GSH in combination with other substrates. The early observations were interpreted as co-transport of GSH together with substrate. However, in light of our findings (chapter 2) and those recently made by others, this view is challenged. It is now clear that some substrates can stimulate transport by MRP1 and MRP2, without being transported. With hindsight, results interpreted in the past as co-transport may be just as well explained by co-stimulation. To accommodate our recent results we put forward a model that suggests (minimally) two ligand-binding sites in MRP2; one site from which substrate is transported and a second modulatory site. In the context of this model, the complex interactions that we observed between ligand pairs can be explained by their relative affinities to the two different sites (chapter 2). It is likely that the true picture is even more complex. MDR1 P-glycoprotein contains 4 distinct binding sites, one of which is thought not to be transport-competent. Clearly, studies with additional substrates will allow the further dissection of the complex binding sites of MRPs. Ultimately, high resolution structures of MRPs in their ligand-bound conformation along the transport pathway are required to settle this issue. The enormous technical complications involved in obtaining high-resolution structures of human drug transporters are a major obstacle that must yet be conquered. However, the recent reports on the structures of bacterial drug transporters and complex ion channels gives hope that in the not too far future this will be accomplished.

**Does MRP3 contribute to clinical multidrug resistance of tumors?**

Our basic interest in MRPs is due to their possible role in clinical multidrug resistance of tumors. The involvement of MRP3 in clinical multidrug resistance was suggested by correlation studies that found a relationship between its expression and resistance of lung and bladder tumors to doxorubicin, etoposide and VCR. Additionally, the homology between MRP3 and MRP1, a bona fide drug transporter, further points to a possible overlap in their substrate specificity. Our studies (chapter 3) on the contribution of MRP3 to drug resistance revealed that MRP3 mediates resistance to the synthetic epipodophyllotoxin derivatives, etoposide and teniposide, and to MTX (as discussed in chapter 1), but not to other drugs from the major anti-neoplastic classes. The Mrp3(-/-) mice are not hypersensitive to etoposide (chapter 5) and at face value, these results suggest that MRP3 is not likely to contribute to multidrug resistance of cancer. Nevertheless, many

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of the anticancer drugs (similar to other xenobiotics) are extensively metabolized by phase-I and phase-II systems. It is possible that the conjugated metabolites formed by these reactions are transported by MRP3 as we demonstrated for etoposide glucuronide \(^{22}\) (chapter 3). Therefore, functional coupling of drug metabolism to MRP3-mediated transport can potentially result in decreased toxicity to tissues. Several anticancer drugs (e.g. SN-38 \(^{23}\)) are extensively glucuronidated and may be used to test this hypothesis.

**What are the physiological roles of MRP3?**

A large part of the work presented in this thesis was aimed at elucidating the roles of MRP3 and MRP4 in physiological processes. The physiological roles of a given transporter are directly derived from its tissue distribution and substrate specificity; e.g. to study the physiological roles, one must initially study the substrate specificity.

The substrate specificities of MRP3 and MRP4 were predominantly studied with vesicular transport assays to avoid the complication associated with the negative charge of MRP substrates that prevent them from entering cells freely. Our studies with MRP3 indicate that conjugates, preferably glucuronidated or sulfated ones, are good substrates. The highest affinity substrates that we have identified to date are glucuronide conjugates of the two bile acids HCA and HDCA and of several steroids (unpublished results). Collectively, this would suggest that MRP3 physiological substrates may be conjugates of sterol-like structures.

The finding that rat Mrp3 transports taurocholate with high affinity fits well with this idea \(^{24}\). The secondary bile acids glycocholic and taurocholic acids are sterol-derived conjugates of cholic acid with glycine or taurine, respectively. Our studies with human MRP3 in vesicular transport assays and in cells that have been transfected with an ASBT cDNA construct, to allow entry of bile acids, are consistent with low affinity bile acid transport \(^{25}\). However, in these studies we find that sulfated bile acids are high affinity substrates of MRP3. Sulfated bile acids are formed extensively during cholestatic liver disease suggesting a possible role for MRP3 in this disease state.

To test if Mrp3 plays a critical role in bile acid physiology *in vivo* we generated Mrp3 deficient mice. The Mrp3\(^{(-/-)}\) are viable and display no overt phenotype (chapter 5). Trans-ileal transport of taurocholate in these mice is undistinguishable from controls. Under cholestatic liver conditions induced by common bile duct ligation serum bile acid levels are substantially increased, but the levels in mutant and control mice are not different. In these experiments we additionally find that the level of serum bilirubin glucuronide in the mutant mice is reduced to 50% of those in control mice. Our results with the Mrp3\(^{(-/-)}\) suggest that Mrp3 does not play a critical role in the *in vivo* transport of bile acids under normal and cholestatic states and again point to a role for Mrp3/MRP3 in the transport of glucuronide conjugates. We are testing this idea further with drugs
for which we have evidence from *in vitro* transport assays that their glucuronide conjugate is transported by MRP3.

The idea that MRP3 plays a role in the detoxification pathway of conjugates was discussed above. The question remains whether MRP3 has other functions? MRP3 is expressed in the adrenal gland (chapter 5 and 26) and other steroidogenic tissues suggesting, in combination with the *in vitro* transport results, a possible role in steroid metabolism. The roles of Mrp3 in the lung, spleen, and pancreas are unclear at present.

**What are the physiological roles of MRP4?**

The substrate specificity of MRP4, initially thought to be restricted, is expanding rapidly and with it our view on the possible functions of this transporter. The initial studies in our group indicated that cyclic nucleotides, suggested by others to be high affinity substrates 27, are low affinity substrates 28. This has prompted us to search for other endogenous high affinity substrates for MRP4. Our studies on MRP4 (chapter 6 and chapter 7) suggest three exciting classes of endogenous molecules as possible high affinity endogenous substrates: steroid conjugates, bile acids, and prostaglandins.

Briefly, we have shown that MRP4 transports DHEAS, a major circulating steroid in humans, with high affinity and is inhibited by several other steroid conjugates 29. Our finding that MRP4 is competitively inhibited by bile acids 29 was further extended recently by Rius et al. 6, who demonstrated that MRP4 transports the monovalent bile acid taurocholic acid in a GSH-dependent manner. In view of these findings and the basolateral localization of MRP4 in hepatocytes 6 the possible role of MRP4 in mitigating cholestatic liver disease requires further investigation.

Finally, we recently demonstrated that MRP4 transports PGE₁ and PGE₂ with high affinity and is inhibited by other prostanoid derivatives 30. A major question that our initial study has left unanswered is whether MRP4 can transport *de novo* synthesized prostanoids. The availability of the Mrp4<sup>(−/−)</sup> mice makes it possible to test the roles of Mrp4 in the above-mentioned processes.

**Concluding remarks**

The first decade of MRP research has been an exciting one. We now possess a basic understanding of these transporters and have techniques and tools to study them. However, we are still a long distance from fully answering the main questions raised in the beginning of this section, even for the relatively well-studied MRP2-4. The second decade will be just as exciting.
Conclusions and perspectives

References


