MRP2-4, from drug resistance to physiology

Zelcer, N.

Citation for published version (APA):
Summary

Cells are continuously exposed to environmental and dietary toxic compounds, metabolic waste products and during chemotherapy to drugs. To counteract the potential damage that these compounds can cause, cells and organisms evolved different protection mechanisms, among them membrane efflux transporters. The work in this thesis focuses on three transporters of the multidrug resistance protein family, MRP2-4.

MRPs are promiscuous transporters and have a remarkable ability to transport diverse and structurally unrelated substrates. Nevertheless, the different MRPs each have a unique substrate specificity and preference. To accommodate these seemingly contradicting requirements MRPs contain a complex substrate-binding site. However, how MRPs transport their substrates is still unknown. In chapter 2 of this thesis we address this question using MRP2 as a model transporter. Using transport assays with vesicles derived from Sf9 insect cells overproducing MRP2, we studied the interactions of drugs, organic anions, and bile acids with three MRP2 substrates: estradiol-17-β-D-glucuronide (E$_2$17βG), methotrexate, and glutathione-S-dinitrophenol. We observed complex inhibition and stimulation patterns that were different from those observed with the related transporters MRP1 and MRP3. Interestingly, the rate of E$_2$17βG transport by MRP2 increases sigmoidally with substrate concentration indicative of homotropic cooperativity. Half maximal transport was obtained at 120 μM E$_2$17βG, in contrast to values < 20 μM for MRP1 and 3. MRP2 stimulators, such as indomethacin and sulfanitran, strongly increased the affinity of MRP2 for E$_2$17βG (half maximal transport rates of 65 and 16 μM E$_2$17βG, respectively) and shifted the sigmoidal dependence of transport rate on substrate concentration to a more hyperbolic one, without substantially affecting the maximal transport rate. Sulfanitran also stimulated MRP2 activity in cells, i.e. the transport of saquinavir through monolayers of Madin-Darby canine kidney II cells. Some compounds that stimulate E$_2$17βG transport, such as penicillin G or pantoprazole are not detectably transported by MRP2, suggesting that they allosterically stimulate transport without being cotransported with E$_2$17βG. We propose a model to explain our observations in which MRP2 contains two similar but non-identical ligand binding sites: one site from which substrate is transported and a second site that regulates the affinity of the transport site for the substrate. This model is based on our studies with MRP2, but is likely to be relevant for the other MRPs as complex allosteric interactions have been reported for MRP1-4 as well. The interactions that occur between substrates within the binding sites of MRPs are a potential source for adverse and advantageous drug interactions and speculatively, for regulation of these transporters by endogenous compounds.

Our studies on MRP3 reported in this thesis were aimed at determining the possible role of MRP3 in multidrug resistance and its physiological role. In chapter 3 we study the possible...
contribution of MRP3 to multidrug resistance of cancer cells. We expressed MRP3 in a murine fibroblast-like cell line generated from the kidneys of mice that lack Mdr1a/b and Mrp1. These cells are hypersensitive to anticancer drugs due to the loss of the major drug transporters Mdr1a/b and Mrp1, and are therefore a suitable cell model to test the contribution of an exogenously introduced transporter to multidrug resistance. We tested stable clones overproducing MRP3 in cytotoxicity assays against a panel of anticancer drugs and find that these cells were resistant only to the epipodophyllotoxins etoposide and teniposide. These cells were not resistant to vincristine, doxorubicin, and cisplatin, as suggested by others. The resistance to etoposide was associated with reduced cellular accumulation and enhanced efflux of this drug and was not affected by depleting cells of glutathione but was reversed by several common organic anion transport inhibitors. Membrane vesicles from infected insect cells expressing MRP3 mediated ATP-dependent transport of E$_2$17βG, leukotriene C4, dinitrophenyl S-glutathione but not glutathione itself, and of etoposide glucuronide, a major metabolite of etoposide in vivo (Km of 11 μM). The transport of E$_2$17βG by MRP3 was inhibited in a concentration-dependent manner by both etoposide and methotrexate. Even though etoposide glucuronide is an excellent substrate for MRP3, this compound is not involved in the drug resistance of our MRP3 cells to etoposide as these cells extrude unmodified etoposide and not etoposide glucuronide. It is still unclear how MRP3 transports unmodified etoposide itself. Our results suggest that MRP3 most likely does not play a major role in multidrug resistance of cancer cells to chemotherapy.

Several lines of evidence suggested that human and rat MRP3/Mrp3 may be involved in transport of bile acids in vivo (summarized in chapter 1, MRP3 section). In chapter 4 we studied the transport of bile acids by human MRP3 in vesicular transport assays with membrane vesicles from insect cells expressing MRP3. In these assays we find that MRP3 transports glycocholate and taurocholate in a time- and ATP-dependent manner. Furthermore, sulfated bile salts were high-affinity competitive inhibitors of etoposide glucuronide transport by MRP3 (IC$_{50}$ = 10 μM). Taurochenodeoxycholate, taurocholate and glycocholate inhibited transport only at higher concentrations (IC$_{50}$ = 100, 250 and 500 μM, respectively). A major complication of studying transport of bile acids in intact cells is that they do not passively diffuse across the plasma membrane due to their anionic charge at physiological pH. To overcome this obstacle, we used mouse fibroblast-like cell lines derived from mice with disrupted Mdr1a, Mdr1b and Mrp1 genes to generate transfectants that express the murine apical Na$^+$-dependent bile-salt transporter (Asbt) and MRP3. Uptake of glycocholate by these cells is Na$^+$-dependent, with a $K_m$ and $V_{max}$ of 29±7 μM and 660±63 pmol/min/mg protein, respectively, and is inhibited by several organic-anion transport inhibitors. Expression of MRP3 in these cells limits the accumulation of glycocholate and increases the efflux from cells preloaded with taurocholate or glycocholate. We find therefore that MRP3 transports both taurocholate and glycocholate, albeit with low affinity, in contrast with the high
Summary

Affinity transport reported for rat Mrp3. Our results suggest that MRP3 is unlikely to be the principal basolateral bile acid transporter of hepatocytes, ileocytes and cholangiocytes, but that it may have a role in the removal of bile acids from the liver in cholestasis.

To further study the contribution of Mrp3 to bile acid transport under normal and cholestatic conditions and to drug resistance we generated mice lacking Mrp3. The generation and initial characterization of the Mrp3(-/-) mice is reported in chapter 5. The Mrp3(-/-) mice are viable, fertile and show no overt phenotype. Using the Mrp3(-/-) as null controls we studied the distribution of Mrp3 in wildtype mice. We detected Mrp3 in the liver, throughout the gastro-intestinal tract, spleen, lung, and adrenal gland. Hepatic levels of Mrp3 in the mice are high, in strong contrast to the low levels observed in livers of rats. The Mrp3(-/-) mice are not hypersensitive to etoposide although MRP3 mediates resistance to this anticancer drug (chapter 3). Trans-ileal transport of 1 mM taurocholate is not impaired in the Mrp3(-/-), suggesting that Mrp3 is dispensable for the reclamation of taurocholate in the terminal ileum. Ligation of the common bile duct (BDL) for 3 days results in elevated levels of bile acids in both Mrp3(-/-) and control mice, however no difference between the two groups of mice was seen. Additionally, BDL results in a strong decrease in hepatic Mrp2 levels concomitant to an increase in serum levels of glucuronate-conjugated bilirubin. The level of bilirubin glucuronide in the serum of Mrp3(-/-) is only 50% of that seen in the control mice suggesting that Mrp3 transports this substrate. Unlike in BDL rats, in which a strong increase in hepatic Mrp3 has been reported (± 30-fold), we do not see an increase in hepatic Mrp3 in BDL mice. Our results with the Mrp3(-/-) mice suggest that Mrp3 does not play a crucial role in the extrusion of bile salts from the liver under conditions of impaired bile flow, cholestasis. However, Mrp3 is important for the removal of bilirubin glucuronide from the liver, when its secretion into bile is impaired, for subsequent excretion in the urine.

The last section of this thesis focuses on MRP4. The studies reported here for MRP4 were aimed at finding physiological high affinity substrates for this transporter. The initial proposal that MRP4 is an important player in the transport of cyclic nucleotides is disputable in light of the very low affinity of MRP4 for cAMP and cGMP found in our laboratory. In chapter 6 we report that MRP4 may be involved in the transport of bile acids and conjugated steroids. This study was based on the recent report that Mrp4 is induced in the livers of Fxr(-/-) mice, which have increased levels of serum bile acids. Since MRP4, like MRP1-3, also mediates transport of a model steroid conjugate substrate, E217βG, we tested whether MRP4 may be involved in the transport of steroid and bile acid conjugates. Bile salts, especially sulfated derivatives, and cholestatic estrogens inhibited the MRP4-mediated transport of E217βG. Inhibition by estradiol 3,17-disulphate and taurolithocholate 3-sulphate was competitive, suggesting that these compounds are MRP4 substrates. Furthermore, we found that MRP4 transports dehydroepiandrosterone 3-sulphate (DHEAS), the most abundant circulating steroid in humans that is made in the adrenal gland. The
ATP-dependent transport of DHEAS by MRP4 showed saturable kinetics with $K_m$ and $V_{max}$ values of 2 µM and 45 pmol/mg protein/min, respectively. As the transporter responsible for the secretion of DHEAS from the adrenal gland is not yet known, we further studied the possible involvement of other members of the MRP family of transporters in the transport of DHEAS. We found that MRP1 transports DHEAS in a glutathione-dependent manner with $K_m$ and $V_{max}$ values of 5 µM and 73 pmol/mg protein/min, respectively. No transport of DHEAS was observed in membrane vesicles containing MRP2 or MRP3. Our findings suggest a physiological role for MRP1 and MRP4 in DHEAS transport and a possible involvement of MRP4 in transport of conjugated steroids and bile acids.

We extended our studies on the substrate specificity of MRP4 in chapter 7 and report that MRP4 may be involved in prostanoid transport. Prostaglandins are involved in a wide variety of physiological and patho-physiological processes, but the mechanism of prostaglandin release from cells is not completely understood. In inside-out membrane vesicles derived from insect cells or HEK293 cells, MRP4 catalyzed the time- and ATP-dependent uptake of prostaglandin E1 (PGE1) and PGE2. In contrast, MRP1, MRP2, MRP3, and MRP5 did not transport PGE1 or PGE2. The MRP4-mediated transport of PGE1 and PGE2 displayed saturation kinetics, with $K_m$ values of 2.1 and 3.4 mM, respectively. Further studies showed that PGF1α, PGF2α, PGA1, and thromboxaneB2 were high-affinity inhibitors, and therefore presumably substrates, of MRP4. Furthermore, several nonsteroidal antiinflammatory drugs were potent inhibitors of MRP4 at concentrations that did not inhibit MRP1. We extended these studies to cells that express the prostaglandin transporter (PGT) in which we could study the effect of MRP4 on the accumulation and release of prostaglandins. The steady-state accumulation of PGE1 and PGE2 in these cells was reduced proportionally to MRP4 expression. Inhibition of MRP4 by an MRP4-specific RNA interference construct or by indomethacin reversed this accumulation deficit. Together, these data suggest that MRP4 can release prostaglandins from cells after they are taken up by PGT, and that in addition to inhibiting prostaglandin synthesis, some nonsteroidal anti-inflammatory drugs might also act by inhibiting their release by MRP4.