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Minireview

On the putative co-transport of drugs by multidrug resistance proteins

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Abstract Experiments with multidrug resistance-associated protein 1 (MRP1) showed 10-years ago that transport of vincristine (VCR) by MRP1 could be stimulated by GSH, and transport of GSH by VCR. Since then many examples of stimulated transport have been reported for MRP1, 2, 3, 4 and 8. We discuss here three models to explain stimulated transport. We favour a model in which a large promiscuous binding site can bind more than one ligand, allowing cooperative/competitive interactions between ligands within the binding site. We conclude that there is no unambiguous proof for co-transport of two different ligands by MRPs, but that cross-stimulated transport can explain the published data.

Keywords: ABC transporters; Homotropic cooperativity; Heterotropic cooperativity; Transport kinetics; MRPs

1. Introduction

Drug transporters belonging to the ATP-binding cassette (ABC) transporter families are able to transport drugs against steep drug concentration gradients at the expense of ATP hydrolysis. These transporters were discovered by their ability to make cells resistant to large amphipathic anticancer drugs, arsenite or toxic organic anions. Some of these transporters are now also known to play an important role in the defense of mammals against xenotoxins. The ABCB1 P-glycoprotein, the ABCG2 Breast Cancer Resistance Protein, and the ABCC2 Multidrug Resistance Protein 2 (MRP2) are present in the apical membrane of the gut mucosa and they inhibit entry of xenotoxins into the body. These transporters also guard vital body compartments against toxins, such as the brain (ABCB1), the fetus (ABCB1 and ABCG2), the germ line (ABCB1, ABCC1), the haemopoietic stem cells (ABCB1, ABCG2, ABCC1), etc.

One of the largest sub-families of the ABC transporters, able to affect drug disposition, is the ABCC (MRP) family. There are now 9 MRP family members and 8 of these (MRP1–8) are known to be organic anion transporters. Only MRP9 (ABCC12) is still without known substrate. Between them the 8 MRPs can transport a remarkable range of organic anions, including anionic drugs and drugs conjugated to glutathione, sulfate or glucuronate. In addition, selected MRPs may transport a variety of endogenous compounds, such as leukotriene C4 (MRP1), bilirubin glucuronides (MRP2, MRP3), prostaglandins E1 and E2 (MRP4), cGMP (MRP4, MRP5, MRP8), and several glucuronosyl-, or sulfatidyl steroids. No two MRPs have exactly the same substrate specificity or tissue distribution and the precise function of most of the MRPs remains to be established. As the MRPs have been reviewed by us[1] [1–5,43] and others [6–12] in recent years, we focus here on one controversial issue that should be of interest to biochemists: the apparent ability of some MRPs to mediate co-transport of two compounds.

2. Initial experiments supporting co-transport

Early work on MRP1-mediated drug resistance showed that resistance to neutral drugs, such as anthracyclines or Vinca alkaloids, required cellular GSH. This led to the suggestion that MRP1 exports these drugs from the cell together with GSH. Apparent support for this idea came from vesicular transport experiments mainly done in the labs of Deeley and Cole and thoroughly reviewed in Deeley and Cole [13]. Loe et al. [14,15] showed that vincristine (VCR) transport by MRP1 is dependent on physiological (mM) concentrations of GSH. Reciprocally, VCR stimulates GSH transport and lowers the K_m for GSH from >1 mM to 100 μM. The presence of GSH increases the affinity of MRP1 for VCR 50-fold. For technical reasons it was not possible to demonstrate that VCR and GSH are co-transported by MRP1, but the co-transport interpretation made sense. Molecules, such as LTC4, in which an organic moiety is covalently linked to GSH, are efficiently transported by MRP1. Co-transporting an organic moiety, such as VCR, together with GSH looked like a plausible alternative. However, other models can explain these results as well, as we show below.

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3. Transport models

The stimulatory effect of compound M (modulator) on the transport of compound S (substrate) can be explained by the three different models illustrated in Fig. 1:

A. Co-transport

Compound M is required for transport of S and vice versa. Transport occurs either from a single site (as drawn in Fig. 1A), or from separate transport sites that both must be occupied to get coupled transport. The stoichiometry of M and S cotransport is defined and a mechanistic property of the system.

B. Heterotropic/homotropic cooperativity.

Compound M binds to an allosteric site in the transporter and binding of M increases the affinity of the transport site for S (heterotropic cooperativity or stimulated transport). M may not be transported at all, as shown in Fig. 1B. If S can also bind to the M-site, it may stimulate its own transport (homotropic cooperativity).

C. Membrane effects on transporter structure.

Compound M alters the membrane environment of the transporter resulting in an increased affinity of the transport site for S, as shown in Fig. 1C. This possibility should be especially considered when one studies amphipathic modulators known to partition substantially into the membrane [16].

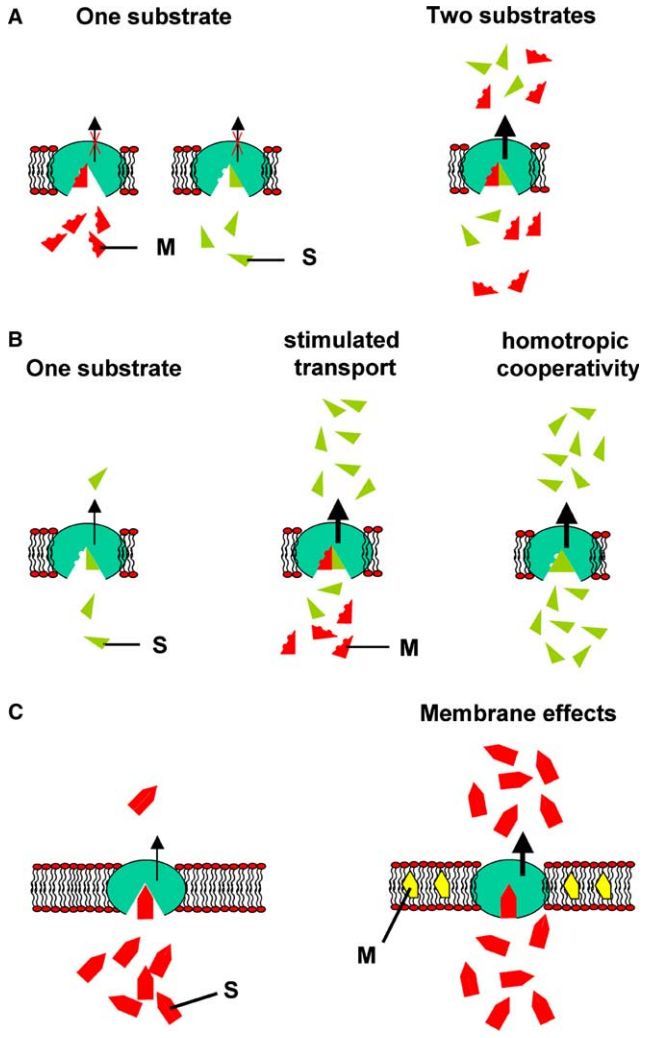

Fig. 1. Models explaining stimulated transport by MRPs. See text for explanation. M is a modulator binding to a site modulating the transport of substrate S, but it can also be a (co-) substrate as in panel A.

4. An evaluation of transport models

Each of the models in Fig. 1 makes predictions, although we admit that none of the predictions is strong enough to rigorously eliminate a model. The simple version of model A predicts that M and S are transported in a fixed stoichiometry (symport). This does not need to be 1:1, as it is conceivable that two M could be transported for one S. Pure co-transport without allosteric activation of one transport site by the other, is most easily pictured in a single large cavity binding both M and S, as shown in Fig. 1A. It should be noted, however, that there are alternative versions of model A (Fig. 1A) in which precise stoichiometry is lost:

a. Compound M is not strictly required for transport of S, but two solutes M and S are needed to complete the translocation cycle (single turnover). The stoichiometry of M and S co-transport will depend on the affinity constants of the transporter for these solutes and the ratio of M and S in the medium, and thus reflects a kinetic property. This can still be co-transport, but mechanistically this is very different from the obligatory co-transport pictured in Fig. 1A.
b. The observed stoichiometry could be affected by differential leak, i.e., if one of the two components has a greater tendency for futile cycling than the other.

Model B is the most flexible. It can explain apparent co-transport by assuming that compounds S and M are both substrate and modulator and that S and M can alternate in binding to the S and M sites.This is shown schematically in Fig. 2B. Some similarity between S and M sites follows from the published results: as will be discussed in Section 6, $E_2$17βG binds both to the S and M sites of MRP2 and many MRP2 substrates will stimulate transport of low concentrations of $E_2$17βG [17,18]. Likewise, GSH is a substrate of MRP1 and stimulates transport of several other substrates. The fact that the increased transport of both VCR and GSH by MRP1, if both compounds are present, is accompanied by a major increase in affinity for GSH is compatible with an altered MRP1 conformation when VCR is bound [13]. The compounds that stimulate $E_2$17βG transport by MRP2, also do so by increasing the affinity for $E_2$17βG without changing V_{max} (see Section 6). Obviously, if the binding sites for S and M resemble each other, the same compound (S) could bind to both sites resulting in homotropic cooperativity (Fig. 1B). Similarly M may compete with S at higher M concentrations and thereby inhibit transport of S, as shown for indomethacin in Fig. 4.

Model C makes the strong prediction that compound M should affect membrane structure. It seems doubtful whether the very hydrophilic and acidic GSH would partition strongly in the membrane and thereby affect the transport. On the other hand, anionic molecules with a significant hydrophobic functionality (many MRP substrates) are likely to do so.

It has been difficult to study membrane effects in drug transporters, but the potential importance of such effects can be gleaned from a different class of ABC-transporter, the bacterial transporter Opu A (reviewed in [19]). When the bacteria are transferred to hyperosmotic medium, this transporter catalyses uptake of glycine betaine resulting in an increase of the inter-

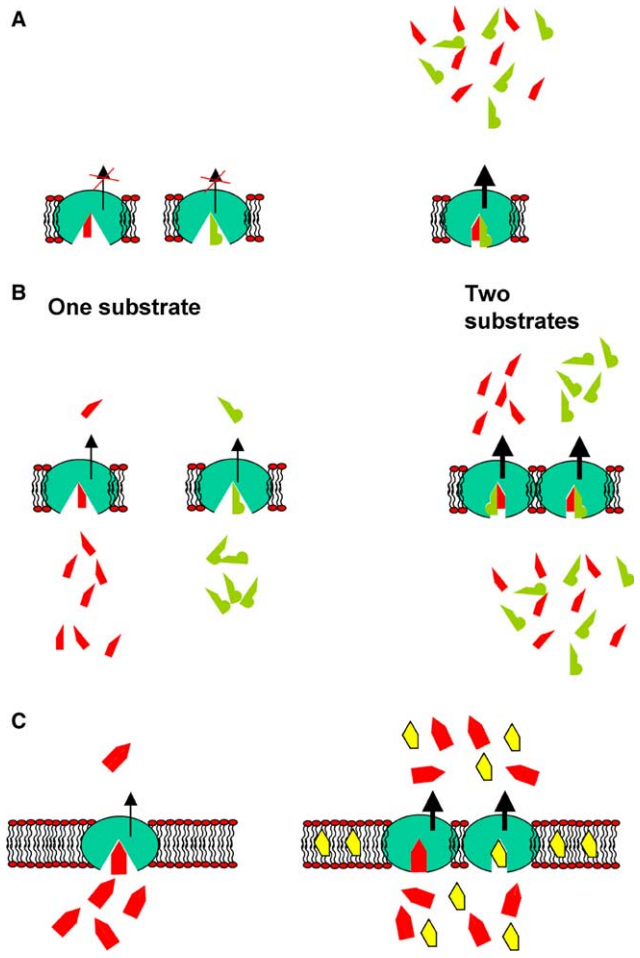

Fig. 2. Models showing how apparent cotransport would be explained according to the models presented in Fig. 1. (A) Obligatory cotransport. Compound M is required for transport of S and vice versa. (B) Heterotropic cooperativity. Compounds S and M are both substrate and modulator and S and M alternate in binding to the S and M sites. (C) Membrane effects. Compounds S and M are both substrates. M modulates transport of S and M by altering the membrane environment of the transporter, resulting in increased transport of both S and M.

nal osmolality. Vesicular transport studies have shown that the relation between transport rate and delta osmolality (the positive difference in osmolality outside and inside the vesicles) is not hyperbolic, but an S-curve. The lag at low delta osmolality, called the activation threshold, is dependent on the composition of the membrane in which the transporter is embedded. The activation threshold increases with an increasing fraction of anionic lipid and this appears to be due to multiple (electrostatic) interactions between anionic lipid and transporter protein. By raising ionic strength these interactions are broken and the transporter is immediately switched on. A larger anionic lipid fraction results in more extensive lipid–protein interactions, which require more salt to disrupt the electrostatic interactions, explaining the effect of lipids on activation threshold [19]. Importantly, not only charged lipids but also charged amphipaths affect the activation of the transporter. This elegant system shows that some ABC-transporters are exquisitely sensitive to the composition of the membrane in which they are embedded and that changes in membrane composition can affect transport by changing transporter conformation.

Obviously, the models presented in Fig. 1 are three extreme versions. There could be more than one transport site without co-transport. There could be two transport sites showing allosteric interaction. There could be membrane effects superimposed on models A or B. The models only provide a handle to analyze the data.

5. MRP1

Although early experiments suggested that MRP1 co-transports vincristine and GSH, subsequent work in the labs of Cole and Deeley [13] has shown that transport by MRP1 is more complex than initially thought. Four observations stand out:

1. GSH can also stimulate transport of some organic anions by MRP1. Examples are etoposide glucuronide [20] and estrone-3-sulfate [21]. Transport of 4-(methylnitrosamino)-1-(3-pyridyl)-butanol-*O*-glucuronide (NNAL-*O*-gluc) [22] and DHEAS [23] is even completely dependent on GSH. The GSH is therefore not merely required to provide a negatively charged moiety. It is also not required to provide a sulfur atom, as GSH analogs not containing sulfur, like ophthalmic acid, can completely replace GSH.
2. Whereas transport of estrone-3-sulfate and NNAL-*O*-gluc was strongly stimulated by GSH, these substrates had no detectable effect on GSH transport [21].
3. Loe et al. [24] found that Verapamil stimulates transport of GSH by MRP1. Whereas no GSH transport was detectable in the absence of Verapamil, vesicular uptake of GSH was substantial (K_m = 0.1 mM; V_{max} = 55 pmol/mg protein/min) in the presence of 100 μM Verapamil. As no transport of Verapamil by MRP1 was detectable with and without GSH, there appeared to be no co-transport of GSH and Verapamil. The authors point out, however, that the hydrophobic Verapamil could undergo futile recycling in the membrane, or be released on the *cis*-side of the membrane. Verapamil was also unable to inhibit transport of other MRP1 substrates, such as LTC4, unless GSH was present. Analysis of Verapamil analogs yielded one that inhibited LTC4 transport up to 90% in the presence of GSH, without significantly stimulating GSH transport by MRP1 [25], showing that it is possible to dissociate the two effects of Verapamil analogs.
4. Leslie et al. [26] found that several flavonoids stimulate GSH transport by MRP1 very strongly. Like Verapamil, the flavonoids do not appear to be transported by MRP1. "Our results suggest that flavonoids stimulate MRP1-mediated GSH transport by increasing the apparent affinity of the transporter for GSH but provide no evidence that a co-transport mechanism is involved" [26].

The initial results indicating co-transport of neutral MRP1 substrates with GSH still stand, but given the newer results discussed in points 1–4, other interpretations of these experiments should be considered. As Deeley and Cole [13] put it: "Overall, the data are consistent with the existence of a site or sites on MRP1 capable of binding free GSH and certain non-sulfhydryl-reducing analogues. They also indicate that there is positive cooperativity between the interaction of GSH (or related molecule) and the second substrate with the protein. What remains unclear at present is why in some

cases both the second substrate and GSH are transported, while in others, it appears that transport of only one or the other substrate occurs.''

6. MRP2

Stimulated transport by MRP2 was first observed by Bakos et al. [27]. Using *N*-ethylmaleimide glutathione (NEM-GS) as substrate, they found up to 3-fold stimulation of transport by sulfinpyrazone and penicillin G and a lower stimulation by indomethacin. GSH had no effect on NEM-GS transport. At higher sulfinpyrazone or indomethacin concentrations the stimulated NEM-GS was inhibited. This inhibition is not surprising as Evers et al. [28] had shown that sulfinpyrazone is a transported substrate of MRP2. Substrates should compete with other substrates at high concentrations.

Huisman et al. [29] showed that stimulated transport by MRP2 is not a peculiarity of the vesicular transport system, because it also occurs in intact cells: they used MDCKII cells transfected with a MRP2 construct and found that probenecid stimulates transport of the HIV protease inhibitor saquinavir by MRP2 up to 7-fold. Very high (10 mM) concentrations of probenecid completely inhibited the stimulated transport. Stimulation of MRP2-mediated transepithelial saquinavir transport was also found for sulfinpyrazone [29] and sulfanitran [18], whereas probenecid can also strongly stimulate transport of the taxanes paclitaxel and docetaxel by MRP2 [30]. These results show that the effects of MRP2-stimulating drugs could be clinically relevant and result in drug–drug interactions affecting plasma levels of taxanes or HIV protease inhibitors [30].

The study of stimulated transport by MRP2 received a further boost when Bodo et al. [17] and Zelcer et al. [18] independently found an unusual substrate concentration dependence of the transport of estradiol-17β-glucuronide ($E_2$17βG) by MRP2. As shown in Fig. 3, a plot of initial transport rate versus substrate concentration shows an S-curve, rather than the standard hyperbolic substrate saturation curve obtained thus far for other MRPs. This suggested that substrate binds both to the transport site and to a modulatory site which affects the transport rate allosterically by homotropic cooperative interaction [18]. At 1 μM $E_2$17βG, transport by MRP2 is low and this provided an opportunity to search for compounds able to stimulate transport by binding to the putative modulatory site. Many were found [17,18]. These included compounds found earlier [27] to stimulate NEM-GS transport, such as penicillin G, sulfinpyrazone and probenicid, but also other compounds such as pantoprazole, saquinavir, indomethacin, furosemide, and glibenclamide. The most spectacular stimulation (30-fold) was found with sulfanitran (Fig. 3). Several of these stimulating compounds are known to be transported MRP2 substrates themselves and one would expect them to inhibit transport at higher concentrations by competing with $E_2$17βG for the substrate site. This was indeed observed for indomethacin, glibenclamide and sulfinpyrazone (Fig. 4). Sulfanitran did not inhibit up to 1 mM (Fig. 4), but this compound may not be transported at all by MRP2 [18]. This also holds for ethinylestradiol-sulfate, which is also able to stimulate $E_2$17βG transport >10-fold [31].

Bile salts also modestly stimulate $E_2$17βG transport 2–3-fold [17,18] and inhibit at higher concentrations. Bodo et al. [17] found that glycocholate transport was also stimulated about 2-fold by 100 μM $E_2$17βG. This led them to propose that bile salts and $E_2$17βG are co-transported by MRP2 [17]. We prefer

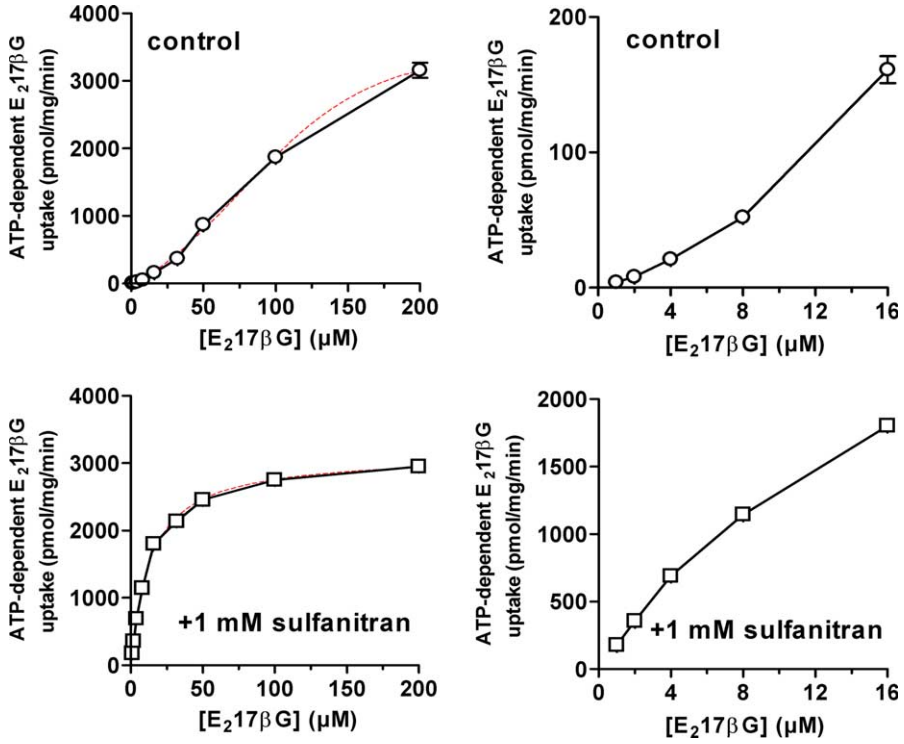

Fig. 3. Concentration-dependent $E_2$17βG transport by MRP2 in the presence and absence of 1 mM sulfanitran. Transport was measured in vesicles from Sf9 cell overproducing human MRP2. Taken from Zelcer et al. [11].

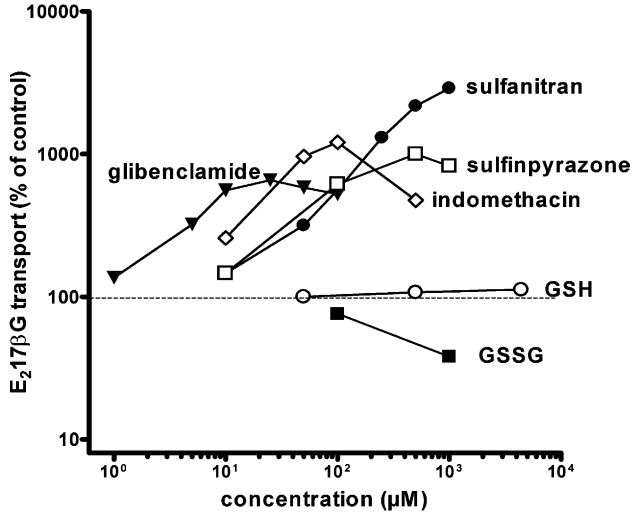

Fig. 4. Effect of various compounds on $E_2$17βG transport. The conditions were the same as in Fig. 3, but a fixed 1 μM concentration of $E_2$17βG was used.

another interpretation of these experiments and we return to this point below.

Stimulation of MRP2-mediated transport is very dependent on substrate. Only modest stimulatory effects were observed with 15 nM GS-DNP (maximally 3-fold), 100 μM MTX (maximally 2-fold), 4 μM NEM-GS (maximally 4-fold) [18,27], and glutathionyl-ethacrynic acid [31]. More pronounced stimulations were observed for transport of saquinavir [18,29] and taxanes [30] in intact cells, but these results are not directly comparable with those of vesicular transport studies.

Not all known substrates of MRP2 are able to stimulate $E_2$17βG transport. Notable exceptions are GSSG (Fig. 4) and GS-DNP, which only inhibit and MTX, which has no effect at low $E_2$17βG concentrations, but inhibits transport at saturating $E_2$17βG concentration, as expected. An interesting compound without any effect on transport of $E_2$17βG is GSH (Fig. 4). This is in stark contrast to the pronounced stimulatory effect of GSH on transport of several substrates by MRP1, as discussed in the preceding section. This contrast is most strikingly illustrated by the transport of NNAL-*O*-gluc by MRP1 and MRP2 [22]. Transport by MRP1 is completely dependent on GSH (or analogs), whereas transport by MRP2 is nearly completely inhibited by 3 mM GSH.

Finally, we note that cooperativity has only been shown for the MRP2 substrate $E_2$17βG. We expect some other substrates to be able to stimulate their own transport as well, because they bind with higher affinity to the M site than to the S site, but these remain to be found.

7. MRP3

MRP3 is rather similar to MRP1 and MRP2 in putative structure and in amino acid sequence. Like MRP1 and 2, MRP3 is also able to transport compounds conjugated to GSH, glucuronides or sulfate, but it appears to be unable to transport GSH itself [32] under any condition, unlike MRP1, 2, 4 and 5. Most of the compounds found to stimulate MRP1- or MRP2-mediated transport were found to have no effect on MRP3, or to inhibit this transporter [18,30,33,34]. Sulfanitran, the most spectacular stimulator of the transport of 1 μM $E_2$17βG by MRP2, stimulates transport by MRP3 less than 2-fold [18]. The most substantial stimulation of MRP3-mediated transport of $E_2$17βG was observed with benzbromarone [34] and ethinylestradiol-sulfate [31], but it did not exceed a factor 4. Interestingly, ethinylestradiol-sulfate completely inhibits the stimulated transport at higher concentrations [31]. This extends results obtained with MRP1 suggesting that compounds that do not appear to be transported at all can nevertheless inhibit transport of other substrates.

8. MRP4

Lai et al. [35] reported that MRP4-mediated export of cAMP from intact cells was inhibited by GSH depletion and they also found increased sensitivity in depleted cells to drugs exported by MRP4. In contrast, we have not found any effect of GSH depletion on MRP4-mediated cyclic nucleotide export from HEK293 cells [36] and we have not observed any requirement for GSH in vesicular transport by MRP4 of steroid- and bile acid-conjugates [23] or prostaglandins E1 and E2 [37]. More recently, however, Rius et al. [38] discovered that the mono-anionic bile salt taurocholate is only transported by MRP4 in the presence of GSH. Just as in the case of GSH-stimulated transport by MRP1, the GSH could be replaced by the GSH analogs methyl-SG and ophthalmic acid. Transport was inhibited by several other mono-anionic bile salts, not available in radioactive form, suggesting that MRP4 is a general mono-anionic bile salt transporter if GSH is present. Interestingly, Rius et al. [38] also found transport of labeled GSH in the presence of 5 μM taurocholate; no transport of GSH was found in the absence of taurocholate. The authors calculate that their rate of GSH transport in the presence of taurocholate is similar to the rate of transport of taurocholate in the presence of GSH and they conclude that "these rates are consistent with co-transport of GSH and taurocholate" [38]. However, they only measured GSH transport at a single concentration of taurocholate. This is inadequate in our opinion to decide whether stimulated transport could be co-transport or not. There were also technical problems in the assay, as the authors emphasize themselves: at a concentration of 5 mM radioactive GSH the background is enormous and about 70% of the actual reaction measured. It remains therefore unclear how much taurocholate stimulates GSH transport in this system, and what the dependence of the stimulation is on taurocholate concentration. Furthermore, a peculiarity of the Chinese hamster lung fibroblast V79 cell system used by Rius et al. [38] should be mentioned here: The rate of taurocholate transport (in the presence of GSH) was only 2-fold higher in the MRP4 transfectants than in the control cells. The K_m values for taurocholate and GSH (for taurocholate transport) of the transfectants and control cells are similar, and Rius et al. [38] therefore do not correct their transfectant values for the rates found in the control cells. However, they clearly show that GSH transport in the control cells in the presence of taurocholate was 7-fold lower (rather than 2-fold lower) than in the transfectants. Hence, there is an obvious problem in determining the stoichiometry of GSH and taurocholate transport given the very different contribution of control cells and transfectants to GSH and taurocholate transport.

On the basis of these arguments, we consider the conclusion that MRP4 co-transports GSH with monoanionic bile salts [38] not adequately supported by data.

In a recent paper Van Aubel et al. [39] report (vesicular) transport of urate by MRP4. They conclude that transport shows homotropic cooperativity with a Hill coefficient of 1.7. Whereas urate inhibited MTX transport, it stimulated cGMP transport nearly 2-fold. The authors also conclude that cGMP transport shows homotropic cooperativity and that this is abolished by urate stimulation. We find these results hard to interpret. Urate transport by MRP4 is only twice the (high) background transport rate in the Sf9 system. It is impossible in our opinion/experience to draw such far-reaching conclusions from such experimental data. Moreover, substantial cooperativity has not been observed for the high cGMP transport by human erythrocyte vesicles [40], which is due to MRP4 (H. Yamaguchi, C. de Wolf and P. Borst, unpublished).

9. MRP5–8

For MRP5, 6, and 7 no evidence for stimulated transport has been reported, but the issue has not been pushed. As far as we know, no systematic attempt has been made to look for stimulatory effects on these transporters in the same way as done for MRP1 and MRP2. Chen et al. [41] recently reported that transport of 1 μM $E_2$17βG by MRP8 is stimulated up to 6-fold by dehydroepiandrosterone-3-sulfate (DHEAS) and to a lesser extent by some other steroid sulfate. DHEAS is also a substrate of MRP8, but its transport is not stimulated by $E_2$17βG.

10. At MRP2

The *Arabidopsis thaliana* vacuolar MRP, At MRP2, has been studied in detail by Liu et al. [42]. Interestingly, they found that this plant MRP transports $E_2$17βG and that transport is stimulated by glutathionyl-dinitrophenol (DNP-GS), the stimulation mainly being due to a lowering of Km (3-fold) with little effect on V_{max}. Conversely, $E_2$17βG decreased the K_m for DNP-GS 5-fold without effect on V_{max}. Whereas GSH also stimulated $E_2$17βG transport, $E_2$17βG completely inhibited GSH transport. The authors conclude from these and many other experiments that DNP-GS and $E_2$17βG are not co-transported, but that these substrates stimulate "each other's transport via distinct but coupled binding sites [42] The authors come up with a complex model for AtMRP with 4 transport and 3 allosteric sites. We think, however, that the model in Fig. 4 with one promiscuous binding site allowing binding of multiple ligands in (partially overlapping) sites can explain their data.

11. Is there hard evidence for co-transport of substrates by MRPs?

It should be clear from the model section that co-transport (model A in Fig. 1) is hard to prove, because model B often provides a satisfactory explanation for the stimulated transport observed (Fig. 2). Conversely, it is hard to disprove that co-transport exists, as the predictions of the co-transport model are not strong. Nevertheless, we are going to argue here that all data purporting to show co-transport fail to make the point.

For MRP1 the possibility of co-transport was first evoked by Loe et al. [15]. They found cross-stimulation of vincristine (VCR) and GSH in vesicular transport studies, but were careful to point out the pitfalls in the co-transport interpretation: "Reliable estimation of the stoichiometry of VCR and GSH transport is difficult because of the extremely brief period for which VCR uptake is linear. However, if the GSH-stimulated uptake of VCR at the earliest time point of 8 s is taken as a minimal estimate of initial rate, a value of ~20 pmol/min/mg is obtained. This is approximately the same as the rate of uptake of GSH uptake calculated over a 5-min period. In addition to the caveat mentioned above, these data are based on determinations at only one concentration of drug and GSH. Consequently, they are certainly not sufficiently reliable to conclude that there is a 1:1 stoichiometry with respect to GSH and VCR transport. However, they do indicate that the transport rates of both compounds are not vastly different, and that a true cotransport mechanism may be involved." [15].

Nearly all subsequent experiments showing stimulated transport by MRP1, did not fit the co-transport model at all, as discussed in Ref. [13], and with hindsight it seems likely that the VCR-GSH cross-stimulation is an example of model B in Fig. 2, in which the rates of transport of S and M happened to be similar at the drug concentrations chosen.

The apparent co-transport of GSH and bile salt by MRP4 has been discussed in Section 8. The only other example of possible co-transport comes from our lab. Evers et al. [28] studied transepithelial vinblastine (VBL) transport mediated by MRP2 in MDCKII cell monolayers and noted an increased efflux of GSH stimulated by VBL. Over a VBL concentration range of 10–50 μM they found a more or less constant ratio of VBL/GSH transport varying between 2.1 and 3.1. This is the only case in the literature in which a constant ratio of drug/GSH transport was found over a range of drug concentrations. Evers et al. [28] tentatively concluded that "transport of VBL is associated with GSH export." They attributed the high VBL/GSH ratio to technical problems in determining the exact rate of VBL transport, caused by the high diffusion rate of the relatively hydrophobic VBL through the plasma membrane.

With hindsight, however, the results of Evers et al. [28] with VBL can also be explained with model B of Fig. 1 and some of their other results actually fit model B better than model A of Fig. 1. For instance, low concentrations of sulfinpyrazone stimulate apical GSH efflux from the MDCKII-MRP2 monolayer, but higher concentrations completely inhibit GSH efflux, whereas drug transport is still going up. This can be explained [28] by assuming that sulfinpyrazone can bind to both M and S sites and displaces GSH at S at high drug concentrations.

Membrane effects (Figs. 1 and 2C) should be kept in mind as an important confounder, but they do not provide a plausible explanation for apparent co-transport, as it is hard to see why substrate S cannot stimulate its own transport by the same membrane effects that it contributes to transport of M. However, apparent homotropic cooperativity, as observed in the transport of $E_2$17βG by MRP2, could in principle also be caused by membrane effects rather than by model B. The fact that rat Mrp2 shows much less cooperativity [43] than human MRP2 when transporting $E_2$17βG in Sf9 cell membranes, is more easily explained by model B, however, although the minor differences in the results for rat Mrp2 reported from different

labs, could reflect membrane effects resulting from differences in growth conditions of the Sf9 cells.

12. The potential physiological significance of stimulated transport by MRPs

Substantial stimulated transport has been found for MRP1, MRP2 and MRP4 and significant effects have been observed for MRP3. Given the apparent similarity in overall topology and function of MRPs, we expect that some form of stimulated transport will be found for each MRP, but a more systematic search still needs to be done. How large the effects could be in vivo, is still anybody's guess, even in the two cases studied most, MPR1 and MRP2. The clear stimulation of bile flow in the rat by benzylpenicillin, presumably due to stimulated transport of GSH by Mrp2 as the effect is absent in Mrp2 null rats, is one example of stimulated transport in an intact animal [44]. More might be uncovered by directed searches. If transport of compound S is totally dependent on the presence of a modulator M that is not routinely tested, as is done for GSH, transport of S may be missed in vesicular transport assays.

Much more work is required to understand the evolution of stimulated transport. A case in point is the stimulated transport of $E_2$17βG by human MRP2 (Figs. 3 and 4). As mentioned above and discussed in detail elsewhere [43], the striking homotropic cooperativity of the human MRP2 (Fig. 3) is hardly present in rat Mrp2, and the 30-fold stimulation of transport of low concentrations of $E_2$17βG by compounds like sulfanitran (Fig. 1) is reduced to 1.5-fold for rat Mrp2 (our unpublished results). One could argue that this result shows that stimulated transport is not important, because it is not conserved between rat and man. In contrast, we think that the rat–human comparison shows that stimulated transport is not an unavoidable consequence of MRP2 transport properties, but something selected for in human evolution.

So what is the selection process? What could be the advantage of stimulated transport? Some inspiration may come from the P450 system. The allosteric phenomena in cytochrome P450-catalyzed mono-oxygenation have been studied in detail [45] and interpreted on the basis of available crystal structures of bacterial P450 isoforms [46]. The most abundant P450 isoform metabolizing xenobiotics in human liver, P450 3A4, shows homotropic cooperativity towards several steroids. Heterotropic cooperative effects on steroid oxidation have been observed with other steroids and with unrelated compounds, such as α-naphthoflavone. Two models have been proposed to explain the cooperative effects: one with a distinct allosteric site, the other in which substrate and activator bind simultaneously to the same active site, changing the dimensions of the site and the position of the substrate: both the crystallographic analysis of protein–ligand complexes [46] and mutagenesis studies [47] support the second model. Especially persuasive are the mutagenesis studies of Harlow and Halpert [47]. By replacing residues in the active site of P450 3A4 thought to bind modulator by more bulky residues, they were able to abolish homo- and heterotropic cooperativity and create an enzyme that displayed hyperbolic steroid hydroxylation kinetics.

There is ample evidence for allosteric effects on P450-catalyzed reactions in vivo [45,48] and it is therefore reasonable to ask why nature opts for such a complex mechanism to dispose of xenotoxins and endogenous metabolites. This question was addressed in depth by Atkins et al. [49]. They argue "...that detoxification enzymes such as CYPs may evolve, not only to metabolize a wide range of structurally unrelated xenobiotics, but also to minimize the probability of bioactivation of previously unencountered xenobiotics by distributing the toxic load into multiple products." Atkins et al. [49] refer "to this probabilistic process as "distributive catalysis." Distributive catalysis will provide a detoxification advantage if the toxicity of a compound and its metabolites exhibit sublinear, or threshold, dose–response relationships. In that case a low rate of oxidation may distribute the toxin load between unaltered toxin and toxic metabolites in such a fashion that the toxicity of the sum is less than that of the same concentration of unaltered toxin or metabolite. At high toxin concentrations the distributive advantage is best accomplished with faster turn-over, as achieved by allostery" [49]. Atkins et al. [49] provide detailed calculations to show that allosteric systems can indeed provide toxicological advantages if the toxin and its metabolites exhibit threshold (or sublinear) dose–response relationships. The advantages are modest, however, and they only exist over a limited toxin concentration range. It is not obvious to us that such a complex mechanism would be selected in evolution on this modest basis.

How do these P450 considerations translate to MRPs? Transporters are no oxygenases, but the common theme is promiscuity and the risk it entails. With P450 it is the generation of toxic compounds from innocuous ones; with MRPs it is the risk of exporting useful compounds from the cell in addition to waste products, toxins, drug conjugates, etc. Homotropic cooperativity would limit loss of valuable organic anions, heterotropic cross-stimulation of transport of toxic compounds would help to limit the total toxin load [33].

These considerations do not explain why MRPs can be activated by compounds that they do not seem to transport at all, like the stimulation of $E_2$17βG transport by sulfanitran (Fig. 3). It is possible that there are endogenous compounds of this class that stimulate as well, as several aromatic compounds with a sulfoxide (S═O) or tosyl (O═S═O) group were found to stimulate MRP2-mediated transport, e.g., sulfinpyrazone and glibenclamide (Fig. 4). However, another simpler explanation would be that the selection in evolution is not for transport regulation, but for a promiscuous binding site accommodating a large range of compounds. Binding compound M in this site may improve binding of S. This could occur in several different ways: M could provide additional interaction sites for S, it could shield residues unfavorable for binding S, or M could affect the binding site of S allosterically.

This model leans heavily on the results obtained with P450, which documents a single cavity in which S and M (or S1 and S2) reside together. However, our interpretation is very different from that of Atkins et al. [49]: it is not toxicological sophistication that drives the evolution of a binding site that can accommodate more than one ligand, but the inability of nature to create a promiscuous binding site that binds only one ligand at a time. Although space does not permit the discussion of bacterial systems with promiscuous drug binding sites, the results obtained in these systems fit the interpretation offered here. This holds both for pumps, for which cooperative drug binding has been shown [50] and for the repressors that control the synthesis of the pumps. These repressors bind the same range of drugs as the pumps they control and their promiscuous binding sites have been studied in detail by crystallography [51].

The interpretation offered here for stimulated transport is compatible with all published data. Whether an allosteric site is separate from the substrate binding site, or part of a larger substrate binding site, does not have any consequences for transport kinetics. Both models can fully account for homo- and heterotropic cooperativity, as discussed in the P450 literature, and for modulator effects on K_m and V_{max} of substrate. The essential difference between the two models is that a separate allosteric site must have been selected for its regulatory advantages, whereas a site that is part of the substrate site might have arisen as byproduct of the selection for a large promiscuous substrate-binding site. For the moment we prefer the second one, because it is the simpler one.

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