The effect of glutathione on the ATPase activity of MRP1 in its natural membranes

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Abstract The transport mechanism by which the multidrug resistance protein 1 (MRP1) effluxes cytotoxic agents out of cells is still not completely understood. However, the cellular antioxidant glutathione (GSH) has been shown to have an important role in MRP1-mediated drug transport. In this study we show that GSH stimulates the ATPase activity of MRP1 in a natural plasma membrane environment. This stimulation was dose-dependent up to 5 mM. The MRP1 substrates vincristine and daunorubicin do not induce MRP1 ATPase activity. In addition, the effect of GSH on the MRP1 ATPase activity is not increased by daunorubicin or by vincristine. In contrast, a GSH conjugate of daunorubicin (WP811) does induce the ATPase activity of MRP1. In the presence of GSH the effect of WP811 was not significantly increased. Finally, (iso)flavonoid-induced MRP1 ATPase activity is not synergistically increased by the presence of GSH. In conclusion, we show that GSH has no apparent influence on the ATPase reaction induced by several MRP1 substrates and/or modulators. The subclasses of molecules had different effects on the MRP1 ATPase activity, which supports the existence of different drug binding sites.

Key words: Multidrug resistance; Multidrug resistance protein 1; Glutathione; (Iso)flavonoid; Flavopiridol; ATPase

1. Introduction

ATP-dependent transport proteins play an important role in biology and medicine. A variety of ABC transporters have been characterized [1]. Several of these proteins can cause cellular resistance against natural toxins or toxic agents used in medicine. One transporter protein is the *MRP-1*-encoded multidrug resistance protein 1 (MRP1) [2,3]. Overexpression of MRP1 causes so-called multidrug resistance (MDR) [4], which may contribute to less effective chemotherapeutic treatment of diseases.

In recent years, the substrate specificity of MRP1 has been the topic of intensive research. MRP1 appeared to mediate the transport of a broad range of drugs across cellular membranes [5,6], and has been characterized as a transporter of neutral and anionic compounds, such as glutathione-conjugated drugs, sulfates and glucuronides [4,7]. In addition, antifolates, such as the anticancer agent methotrexate, were recently described as MRP1 substrates [8]. Therefore, MRP1 is considered to be a multiple organic anion transporter [7,9].

The main cellular antioxidant glutathione (GSH) has been reported to have an important function in MRP1 activity. The presence of millimolar levels of cellular GSH has turned out to be necessary for MRP1-mediated efflux of cytotoxic agents, such as the anthracyclines [10,11]. Several other observations have led to the assumption that potential MRP1 substrates have to form conjugates with GSH, in order to make their transport by MRP1 possible [7,9,12–14]. Therefore, MRP1 has been considered a member of the GS-X pump family [15]. Alternatively, GSH has been described as a required co-substrate, which would be transported concomitantly with MRP1 substrates [16–19].

Available evidence suggests that transmembrane domains as well as cytoplasmic domains of MRP1 may recognize MRP1's various hydrophobic and hydrophilic substrates [20]. In analogy to P-glycoprotein (Pgp), the ATP hydrolysis necessary for transport is supposed to be carried out at nucleotide binding domains in MRP1. Also, in the light of recent studies of the action of potential substrates on the ATPase reaction of MRP1 [21–24] it appears that several GSH-conjugated compounds, such as dinitrophenyl *S*-glutathione, as well as several unconjugated compounds, such as (iso)flavonoids, are able to interfere with MRP1 ATPase activity. The role of GSH itself in MRP1-associated ATPase activity has been studied in two articles, in which purified MRP1 was reconstituted into artificial lipid membranes [23,24]. In those papers, however, conflicting data were presented concerning the induction of ATPase activity by reduced GSH. As indicated by one of the authors the lipid environment might be of influence on the ATPase activity of MRP1 [24] and, consequently, on the outcome of these studies. This has earlier been demonstrated for Pgp-associated ATPase activity [25]. Therefore, it seems of great importance to study MRP1-associated ATPase activity in reconstituted lipids as well as in a natural membrane environment.

In the present work we investigated the role of GSH in facilitating MRP1-mediated drug transport at the level of the ATPase reaction of MRP1. In brief, we studied the effects of GSH on MRP1 ATPase in its natural (lipid) environment. The effects of several established MRP1 substrates, such as daunorubicin (DNR), vincristine (VCR), and a DNR–GSH conjugate (WP811) on MRP1 ATPase activity were investigated. In addition, we have determined the effect of GSH on (iso)flavonoids–MRP1 interactions. (Iso)flavonoids, among which the currently tested anticancer agent flavopiridol [26], are among the first identified inhibitors of MRP1-mediated

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Abbreviations: GSH, reduced glutathione; MRP1, multidrug resistance protein 1; DNR, daunorubicin; VCR, vincristine; DTT, dithiothreitol; Pgp, P-glycoprotein

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drug transport, and also increase MRP1 ATPase activity [21,22,27,28]. Therefore, we included these compounds to analyze drug–MRP1 interactions.

2. Materials and methods

2.1. Chemicals

The synthetic flavone derivative flavopiridol (L86-8275, NSC 649890) (−)*cis*-5,7-dihydroxy-2-(2-chlorophenyl)-8[4-(3-hydroxy-1-methyl)-piperidinyl]-4*H*-benzopyran-4-one was kindly provided by Dr. Bolen (Hoechst Marion Roussel, Cincinnati, OH, USA). Reduced GSH, malachite green base, ammonium molybdate, probenecid, benzbromarone, Triton N101, digitonin, ATP, verapamil hydrochloride, dithiothreitol (DTT) and the flavonoid kaempferol were from Sigma (St. Louis, MO, USA). The flavonoid apigenin was from Extrasynthese (Genay, France). The isoflavonoid genistein was from ICN Biomedicals (Zoetermeer, the Netherlands). The (iso)flavonoids were dissolved as a stock solution of 40 mM in DMSO (Across Chimica, Belgium) and stored at −20°C. Before experiments 1:1 dilutions were made in ethanol, followed by dilution in ethanol/water (1:3, v/v) to the appropriate concentrations. The maximal concentration of DMSO and ethanol, which was also added to the controls, was 0.5% (v/v). Daunorubicin hydrochloride was from Specia (Paris, France). The GSH conjugate of daunorubicin, WP811, was synthesized as described earlier [29]. WP811 was dissolved in DMSO (100 mM) and stored at −20°C. Doxorubicin hydrochloride was from Laboratoire Roger Bellon (France).

2.2. Cell lines

The human small cell lung cancer cell line GLC_4 and its adriamycin-selected MRP1-overexpressing subline GLC_4/ADR [30], as well as the human ovarian carcinoma cell line 2008 and its stable MRP1 transfectant 2008/MRP1 (clone 4) [31], were cultured in RPMI 1640 medium (Flow Labs., Irvine, UK), supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco, Paisley, UK). GLC_4/ADR cells were cultured in the presence of 1.2 μM doxorubicin until 7–14 days before the experiments. As reported earlier, all of these cell lines do not express the MRP homologues MRP2–5 or MDR1–3 [32].

2.3. Antibodies

The preparation of the monoclonal antibody MIB6, against MRP1, has been described elsewhere ([22] and Scheffer et al., in preparation). MIB6 has been reported to specifically inhibit MRP1-associated ATPase activity, as well as MRP1-mediated drug transport into inside-out membrane vesicles.

2.4. Plasma membrane vesicles

Plasma membrane vesicles were prepared as described earlier [33]. First, cells were harvested by centrifugation ($275\times g$, 5 min) and subsequently washed in phosphate-buffered saline. Cell pellets were resuspended in 100 mM KCl, 5 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride and 50 mM HEPES–KOH (pH 7.4) for 1 h at 0°C, followed by ultrasonication at 20% of the maximum power of an MSE sonicator (Soniprep 150) (three bursts of 15 s) Then, the suspension was centrifuged at $1500\times g$ for 10 min. The post-nuclear supernatant was layered on top of a sucrose cushion (46% m/v). After ultracentrifugation ($100\,000\times g$, 60 min) the interface was collected, washed in 100 mM KCl–50 mM HEPES–5 mM $MgCl_2$ buffer (pH 7.4) and ultracentrifuged again ($100\,000\times g$, 30 min). The final membrane preparation was resuspended in 100 mM KCl–50 mM HEPES–5 mM $MgCl_2$ buffer (pH 7.4) and stored at −80°C at a protein concentration of ∼2 mg/ml.

2.5. ATPase activity determinations

ATPase activity of MRP was measured colorimetrically according to a method described earlier [34] with some modifications. Plasma membranes prepared as described above were incubated for 1 h in KCl–HEPES buffer (pH 7.4) at 37°C, in the presence of 1 mM EGTA, 1 mM sodium azide and 0.1 mM ouabain, with or without 1 mM ATP. During the incubation time the reaction was linear, as was shown before [21]. Subsequently, a mixture of the color reagent (0.034% w/w malachite green base, 1.05% w/w ammonium molybdate) and 0.025% v/v Triton N101 was added. After 1 min sodium citrate was added to a final concentration of 3.6% (w/w). Light absorption was measured 30 min later in an ELISA reader at a wavelength of 595 nm.

3. Results

3.1. Dose-dependent induction of MRP1 ATPase activity by GSH

In order to study the role of GSH in MRP1 ATPase activity, we first measured the concentration dependence of GSH-induced MRP1 ATPase activity. We used membranes prepared from the tumor cell line GLC_4 and its MRP1-overexpressing subline GLC_4/ADR, and from the tumor cell line 2008 and its MRP1 transfectant 2008/MRP1. Fig. 1 shows the GSH induction of ATPase activity of 2008 and 2008/MRP1 membranes and of GLC_4 and GLC_4/ADR membranes. Clear GSH effects were observed in membranes of both the MRP1-overexpressing cell lines, whereas in membranes of parental cells no or a very small ATPase stimulation could be observed. A maximum induction of the ATPase activity in MRP1-containing membranes was observed at a concentration of 5 mM GSH. At this concentration the phosphate production of 2008/MRP1 membranes was 5.9 ± 0.1 nmol/mg protein/min (i.e. 129% of the control) and of GLC_4/ADR membranes 7.5 ± 0.4 nmol/mg protein/min (i.e. 159% of the control). Above GSH concentrations of 20 mM the basal ATPase activity of the membranes was inhibited (not shown). In order to exclude effects of GSSG, potentially formed during incubation with GSH, the effect of 1 mM DTT on the MRP1 ATPase stimulation by 5 mM GSH was meas-

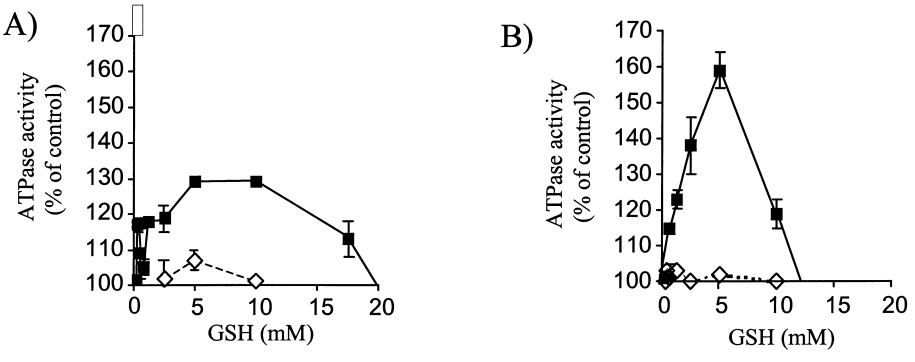

Fig. 1. GSH-induced ATPase activity of MRP1. A: ATPase activity of MRP1 in membranes from 2008 cells (open symbols) and of 2008/MRP1 cells (filled symbols). B: ATPase activity of membranes from GLC_4 cells (open symbols) and from GLC_4/ADR cells (filled symbols). Data are means ± S.D. ($n=3$). ATPase activity is given as percentage of control (without GSH). Absence of error bars means that the S.D. is smaller than the symbol.

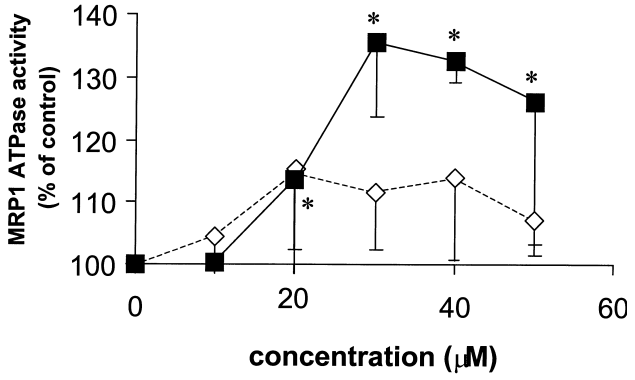

Fig. 2. Induction of MRP1 ATPase activity by DNR and the DNR–GSH conjugate WP811. ATPase activity of GLC_4/ADR membranes was measured in the presence of 10, 20, 30, 40 or 50 μM DNR (open symbols), or WP811 (same concentrations) (closed symbols). Data are means ± S.D. ($n = 3$). Symbols labeled with an asterisk are statistically different from control (100%), as calculated with Student's t-test ($P < 0.05$, $n = 3$). Absence of error bar means that the error bar is within the symbol.

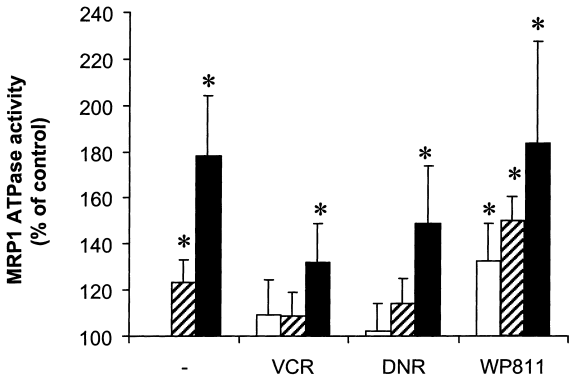

Fig. 3. Effect of GSH on the interaction of DNR, VCR and WP811 with MRP1. MRP1 ATPase activity of GLC_4/ADR membranes was measured in the presence of 30 μM VCR, DNR or WP811 without GSH (open bars), in the presence of 0.5 mM GSH (hatched bars), or in the presence of 5 mM GSH (filled bars). Data are means ± S.D. ($n = 7$). Bars labeled with an asterisk are significantly different from 100% ($P < 0.05$, $n = 7$).

ured. In the presence of DTT no significant difference with the effect of GSH alone was observed (data not shown).

3.2. Inhibition of GSH-induced MRP1 ATPase activity

As shown above, GSH-induced ATPase activity was measured using membranes prepared from the MRP1 transfectant cell line 2008/MRP1, and not in membranes from parental 2008 cells, which indicates the MRP1 specificity of this effect. However, since the MRP1 overexpression and accordingly the MRP1-associated ATPase activity was much higher in membranes from GLC_4/ADR cells, we selected this cell line for further studies. In order to obtain more evidence for the MRP1 specificity of the GSH effect in GLC_4/ADR, we used the earlier described MRP1 antibody MIB6 [22] to specifically inhibit MRP1 ATPase activity. As shown in Table 1, MIB6 was able to inhibit the GSH-induced ATPase activity of GLC_4/ADR membranes. Also, we tested whether we could inhibit this GSH-induced ATPase activity with the earlier described inhibitors of MRP1-mediated drug transport benzbromarone [35] and probenecid [36]. Benzbromarone inhibited the stimulation of ATPase activity by GSH in 2008/MRP1 membranes (not shown) and GLC_4/ADR membranes (Table 1) completely. Interestingly, probenecid did not inhibit the GSH-induced ATPase activity. In contrast, probenecid stimulated MRP1 ATPase activity in the absence or presence of GSH. In membranes prepared from the parent cells no stimulation by probenecid could be observed (data not shown).

Table 1
Inhibition of GSH-induced MRP1 ATPase activity of GLC_4/ADR membranes

Substrate	MRP1 ATPase activity (% of control)
Control	100
GSH	132 ± 4*
GSH+MIB6	110 ± 8#
Benzbromarone	86 ± 5
GSH+benzbromarone	91 ± 9#
Probenecid	124 ± 9*
GSH+probenecid	144 ± 6*#

Concentration of GSH was 1 mM, concentration of MIB6 was 0.35 μg/ml, concentration of benzbromarone was 100 μM and concentration of probenecid was 500 μM.
*Significant difference from the control (Student's t-test, $P < 0.05$; $n = 6$).
#Significant difference from the GSH effect (Student's t-test, $P < 0.05$; $n = 6$).

3.3. Comparison of DNR and WP811 effects on MRP1 ATPase activity

In order to study possible interactions of GSH with MRP1 substrates, we chose to use the established MRP1 substrate DNR. We measured the ability of DNR to induce MRP1 ATPase activity. Also, we compared the effect of DNR on ATPase activity with the DNR–GSH conjugate WP811 [29], since many drug–GSH conjugates have been suggested to be preferred MRP1 substrates [7,9,12,13]. In Fig. 2 the induction of ATPase activity in the presence of different concentrations of both compounds is given. In contrast to DNR, which did not have an effect up to 50 μM, WP811 induced ATPase activity to a significant level at concentrations above 20 μM. A maximum WP811 effect occurred at a concentration of 30 μM WP811.

3.4. Induction of the MRP1 ATPase by MRP substrates in the presence of GSH

Recently, it has been reported that GSH may act as a co-substrate in MRP1-mediated transport of several anticancer agents, including VCR [16] and DNR [17]. This suggests that the presence of GSH is needed to allow efficient interaction of MRP1 with substrates leading to their transmembrane transport. Thus, we measured the ATPase effect of simultaneous incubation of GSH with DNR, VCR and WP811 (Fig. 3). Again WP811 induced the ATPase activity to a significant level, and in the presence of 0.5 mM or 5 mM GSH no increase of the ATPase activity was observed for WP811, DNR or VCR.

3.5. Induction of the MRP1 ATPase by (iso)flavonoids in the presence of GSH

As described earlier, several (iso)flavonoids are able to induce MRP1 ATPase activity [22]. Therefore, we also investigated the effect of GSH on flavonoid-induced MRP1 ATPase activity. In particular, we studied the effect of 0.5 mM and 5 mM GSH on the induction of MRP1 ATPase activity by the (iso)flavonoids genistein, apigenin and flavopiridol (Fig. 4).

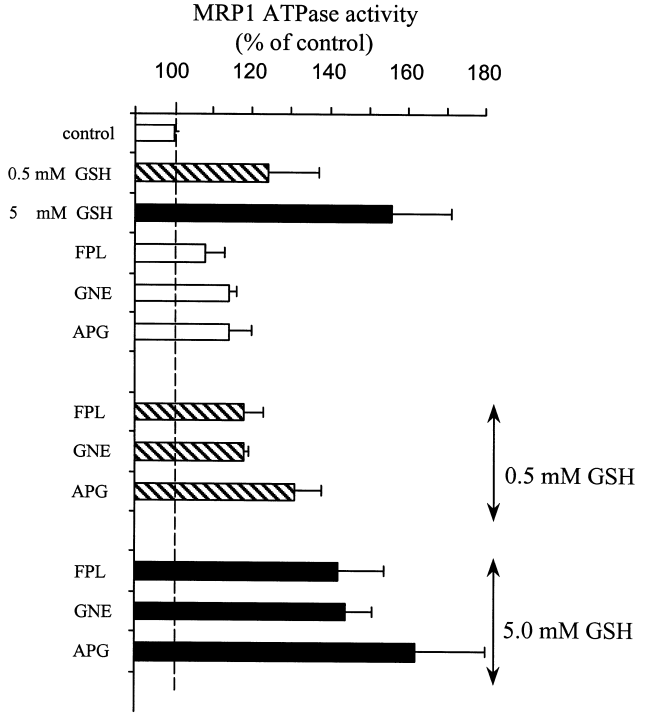

Fig. 4. Effect of GSH on (iso)flavonoid-induced MRP1 ATPase activity. ATPase activity of GLC_4/ADR was measured in the presence of 50 μM of the (iso)flavonoids flavopiridol (FPL), genistein (GNE) or apigenin (APG), in the absence (open bars) or presence 5 mM GSH (filled bars). Data are means ± S.D. ($n = 3$). All effects were significantly different from the control (100%). All (iso)flavonoid effects in the presence of GSH were not statistically different from GSH effects as calculated with Student's t-test ($P < 0.05$, $n = 3$).

All tested (iso)flavonoids induced the ATPase activity in the absence of GSH. In the presence of GSH the induction of ATPase activity was comparable with the GSH controls. There was no indication for an additive or synergistic increase of the MRP1 ATPase activity.

4. Discussion

The multidrug resistance protein MRP1 is a member of the family of ABC transporters [1–3]. Hydrolysis of ATP is required for the transport activity of this protein. MRP1 has been shown to transport major anticancer agents, such as the anthracyclines, vinca alkaloids and etoposide [4,15,27]. Recently, antifolates have also been described as MRP1 substrates [8].

The cellular antioxidant GSH has been shown to play an important role in the transport mechanism of MRP1. Cellular depletion of GSH abolishes the MRP1-mediated efflux of neutral and basic drugs [11], and several studies have provided evidence that MRP1 mediates drug transport with GSH acting as a co-substrate [14,16,17]. Also, transport of drugs as a GSH conjugate has been published [7,9,12–14]. However, recently several GSH-independent interactions of MRP1 have also been reported. The MRP1-mediated transport of antifolates [8] and the induction of the MRP1 ATPase activity by (iso)flavonoids [21,22], both in the absence of GSH, showed that the presence of GSH is not absolutely required for MRP1 function.

Here we report an analysis of the effects of GSH on the induction of ATPase reactions associated with MRP1. We focussed on the influence of GSH on MRP1 in its natural lipid environment, and studied its interactions with several established anticancer drugs and (iso)flavonoids.

Although earlier reports described ATPase activity of partly purified MRP1, which was solubilized in lipids [23,24], we chose to study MRP1 ATPase activity in a more physiological environment. The necessity to provide an experimental basis for the study of these interactions by using natural membranes is very clearly shown for Pgp in which the ATPase activity is significantly influenced by subtle differences in the lipid environment [26]. Moreover, conflicting data between two recent reports on ATPase activity of purified and reconstituted MRP1 were explained by the authors as a possible result of the artificial lipid environments of MRP1 in those studies [23,24]. Therefore, in our study we have used plasma membrane fractions of MRP1-overexpressing cell lines.

Our data clearly show a GSH-induced MRP1-specific ATPase activity that was dose-dependent. The effect of GSH on the MRP1 ATPase was induced by concentrations in the millimolar range, which is in accordance with results by others, who showed that 0.5 mM reduced GSH was able to induce ATPase activity of partly purified MRP1 [23]. Also, drug transport into MRP1-containing membrane vesicles has previously been described to be stimulated by millimolar concentrations of GSH [16,17]. Moreover, such GSH concentrations are present in intact cells, and a lowering of this GSH concentration leads to a decreased drug efflux [11].

A main item we have addressed in this study is whether interactions between GSH and putative or established MRP1 substrates can be seen at the level of ATPase activity. Recently, interactions between VCR or DNR and GSH have been shown in transport assays using inside-out membrane vesicles, in which MRP1-mediated transport of VCR or DNR was stimulated by GSH [16,17]. Also, co-transport of GSH with MRP1-mediated efflux of etoposide has been demonstrated in intact cells [19]. Yet, despite these reported observations we found no evidence for the interaction of VCR or DNR with GSH on the level of MRP1 ATPase activity.

Interestingly, and in contrast to VCR and DNR, some inhibitors of MRP1-mediated transport did stimulate the MRP1 ATPase activity. As described already in a study of (iso)flavonoids [21], probenecid and the DNR–GSH conjugate WP811 also induced MRP1 ATPase activity. Yet, like VCR and DNR, these compounds apparently showed no synergistic increase of their induced MRP1 ATPase activity in the presence of a millimolar GSH concentration.

A third category of MRP1-interacting compounds in our study is presented by benzbromarone. This drug is a potent inhibitor of the MRP1 ATPase activity induced by all putative substrates or modulators tested so far. Benzbromarone does not seem to affect the basal ATPase activity.

With regard to MRP1 interactions of putative substrate/modulator drugs and GSH, the present experiments do not provide evidence that GSH facilitates or increases the ATPase stimulation by these drugs. On the other hand, the clear induction of ATPase activity by the DNR–GSH conjugate WP811 in a micromolar concentration range adds further evidence to the finding that preferred substrates of MRP1 contain a hydrophobic as well as a negatively charged part. WP811 was earlier described as an inhibitor of MRP1-mediated transport [29]. Priebe et al. reported that, in contrast to

DNR, WP811 was able to inhibit the MRP1-mediated uptake of LTC_4 into inside-out membrane vesicles. The competitive inhibition by WP811 of MRP1-mediated LTC_4 transport in turn suggests that WP811 is an MRP1 substrate itself, that has no need of additional GSH. That observation would be consistent with our finding of ATPase induction of MRP1 by WP811 and the absence of a synergistic effect by GSH.

In order to summarize the present data, it may be helpful to discuss the analogies with or differences from the much more studied Pgp ATPase activity. First, Pgp is likely to have a high basal ATPase activity, suggesting no rigid coupling of ATP hydrolysis and drug transport [37]. This might explain the rather small ratio of drug-induced Pgp ATPase activity to basal Pgp ATPase activity as found in most studies [38]. In this respect, there may be a parallel between Pgp and MRP1 for substrates such as the anthracycline DNR; in neither case does the substrate induce a stimulation or inhibition of the ATPase activity ([39] and this study).

Further, the concentration-dependent pattern of MRP1 ATPase induced by, for example, WP811 and GSH is very similar to that seen in most Pgp studies [38,40,41]. In addition, we were not able to observe an increase of MRP1 ATPase activity induced by GSH in the presence of the substrates DNR or VCR. Similar observations have previously been reported for Pgp, in which even an inhibition of ATPase activity was observed upon simultaneous incubation of an ATPase-activating modulator and an established Pgp substrate [42]. Several models of Pgp binding have been proposed for the localization of the drug binding sites in Pgp, all of which shared the feature of having at least two drug binding sites [42,43]. In comparison, different drug binding sites have also been shown to be involved in the interaction of MRP1 with different subclasses of substrates [20]. Despite the limitations of natural membrane preparations as well as purified systems we think that ATPase studies may contribute to our understanding of MRP1. However, it is clearly too early to explain the present data or those of Chang et al. [23] and Mao et al. [24] in terms of drug binding and transport properties of MRP1.

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