Transport of unmodified basic anticancer drugs by the multidrug resistance protein MRP1 is dependent on an intact GSH molecule

Johan Renes\textsuperscript{1}, Elisabeth G.E. de Vries\textsuperscript{2}, Peter L.M. Jansen\textsuperscript{1} and Michael Müller\textsuperscript{1}

Groningen University Institute for Drug Exploration (GUIDE)
\textsuperscript{1}Department of Gastroenterology and Hepatology and
\textsuperscript{2} Department of Medical Oncology,
University Hospital Groningen, Groningen, The Netherlands
Abstract

The multidrug resistance protein MRP1 is a transporter for unmodified basic anticancer drugs but only in the presence of reduced glutathione (GSH). In this study we further characterised MRP1-mediated drug transport. Membrane vesicles were isolated from the MRP1-overexpressing GLC₄/Adr cell line, the multidrug resistant counterpart of the GLC₄ small cell lung cancer cell line. Transport experiments were performed using a rapid filtration technique with ³H-labelled vincristine and daunorubicin as substrates. Both substrates were transported by MRP1 in the presence of GSH but also in the presence of methyl-GS, a short chain alkyl derivative of GSH. The GSH dipeptide fragment cysteinyi-glycine did not stimulate MRP1-mediated transport of vincristine or daunorubicin. Because glucuronides are also substrates for MRP1 we tested whether glucuronic acid could also stimulate MRP1-mediated drug transport. Glucuronic acid did not stimulate MRP1-mediated transport of either vincristine or daunorubicin. From these results we conclude that MRP1-mediated transport of unmodified basic anticancer drugs is most likely specifically dependent on GSH and requires an intact GSH molecule.
**Introduction**

Overexpression of the ATP-binding cassette (ABC) transporter multidrug resistance protein MRP1 causes a multidrug resistant (MDR) phenotype. One of the mechanisms of MRP1-mediated MDR is an ATP-dependent reduction of intracellular levels of, usually basic, chemotherapeutic drugs. However, the first transport studies with membrane vesicles from MRP1-overexpressing cells demonstrated that MRP1 is a transporter for multivalent anionic conjugates, preferentially glutathione S-conjugates (GS S-conjugates). This remained the question how MRP1 could function as a transporter for unmodified basic anticancer drugs.

According to its transport capacity for GSH-, glucuronic acid- and sulphate conjugates, it was suggested that MRP1 might confer drug resistance by export of drug conjugates. Although MRP1 is a transporter for the glutathione S-conjugates (GS S-conjugates) of the alkylating agents chlorambucil and melphalan, MRP1 appears not to confer resistance to these drugs. On the other hand, there is no evidence that chemotherapeutic drugs to which MRP1 confers resistance are substrates for GSH-, glucuronic acid- or sulphate conjugation. In addition, in efflux media from MRP1-overexpressing cells drug conjugates could not be detected. Still, GSH is important in the function of MRP1 in drug resistance. Drug resistant cells overexpressing MRP1 showed increased drug sensitivity after treatment with the GSH-depleting compound buthionine sulfoximine (BSO). In addition, increased efflux of GSH was measured from MRP1 overexpressing cells. These results suggested that GSH plays an important role in the transport properties of MRP1.

Experiments with membrane vesicles from MRP1-overexpressing cells demonstrated that MRP1 is a transporter for the unmodified basic anticancer drugs vincristine and daunorubicin but only in the presence of GSH. The transport process for vincristine is supposed to be driven via a co-transport mechanism with GSH. Dipeptide fragments of the GSH molecule i.e. cysteinyl-glycine and γ-glutamylcysteine did not stimulate MRP1-mediated vincristine transport. This suggests that drug transport by MRP1 requires an intact GSH molecule. Recently, we demonstrated that daunorubicin transport by MRP1 is also dependent on GSH. GSH stimulated MRP1-mediated daunorubicin transport in a dose-dependent manner with a $K_m$ value of 2.7 mM and with maximal stimulation at concentrations ≥ 10 mM. The specificity of this transport process was demonstrated using a MRP1-specific monoclonal antibody that inhibited GSH-stimulated daunorubicin transport by MRP1. In this study we further examined the mechanism of MRP1-mediated drug transport.
Materials and Methods

Cell culture
Culture of the MRP1-overexpressing GLC4/Adr cells, the multidrug resistant counterpart of the human GLC4 small cell lung cancer cell line has been described previously in detail.12

![Figure 1. ATP-dependent transport of [%sup3;H%]vincristine (300 nM) during 5 min into membrane vesicles prepared from GLC4/Adr cells. Transport was measured in the presence of 5 mM of either GSH, methyl-GS (Me-GS) or cysteinyl-glycine (Cys-Gly). MK571 was added to a final concentration of 5 µM. Data represent mean ± S.D. from two experiments with at least triplicate determinations. * P < 0.05 (one-way ANOVA) compared to ATP; ** P < 0.05 (one-way ANOVA) compared to GSH; *** P < 0.05 (one-way ANOVA) compared to Me-GS.](image)

Preparation of membrane vesicles
Membrane vesicles used in the transport studies were prepared as described.10 Briefly, GLC4/Adr cells were harvested, washed with PBS and centrifuged at 180 x g for 10 min at 4°C. The pellet was diluted 40-fold in 1 mM NaHCO3 and stirred gently for 1 h in the presence of 100 U Benzonase. The cell lysate was centrifuged at 100,000 x g and the pellet was suspended in 10 mM tris/250 mM sucrose (TS) buffer and layered on top of a 38% sucrose/10 mM tris solution. This was centrifuged for 1 h at 4°C at 280,000 x g and the interface layer was collected, resuspended in 25 ml TS buffer and centrifuged at 100,000 x g for 30 min at 4°C. The pellet was resuspended in 500 µl TS buffer and vesicles were formed by passing this suspension 20-25 times through a 25 gauge needle. Aliquots of 25 µl were snap frozen in liquid nitrogen and stored at -80°C.

Protein contents were measured with a Bradford-based protein assay (Biorad laboratories, Hercules, CA, USA) and expression of MRP1 protein was determined by immunoblot analysis as described.10
Transport studies
Transport experiments were performed by a rapid filtration technique using $^3$H-labelled vincristine and daunorubicin as substrates as was described earlier. Membrane vesicles (50-100 µg protein) were thawed and added to a transport buffer containing 4 mM ATP or AMP-PCP, a non-hydrolyzable ATP-analogue, 10 mM MgCl₂, 10 mM creatine phosphate, 100 µg/ml creatine kinase, 1 µM bafilomycin A₁, 250 mM sucrose and 10 mM tris pH 7.4. Bafilomycin A₁ is added to prevent aspecific uptake. After 1 min prewarming at 37°C, [³H]vincristine or [³H]daunorubicin was added to final concentrations of 300 nM and 600 nM, respectively, to a final volume of 115 µl. After 5 min incubation, 25 µl samples were taken and diluted in 1 ml ice cold PBS or PBS/1 mM ethidium bromide for daunorubicin. These solutions were filtered through OE66 cellulose acetate filters, pore size 0.2 µm (Schleicher and Schuell, Dassel, Germany) which were presoaked in either PBS (vincristine) or PBS/1 mM ethidium bromide (daunorubicin). Filters were washed with 5 ml PBS/1 mM ethidium bromide, 5 ml PBS/0.1% Tween and 5 ml PBS for daunorubicin experiments and with 5 ml PBS/0.1% Tween and 5 ml PBS for vincristine experiments. Filters were air-dried and radioactivity was counted with liquid scintillation.

Results and Discussion
We previously demonstrated that the unmodified basic anticancer drugs vincristine and daunorubicin are MRP1 substrates only in the presence of GSH. In this study we have further characterised MRP1-mediated drug transport.
GLC₄/Adr membrane vesicles were used because of high MRP1 expression levels and proven capabilities to study transport properties of MRP1. Membrane vesicles from GLC₄/Adr cells were isolated and MRP1 expression was analysed by immunoblot analysis. Compared to membrane vesicles from GLC₄ control cells, MRP1 expression levels were highly increased in GLC₄/Adr membrane vesicles (ref. 10 and data not shown).

Vincristine transport was stimulated about 6-fold by GSH and methyl-GS, a short-chain alkyl derivative of GSH (5 mM final concentration). This increased vincristine transport was reduced by the MRP1-inhibitor MK571 (5 µM final concentration) (Figure 1). The GSH fragment cysteinyl-glycine however, did not stimulate vincristine transport (Figure 1). These results are in agreement with previous results showing that MRP1-mediated vincristine transport requires an intact GSH molecule. Similar to vincristine, transport of daunorubicin was stimulated by GSH and methyl-GS, but not by cysteinyl-glycine. GSH- and methyl-GS-stimulated daunorubicin transport was inhibited by MK571 (Figure 2). These results demonstrate that MRP1-mediated daunorubicin is also dependent on an intact GSH molecule.

Besides GS-S-conjugates, glucuronides are also MRP1 substrates. Transport of 17β-estradiol 17-(β-D-glucuronide) (E₂17βG) is reduced by LTC₄ and photoaffinity labelling of MRP1 by LTC₄ is inhibited by E₂17βG. These results suggest that GS-S-conjugates and glucuronides bind to identical or overlapping binding sites. This prompted us to investigate whether glucuronic acid could also stimulate MRP1-mediated drug transport. Consistent with previous observations, glucuronic acid (5 mM) did not stimulate vincristine transport (Figure 3). We observed that glucuronic acid did not stimulate MRP1-mediated daunorubicin transport, even with concentrations up to 15 mM (Figure 4).
The molecular mechanism of MRP1-mediated drug transport remains to be elucidated. Substrates with highest affinity for MRP1 are anionic compounds such as GS S-conjugates and glucuronides. Unmodified basic anticancer drugs are substrates for MRP1 but only in the presence of GSH. Otherwise, if the drug is anionic there is no need for further modification to be a MRP1 substrate as was shown for the antifolate methotrexate. This suggests that the anionic moiety of a compound is a prerequisite for MRP1-mediated transport. However, in contrast to GSH, the anionic glucuronic acid does not stimulate MRP1-mediated transport of vincristine and daunorubicin. Thus transport of unmodified basic drugs by MRP1 seems not dependent on the presence of an arbitrary anionic compound but is rather specifically dependent on GSH. Since GSH and methyl-GS, but not cysteine containing dipeptide fragments of GSH, stimulate MRP1-mediated drug transport, it is not the reducing capacity of GSH that increases drug transport, this study. Moreover, these results demonstrate that an intact GSH molecule is required for MRP1-mediated drug transport.

Figure 4. ATP-dependent transport of [³H]-daunorubicin (600 nM) during 5 min into membrane vesicles prepared from GLC4/Adr cells. Transport was measured in the absence or the presence of 5-15 mM glucuronic acid (G.A.). Data represent mean ± S.D. from two experiments with at least triplicate determinations.

How GSH facilitates MRP1-mediated drug transport is unclear. Short-chain alkyl derivatives of GSH also stimulate MRP1-mediated vincristine and daunorubicin transport, this study, suggesting that this transport is not initiated by reduction or glutathionylation of MRP1. Rather, GSH may interact with a hydrophilic binding site at MRP1. Indeed, GSH interacts with MRP1 and GSH is a substrate for MRP1. When LTC₄ transport was measured in the presence of vincristine and GSH both compounds acted as competitive inhibitors suggesting that vincristine and GSH have similar binding sites on MRP1 as LTC₄. Thus MRP1-mediated transport of unmodified basic anticancer drugs may depend on occupation of a specific binding site on MRP1 by GSH. This binding site may also be occupied by short-chain alkyl...
derivatives of GSH but not by dipeptide fragments of GSH or glucuronic acid. This model provides an explanation for the results presented in this study. How GSH facilitates drug transport after binding to MRP1 remains speculative. A simple explanation is that interaction of GSH with MRP1 induces drug binding after which both are translocated. The recently observed GSH-mediated increase in MRP1 ATPase activity \(^{18}\) and induction of nucleotide trapping at nucleotide binding domain 2 \(^{19}\) may be involved in this process.

In conclusion, MRP1-mediated transport of unmodified basic anticancer drugs is most likely specifically dependent on GSH and requires an intact GSH molecule.

Acknowledgements
We thank Edith Nienhuis and Inge Krikken for technical assistance. This study was supported by grant RUG 95-1007 from the Dutch Cancer Society.
MRP1-mediated drug transport by intact GSH

References

15. Loe DW, Deeley RG, and Cole SPC. Verapamil stimulates glutathione transport by the 190-kDa

