British Journal of Pharmacology (1999) 126, 681–688
http://www.stockton-press.co.uk/bjp

ATP- and glutathione-dependent transport of chemotherapeutic drugs by the multidrug resistance protein MRP1

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1 The present study was performed to investigate the ability of the multidrug resistance protein (MRP1) to transport different cationic substrates in comparison with *MDR1*-P-glycoprotein (MDR1). Transport studies were performed with isolated membrane vesicles from *in vitro* selected multidrug resistant cell lines overexpressing MDR1 (A2780AD) or MRP1 (GLC_4/Adr) and a *MRP1*-transfected cell line (S1(MRP)).

2 As substrates we used ^{3}H-labelled derivatives of the hydrophilic monoquaternary cation *N*-(4′,4′-azo-*n*-pentyl)-21-deoxy-ajmalinium (APDA), the basic drug vincristine and the more hydrophobic basic drug daunorubicin. All three are known MDR1-substrates.

3 MRP1 did not mediate transport of these substrates *per se*. In the presence of reduced glutathione (GSH), there was an ATP-dependent uptake of vincristine and daunorubicin, but not of APDA, into GLC_4/Adr and S1(MRP) membrane vesicles which could be inhibited by the MRP1-inhibitor MK571.

4 ATP- and GSH-dependent transport of daunorubicin and vincristine into GLC_4/Adr membrane vesicles was inhibited by the MRP1-specific monoclonal antibody QCRL-3.

5 MRP1-mediated daunorubicin transport rates were dependent on the concentration of GSH and were maximal at concentrations $\geqslant$10 mM. The apparent K_M value for GSH was 2.7 mM. Transport of daunorubicin in the presence of 10 mM GSH was inhibited by MK571 with an IC_{50} of 0.4 μM.

6 In conclusion, these results demonstrate that MRP1 transports vincristine and daunorubicin in an ATP- and GSH-dependent manner. APDA is not a substrate for MRP1.

Keywords: Drug resistance; ABC transporter; MDR1; MRP1; ATP-dependent drug transport; GSH; daunorubicin; vincristine; APDA

Abbreviations: ABC transporter, ATP binding cassette transporter; AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate; [^{3}H]-APDA, *N*-(4′,4′-azo-*n*-pentyl)-21-deoxy-[21-^{3}H]ajmalinium; Baf, bafilomycin A_1; FCS, foetal calf serum; GSH, reduced glutathione; GSSG, oxidized glutathione; GS *S*-conjugates, glutathione *S*-conjugates; LTC_4, leukotriene C_4; MDR1, *MDR1*-P-glycoprotein; MRP, multidrug resistance protein; PBS, phosphate-buffered saline; TS, tris/sucrose; V-type ATPase, vacuolar H^+-ATPase

Introduction

Human multidrug resistance is frequently associated with the overexpression of the *MDR1*-P-glycoprotein (MDR1) (Juliano & Ling, 1976) and/or the multidrug resistance protein (MRP1) (Cole *et al*., 1992), two members of the ATP binding cassette (ABC) transporter superfamily (Higgins, 1992). Although they share only 15% amino acid sequence homology, both proteins confer resistance to a broad range of natural product drugs in drug selected cell lines as well as in transfected cells (Loe *et al*., 1996c; Broxterman *et al*., 1995; Gottesman & Pastan, 1993). Currently, it is generally accepted that MDR1 functions as an ATP-dependent transport protein capable of reducing intracellular levels of various natural product drugs (Borst & Schinkel, 1997; Germann, 1996; Ruetz & Gros, 1994a). Most of these drugs, e.g. vinca alkaloids, anthracyclines, epipodophillotoxins, actinomycin D and paclitaxel, have unrelated chemical structures but share the property of being hydrophobic compounds.

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MRP1 was first identified in tumour cells with a multidrug resistant phenotype without overexpression of MDR1 (Cole *et al*., 1992). Although MRP1 is able to confer resistance to drugs which are MDR1 substrates, the substrate specificity of MRP1 seems different from that of MDR1. Transport studies with membrane vesicles isolated from MRP1 overexpressing cells, either *in vitro* selected or transfected, revealed that MRP1 is a transporter of multivalent organic anions, preferentially glutathione *S*-conjugates (GS *S*-conjugates), (Barnouin *et al*., 1998; Loe *et al*., 1996b; 1997; Jedlitschky *et al*., 1996; Müller *et al*., 1994b), but also of sulphate conjugates (Jedlitschky *et al*., 1996) and glucuronides (Jedlitschky *et al*., 1996; Loe *et al*., 1996a). Also oxidized glutathione (GSSG), complexes of reduced glutathione (GSH) with arsenite (Zaman *et al*., 1995) and unmodified compounds in the presence of GSH (Loe *et al*., 1996b; 1997) are MRP1 substrates. In view of its substrate specificity and the ubiquitous expression of *MRP1* in human tissues (Zaman *et al*., 1993) and blood cells (Burger *et al*., 1994), the putative physiological role of MRP1 seems to be cellular extrusion of metabolites of GSH-dependent detoxification reactions (Müller *et al*., 1996).

The molecular basis of the drug specificity of MRP1 is not fully understood. Two models currently exist describing the drug transport mechanism of MRP1. First, MRP1 may be a

transporter of drug conjugates (e.g. GS *S*-conjugates and glucuronide conjugates) (Priebe *et al*., 1998; Jedlitschky *et al*., 1996; Ishikawa *et al*., 1995), however, stable GS *S*-conjugates of vincristine and daunorubicin could not be detected in HPLC-analysed media from *MRP1* transfected cells (Zaman *et al*., 1995). Moreover, there is no evidence that the chemotherapeutic agents to which MRP1 confers resistance, are substrates for GSH- or glucuronic acid conjugation (for review see O'Brien & Tew, 1996; Tew, 1994). Secondly, MRP1 may transport unmodified natural product drugs in the presence of GSH. For example, it has been demonstrated that unmodified vincristine is transported by MRP1 but only in the presence of physiological concentrations of GSH (Loe *et al*., 1996b). Furthermore, resistance to vinca alkaloids and anthracyclines is reversed by GSH depletion in MRP1-overexpressing cells, but not in cells overexpressing MDR1 (Zaman *et al*., 1995; Versantvoort *et al*., 1995). Therefore, GSH appears to play an important role in MRP1-mediated drug resistance. In this study we compared MDR1 and MRP1 with respect to their ability to transport different classes of cationic drugs and investigated the role of GSH in MRP1-mediated transport. We hypothesized that the hydrophobic basic drug daunorubicin has not necessarily to be conjugated with GSH to be a MRP1 substrate (Priebe *et al*., 1998; Ishikawa *et al*., 1995), but is transported by MRP1 in the presence of GSH. We used vincristine as a previously demonstrated MRP1 substrate (Loe *et al*., 1996b) to characterize our experimental system. Furthermore, experiments were performed with the permanently positively charged monoquaternary cationic derivate of the anti-arrhythmic drug ajmaline (*N*-(4′, 4′-azo-*n*-pentyl)-21-deoxy-ajmalinium, APDA) to study the ability of MRP1 to mediate the transport of more hydrophilic cations which are excellent MDR1 substrates *in vitro*.

Methods

Chemicals

N-(4′,4′-azo-*n*-pentyl)-21-deoxy-[21-^{3}H]-ajmalinium [^{3}H]-(APDA) (46 GBq mmol^{-1}) (Müller *et al*., 1994a), was kindly provided by Dr G. Kurz (University of Freiburg, Germany), [G-^{3}H]-vincristine (226 GBq mmol^{-1}) was obtained from Amersham (Little Chalfont, U.K.) and [14,15,19,20-^{3}H(N)]-leukotriene C_4 ([^{3}H]-LTC_4) (4884 GBq mmol^{-1}) and [G-^{3}H]-daunorubicin (46.62 GBq mmol^{-1}) were from NEN (Boston, MA, U.S.A.). Benzonase, grade I protease free, was from Merck (Darmstadt, Germany). ATP, creatine phosphate and creatine kinase were purchased from Boehringer (Mannheim, Germany). The MRP1 inhibitor MK571 was kindly provided by Dr A.W. Ford-Hutchinson (Merck-Frosst, Pointe Claire-Dorval, Quebec, Canada) and the MDR1 inhibitor PSC833 was a kind gift of Novartis (Basel, Switzerland). The monoclonal antibody (mAb) QCRL-3, directed against MRP1, was obtained from Signet Laboratories (Dedham, MA, U.S.A.). β,γ-Methyleneadenosine 5′-triphosphate (AMP-PCP), GSH, the vacuolar H^+-ATPase (V-type ATPase) inhibitor bafilomycin A_1 (Baf) and all other chemicals were from Sigma (St. Louis, MO, U.S.A.).

Cell culture

Culture procedures for the human GLC_4 small cell lung cancer line and its doxorubicin selected multidrug resistant counterpart GLC_4/Adr have been described previously (Zijlstra *et al*., 1987). Culture of the non-small cell lung cancer cell line SW1573/S1, further designated as S1, and of its *MRP1* transfected subline S1(MRP) was performed as reported (Zaman *et al*., 1994) only with stepwise increase of the Geneticin concentration to 0.4 mg ml^{-1} (Gibco, Paisley, U.K.). The human ovarian tumour cell line A2780 was cultured in RPMI1640 medium, supplemented with 10% foetal calf serum (FCS), (Gibco). The multidrug resistant A2780AD line was isolated from the A2780 line by a multistep selection with doxorubicin and maintained in RPMI1640 medium supplemented with 10% FCS as described (Rogan *et al*., 1984).

Preparation of membrane vesicles

Membrane vesicles were prepared as described previously (Müller *et al*., 1994b) with minor modifications. Briefly, cells were harvested, washed with phosphate-buffered saline (PBS, mM: NaCl 137, KCl 2.7, Na_2HPO_4 10.1, KH_2PO_4 1.8, pH 7.4) and centrifuged at $180 \times g$, for 10 min at 4°C. The resulting pellet was diluted 40 fold in hypotonic buffer (1 mM $NaHCO_3$, pH 7.4) and stirred gently in the presence of 100 U Benzonase for 1 h. The cell lysate was centrifuged at $100{,}000 \times g$ for 30 min at 4°C and the remaining pellet was resuspended in 5 ml isotonic TS buffer (10 mM Tris/250 mM sucrose, pH 7.4) and homogenized with a Dounce B homogenizer in the presence of 100 U Benzonase. The crude membrane fraction was layered on top of a 38% (w/v) sucrose solution in 10 mM Tris (pH 7.4) and centrifuged at $280{,}000 \times g$ for 1 h at 4°C in a swing out rotor. The interface layer was collected, diluted to 25 ml in TS buffer and centrifuged at $100{,}000 \times g$ for 30 min at 4°C. The resulting pellet was resuspended in 300–500 μl isotonic buffer. Vesicles were formed by passing the suspension 20 times through a 25 gauge needle. The membrane vesicles were snap frozen in liquid nitrogen and stored at −80°C. Protein content was measured by a Bradford-based Biorad protein assay (Biorad laboratories, Hercules, CA, U.S.A.).

Immunodetection of MRP1 and MDR1

Ten μg protein of each membrane preparation was separated on a SDS/7.5% polyacrylamide gel and transferred to nitrocellulose (Amersham) by electroblotting. MRP1 was detected by the rat monoclonal MRPr1 (1 : 500), kindly provided by Dr R. Scheper, (Free University, Amsterdam, the Netherlands). MDR1 was detected by the monoclonal antibody C219 (1 : 500), (Centocor, Malvern, MA, U.S.A.). MRP2 protein levels were analysed with the monoclonal antibody M_2 III-5, a kind gift of Dr R. Oude Elferink (Academic Medical Centre, Amsterdam, the Netherlands). Primary antibodies were visualized by enhanced chemiluminescence (ECL), (Pierce, Rockford, IL, U.S.A.).

Transport studies

Uptake of [^{3}H]-APDA (300 nM), [^{3}H]-vincristine (300 nM) and [^{3}H]-daunorubicin (600 nM) into membrane vesicles was measured by a rapid filtration technique. Membrane vesicles (50 μg protein) were rapidly thawed and added to the buffer system containing 4 mM ATP or AMP-PCP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 μg ml^{-1} creatine kinase, 10 mM Tris pH 7.4 and 250 mM sucrose. After 1 min prewarming at 37°C, the substrate was added (110 μl final volume). At indicated time points, samples (25 μl) were taken and diluted in 1 ml ice cold stopsolution (PBS). These solutions were subsequently filtered through OE66 cellulose

acetate filters, pore size 0.2 μm (Schleicher & Schuell, Dassel, Germany), presoaked in PBS. Filters were rinsed with 5 ml PBS/0.05% Tween 20 followed by 5 ml PBS. After rinsing, the filters were air dried and radioactivity was counted with a liquid scintillation counter. Experiments with [^{3}H]-daunorubicin were performed similarly, except that the PBS stopsolution contained 1 mM ethidium bromide, the filters were presoaked in PBS/1 mM ethidium bromide and the filters were first washed by 5 ml PBS/1 mM ethidium bromide, followed by PBS/0.05% Tween 20 and 5 ml PBS. This method reduced the background binding of [^{3}H]-daunorubicin with 70–80% and resulted in a signal to noise ratio of 10 : 2–3. Experiments with [^{3}H]-LTC_4 (1.5 nM) were performed with 10 μg protein, NC45 nitrocellulose filters, pore size 0.45 μm (Schleicher & Schuell), and 10 mM Tris/250 mM sucrose buffer (pH 7.4) instead of PBS. If required, PSC833, MK571, GSH and/or Baf were added together with the membrane vesicles to the buffer system.

Time course experiments of [^{3}H]-daunorubicin (600 nM) uptake were carried out with membrane vesicles from GLC_4/Adr cells (100 μg protein in 220 μl reaction volume) in the presence of 1 μM Baf and in the presence or the absence of 5 mM GSH. At indicated time points, samples of 25 μl were taken and treated as described above. Aspecific binding of [^{3}H]-daunorubicin was measured at 4°C in the presence of 4 mM ATP and 5 mM GSH and subtracted from all values obtained at 37°C.

Uptake experiments in the presence of the MRP1-specific monoclonal antibody (mAb) QCRL-3 or the control mAb directed against the T-cell marker CD3 (a kind gift of Dr B.J. Kroesen, University Hospital Groningen) were performed for 1 min as described above. The mAbs were added together with the membrane vesicles to the buffer system.

All transport data are presented as the difference of the values measured in the presence of ATP and in the presence of AMP-PCP. The non-hydrolyzable ATP analogue AMP-PCP did not support uptake, demonstrating that ATP hydrolysis was required.

Results

Immunoblot analysis of MRP1 and MDR1

The levels of MRP1 and MDR1 in membrane subfractions were analysed by immunoblotting. Increased levels of the 190 kDa MRP1 (Müller *et al*., 1994b; Roelofsen *et al*., 1997) were detected in membranes from GLC_4/Adr and *MRP1*-transfected S1(MRP) cells, while membranes isolated from A2780, A2780AD, GLC_4 and S1 cells exhibited lower levels (Figure 1). MDR1 was found to be overexpressed in membranes isolated from A2780AD cells. No expression of MDR1 was found in membrane preparations from the other cell lines. MRP2 was not detected in membranes from any of these cell lines (data not shown).

ATP-dependent uptake of cationic drugs in membrane vesicles from MDR1- and MRP1-overexpressing cells

Transport properties of MDR1 and MRP1 were compared with two hydrophobic basic drugs ([^{3}H]-vincristine and [^{3}H]-daunorubicin) and the more hydrophilic cation ([^{3}H]-APDA). Figure 2a shows ATP-dependent uptake of [^{3}H]-APDA, [^{3}H]-vincristine and [^{3}H]-daunorubicin into membrane vesicles from A2780 and A2780AD cells. ATP-dependent uptake of all three substrates into A2780AD membrane vesicles was increased

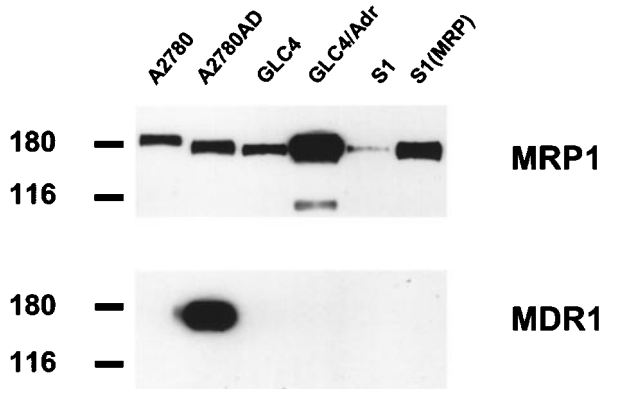

Figure 1 Immunoblot analysis of isolated membrane subfractions. Membrane proteins (10 μg) were resolved on 7.5% SDS–PAGE and transferred to nitrocellulose by electroblotting. Protein levels were analysed with monoclonal antibodies raised against MRP1 (MRPr1) and MDR1 (C219). Primary antibodies were visualized by enhanced chemiluminescence. Sizes of molecular weight markers are indicated in kDa.

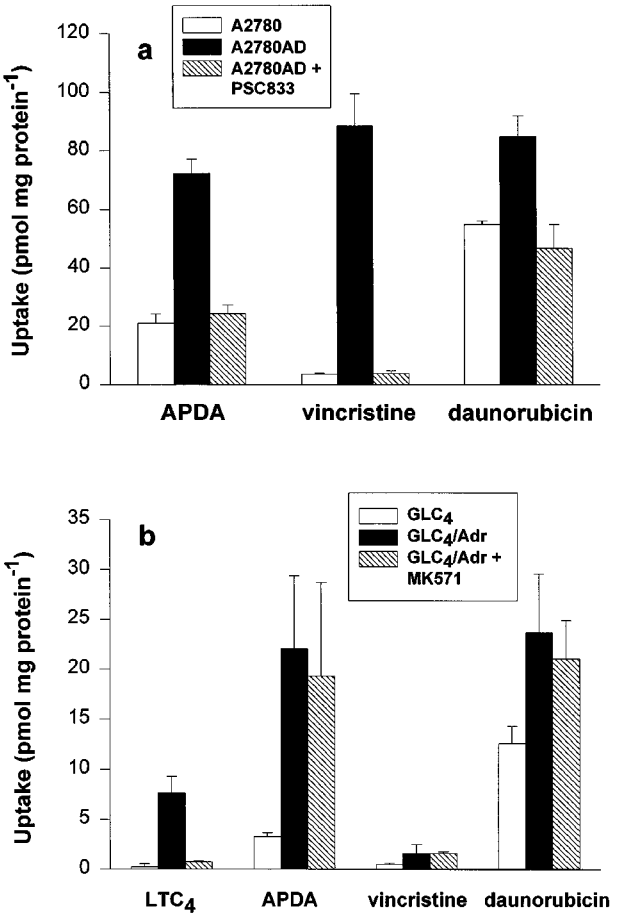

Figure 2 ATP-dependent uptake of cationic drugs into membrane vesicles from MDR1- and MRP1-overexpressing cells. Uptake of [^{3}H]-APDA (300 nM), [^{3}H]-vincristine (300 nM) and [^{3}H]-daunorubicin (300 nM) into membrane vesicles (50 μg) from A2780 and A2780AD cells was measured during 5 min as described under 'experimental procedures'. Uptake into A2780AD membrane vesicles was also measured in the presence of 1 μM of the MDR1 inhibitor PSC833 (a). Similar experiments were performed with membrane vesicles from GLC_4 and from GLC_4/Adr cells either in the absence or the presence of 5 μM of the MRP1 inhibitor MK571 (b). [^{3}H]-LTC_4 was used as control substrate for MRP1-mediated transport. Data shown are means $\pm$s.d. from at least three experiments with at least triplicate determinations.

compared to A2780 membrane vesicles. This uptake was inhibited by the MDR1 inhibitor PSC833. These results indicate that the ATP-dependent transport of [^{3}H]-APDA, [^{3}H]-vincristine and [^{3}H]-daunorubicin into membrane vesicles from A2780AD is mediated by MDR1.

Under the same conditions, transport studies were performed with membrane vesicles from GLC_4 cells and the MRP1-overexpressing GLC_4/Adr cell line (Figure 2b). The cysteinyl-leukotriene LTC_4 was used as a control substrate for MRP1-mediated transport. ATP-dependent transport of [^{3}H]-LTC_4 into membrane vesicles from GLC_4/Adr cells was 16 fold higher than the uptake into GLC_4 membrane vesicles. This was inhibited by the MRP1 inhibitor MK571. In the presence of ATP, uptake of [^{3}H]-APDA and [^{3}H]-daunorubicin into GLC_4/Adr membrane vesicles was significantly higher than into GLC_4 membrane vesicles. [^{3}H]-vincristine uptake was only modestly higher. In contrast to [^{3}H]-LTC_4, uptake of these three cationic compounds into GLC_4/Adr membrane vesicles was not inhibited by MK571. The uptake of [^{3}H]-APDA, [^{3}H]-vincristine and [^{3}H]-daunorubicin into membrane vesicles from S1(MRP) and S1 cells was similar. Furthermore, uptake of all three substrates into S1(MRP) membrane vesicles was not inhibited by MK571 (data not shown). These results indicate that the uptake of [^{3}H]-APDA, [^{3}H]-vincristine and [^{3}H]-daunorubicin *per se* into membrane vesicles from MRP1-overexpressing cells, although ATP-dependent, is not mediated by MRP1.

MRP1-mediated transport of [^{3}H]-vincristine and [^{3}H]-daunorubicin but not of [^{3}H]-APDA in the presence of GSH

Next, we investigated the GSH-dependency of MRP1-mediated transport. The above described non-MRP1-mediated uptake of the monoquaternary cation [^{3}H]-APDA and the weak base [^{3}H]-daunorubicin into GLC_4/Adr membrane vesicles (Figure 2b) might be driven by a proton gradient. To eliminate this uptake we used the vacuolar H^+-ATPase (V-type ATPase) inhibitor bafilomycin A_1 (Baf) to block the potential involvement of proton gradient generating V-type ATPases. Uptake of [^{3}H]-APDA and [^{3}H]-daunorubicin into GLC_4/Adr membrane vesicles in the presence of 1 μM Baf was reduced to 5 and 35% of the control values, respectively, while [^{3}H]-vincristine uptake was only slightly inhibited. In addition, Baf did not alter [^{3}H]-LTC_4 uptake (Table 1). These results indicate that Baf reduces non-MRP1-mediated uptake of cationic compounds into GLC_4/Adr membrane vesicles without affecting the transport activity of MRP1. Therefore, all experiments to investigate the role of GSH in MRP1-mediated cation transport were performed in the presence of 1 μM Baf.

GSH (5 mM) did not affect the uptake [^{3}H]-APDA into GLC_4/Adr membrane vesicles (Figure 3a). However, the uptake of [^{3}H]-vincristine and [^{3}H]-daunorubicin, in the presence of ATP, was stimulated by GSH. This stimulated uptake was inhibited by MK571. With membrane vesicles from S1(MRP) cells, similar results were obtained (Figure 3b). The levels of GSH-mediated uptake of [^{3}H]-vincristine and [^{3}H]-daunorubicin into GLC_4/Adr and into S1(MRP) membrane vesicles correlated with levels of MRP1 protein expression (Figure 1). These data show that MRP1-mediated ATP-dependent transport of daunorubicin appears to be GSH-dependent. To gain more insight into the initial uptake phase, a 2 min time course of [^{3}H]-daunorubicin uptake was measured (Figure 4). Because of a relatively low overexpression of MRP1 in S1(MRP) membrane vesicles, GLC_4/Adr membrane vesicles were used for these experiments. In the presence of 5 mM GSH, the uptake of [^{3}H]-daunorubicin was linear up to 1 min. After 3 min, a steady state uptake of about 30 pmol mg protein^{-1} was reached which did not significantly

Table 1 Uptake of [^{3}H]-LTC_4 and cationic drugs into membrane vesicles from GLC_4/Adr cells in the absence and the presence of Baf

Substrate	*Uptake* (pmol mg protein^{-1}) *Control*	*1 μM Baf*	
[^{3}H]-LTC_4 (1.5 nM)	7.7±1.64	7.8±2.0	ns
[^{3}H]-APDA (300 nM)	22.1±7.34	1.2±1.34	$P<0.001$
[^{3}H]-vincristine (300 nM)	1.6±0.9	0.4±0.02	ns
[^{3}H]-daunorubicin (300 nM)	23.7±5.9	8.4±5.7	$P<0.006$

Uptake of indicated substrates into membrane vesicles from GLC_4/Adr cells (50 μg) were measured for 5 min in the absence or the presence of 1 μM Baf. Data represent mean±s.d. of three independent experiments with quadruplicate determinations. Data were compared by an unpaired two-tailed Student's *t*-test, differences were considered to be significant when $P<0.05$; ns, not significant.

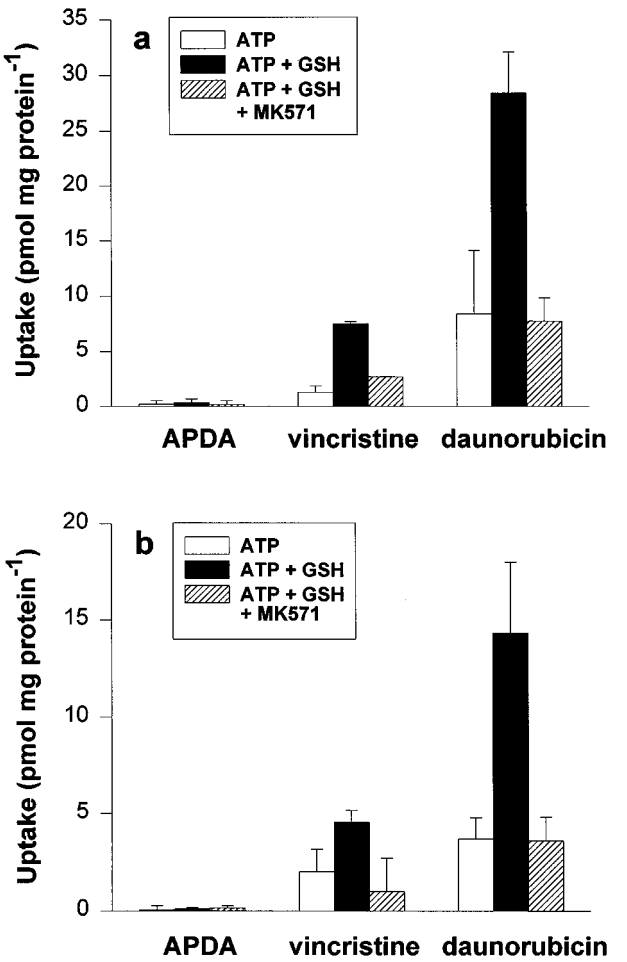

Figure 3 ATP-dependent uptake of cationic drugs in the presence of GSH. (a) represents uptake during 5 min of [^{3}H]-APDA (300 nM), [^{3}H]-vincristine (300 nM) and [^{3}H]-daunorubicin (600 nM) into membrane vesicles prepared from GLC_4/Adr cells in the absence and the presence of 5 mM GSH. Uptake was also measured in the presence of 5 μM MK571. (b) represents the same experiments with *MRP1*-transfected S1 (MRP) cells. All experiments were performed in the presence of 1 μM Baf. Data represent mean±s.d. from at least two experiments with quadruplicate determinations.

change during the next 7 min (data not shown). The non-hydrolyzable ATP analogue AMP-PCP did not stimulate uptake, indicating that GSH-dependent uptake of [^{3}H]-daunorubicin requires ATP hydrolysis. MK571 (2 μM) completely inhibited the GSH-dependent [^{3}H]-daunorubicin uptake.

Inhibition of [^{3}H]-vincristine, [^{3}H]-daunorubicin and [^{3}H]-LTC$_4$ transport with the monoclonal antibody (mAb) QCRL-3

To demonstrate that transport of [^{3}H]-daunorubicin and [^{3}H]-vincristine is specifically mediated by MRP1, uptake of both cations into GLC$_4$/Adr membrane vesicles was measured in the presence of the mAb QCRL-3 which recognizes a conformation dependent MRP1-epitope (Hipfner *et al.*, 1994). [^{3}H]-LTC$_4$ was used as positive control for QCRL-3 inhibition. Results are presented in Figure 5. Transport of all three substrates was inhibited by QCRL-3 with IC$_{50}$'s of 2, 20 and 12 μg ml^{-1} (0.23, 2.3 and 1.4 μg mAb per 50 μg protein) respectively for [^{3}H]-daunorubicin, [^{3}H]-vincristine and [^{3}H]-LTC$_4$. A control mAb directed against the T-cell marker CD3 with an identical isotype as QCRL-3 did not affect [^{3}H]-LTC$_4$ transport and only slightly inhibited transport of [^{3}H]-daunorubicin and [^{3}H]-vincristine (less than 15% at the highest QCRL-3 concentration, data not shown).

Dependency of MRP1-mediated daunorubicin transport on GSH concentration

The effect of different GSH concentrations on MRP1-mediated daunorubicin transport was investigated with GLC$_4$/Adr membrane vesicles. Figure 6 shows that [^{3}H]-daunorubicin transport was stimulated by increased concentrations of GSH with a maximum of 10 mM after which a steady state level was reached. The K_M value was determined to be 2.7 mM.

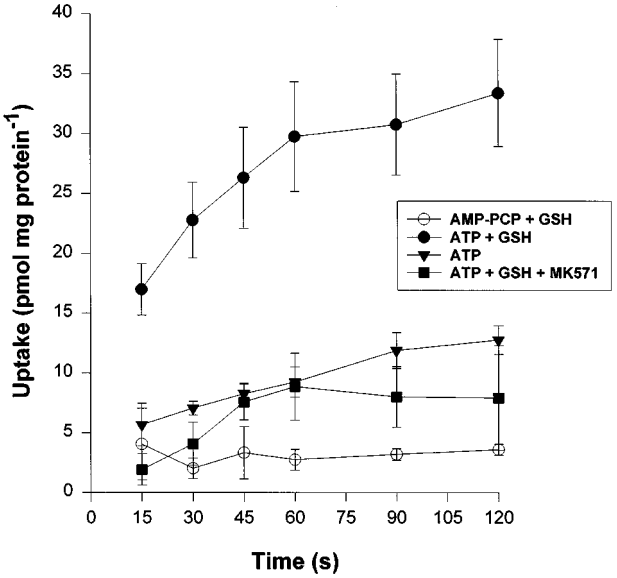

Figure 4 Time course of MRP1-mediated daunorubicin transport. Initial uptake of [^{3}H]-daunorubicin (600 nM) into GLC$_4$/Adr membrane vesicles (100 μg protein, 220 μl final volume) was followed during 2 min with different conditions. All experiments were performed in the presence of 1 μM Baf. GSH and MK571 were added to a final concentration of 5 mM and 2 μM, respectively. Data points are means$\pm$s.d. from at least four independent experiments with quadruplicate measurements. Experiments were performed with two different batches of membrane vesicles.

Determination of IC$_{50}$ value of MK571 for MRP1-mediated daunorubicin transport

The inhibitory effect of MK571 on MRP1-mediated daunorubicin transport was measured in the presence of 10 mM GSH. MK571 effectively inhibited GSH-stimulated [^{3}H]-daunorubicin transport in a dose-dependent manner with an IC$_{50}$ of 0.4 μM (Figure 7).

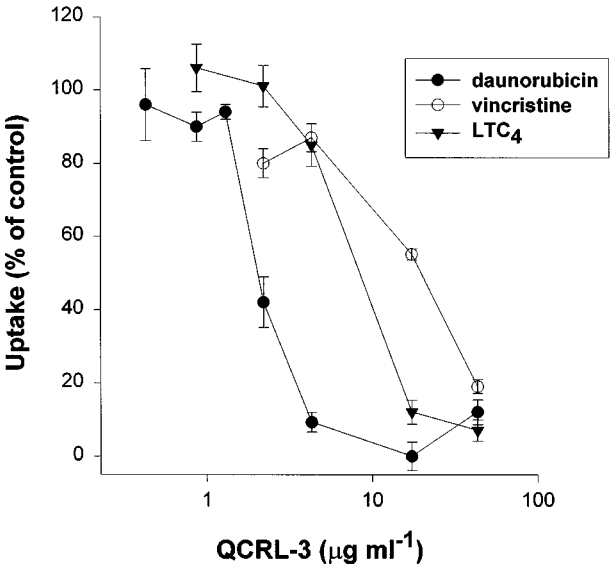

Figure 5 Inhibition of MRP1-mediated transport by the mAb QCRL-3. Uptake of [^{3}H]-daunorubicin (600 nM), [^{3}H]-vincristine (300 nM) into GLC$_4$/Adr membrane vesicles (50 μg) was measured for 1 min in the presence of 1 μM Baf, 5 mM GSH and increasing concentrations of the MRP1 specific mAb QCRL-3. [^{3}H]-LTC$_4$ (1.5 nM) was used as control for MRP1 inhibition by QCRL-3. Data are plotted as percentages of control. Uptake rates of control experiments were 19, 4.3 and 3.9 pmol mg protein^{-1} min^{-1}, respectively, for [^{3}H]-daunorubicin, [^{3}H]-vincristine and [^{3}H]-LTC$_4$. Data points represent means$\pm$s.e.mean of triplicate measurements in a single experiment.

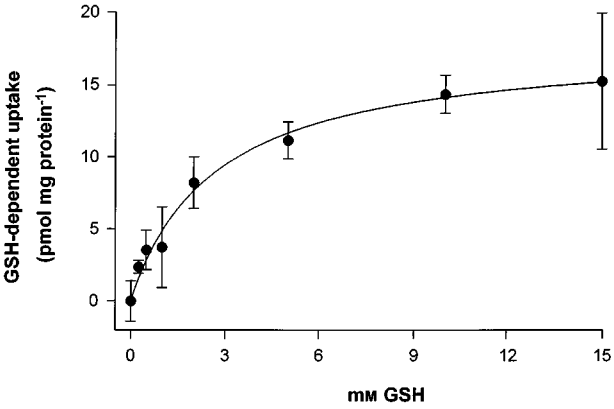

Figure 6 Dependency of MRP1-mediated daunorubicin transport on the GSH concentration. Uptake of [^{3}H]-daunorubicin into GLC$_4$/Adr membrane vesicles was measured for 1 min as described at Figure 5 in the presence of different GSH concentrations. Data points represent the difference between ATP-dependent uptake and ATP-dependent uptake in the presence of GSH. Data points are means$\pm$s.e.mean of at least triplicate measurements in a single experiment. Two additional experiments showed the same results. Curve fitting and calculation of the K_M value was performed with the Graphpad Prism^{TM} program. The initial ATP-dependent uptake of daunorubicin in the absence of GSH was 9.8$\pm$1.4 pmol mg protein^{-1}.

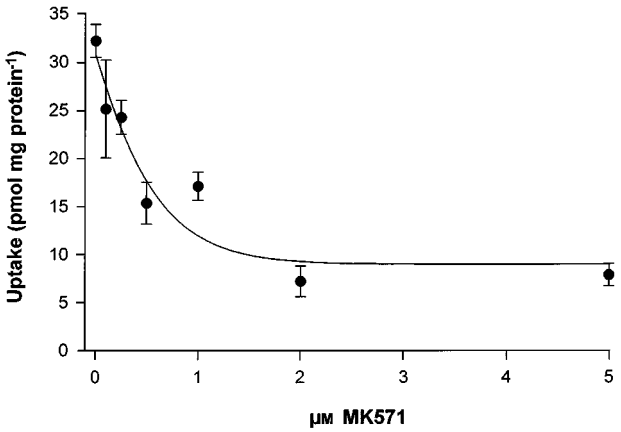

Figure 7 Determination of IC_{50} for MK571 of GSH-dependent daunorubicin transport by MRPI. Uptake of [^{3}H]-daunorubicin into GLC_4/Adr membrane vesicles was measured in the presence of 10 mM GSH with different concentrations of MK571 as described at Figure 5. Data points represent means$\pm$s.e.mean of triplicate measurements in a single experiment. The experiment was performed twice with similar results. Curve fitting and calculation of the IC_{50} value was performed with the Graphpad Prism^{TM} program.

Discussion

MDR1 and MRP1, two distantly related members of the large superfamily of ABC-transporter proteins, are both able to confer resistance of cells against a broad spectrum of natural product drugs (Loe *et al*., 1996c; Broxterman *et al*., 1995; Gottesman & Pastan, 1993). MDR1-related multidrug resistance is most likely due to the ATP-dependent extrusion of unmodified drugs from the cells mediated by the MDR1 protein (Dong *et al*., 1996; Urbatsch *et al*., 1995; Sharom *et al*., 1995; Shapiro & Ling, 1994; Ruetz & Gros, 1994b; Horio *et al*., 1988). Also MRP1-mediated multidrug resistance depends on the transport capacity of MRP1, but the mechanism by which this protein mediates drug transport appears to differ from MDR1. Most MRP1 substrates are organic compounds that need to be conjugated with e.g. GSH before being transported. Also some organic cationic drugs appear to be MRP1 substrates. Although these substrates are not known to form GS *S*-conjugates, it has been shown that the presence of GSH is required for MRP1-associated resistance against the cytotoxicity of these drugs (Versantvoort *et al*., 1995; Zaman *et al*., 1995). This suggests that for MRP1-mediated extrusion of these cations, GSH is required without a need for conjugation. This prompted us to investigate the role of GSH in MRP1-mediated organic cation transport.

We have used three cationic substrates: two more hydrophobic basic drugs [^{3}H]-vincristine and [^{3}H]-daunorubicin and the permanently positively charged organic cation [^{3}H]-APDA. These drugs are substrates for MDR1 as demonstrated by us in this study and by others previously (Müller *et al*., 1994a; Tamai & Safa, 1990; Kamimoto *et al*., 1989). To test the ability of MRP1 to transport these substrates *in vitro* we have used membrane vesicles prepared from the GLC_4/Adr cell line. This cell line was used for two reasons. First, GLC_4/Adr exhibits a very high overexpression of MRP1. This high level could not be found in *MRP1*-transfected cell lines such as S1(MRP) or even not in *Spodoptera frugiperda* or *Trichoplusia ni* (High Five^{TM}) insect cells, overexpressing MRP1 by using the baculovirus expression system (Renes *et al*., unpublished data). Furthermore, it has been demonstrated recently that GLC_4/Adr cells exclusively overexpress *MRP1* but not the isoforms *MRP2*, *MRP3*, *MRP4* and *MRP5* (Kool *et al*., 1997).

We showed that [^{3}H]-APDA and [^{3}H]-daunorubicin, and to a much lower extent [^{3}H]-vincristine, were taken up in membrane vesicles from GLC_4/Adr cells in the presence of ATP but not in the presence of AMP-PCP. However, this uptake was not inhibited by the leukotriene D_4 receptor antagonist and MRP1-inhibitor MK571 (Gekeler *et al*., 1995; Jones *et al*., 1989). This is a clear indication that MRP1, in contrast to MDR1, is not directly involved in ATP-dependent transport of these cationic substrates. We have recently demonstrated that GLC_4/Adr cells have a more pronounced Golgi apparatus and contain more intracellular vesicular structures than GLC_4 cells (van Luyn *et al*., 1998). We therefore speculated that addition of ATP might activate V-type ATPases that are present in membranes of certain intracellular organelles. These V-type ATPases generate a proton-gradient by hydrolyzing ATP and are involved in acidification of intracellular compartments of the endocytotic and exocytotic pathway (for review see Gluck *et al*., 1996; Mellman *et al*., 1986). The crude membrane preparations we used contain plasma membranes as well as intracellular organelles, including acidic vesicular structures which could be responsible for the MRP1-independent uptake of cations. The macrolide antibiotic bafilomycin A_1 (Baf) is known to function as a specific inhibitor for V-type ATPases (Bowman *et al*., 1988). Addition of Baf blocked ATP-stimulated uptake of the cations into GLC_4/Adr membrane vesicles but it did not influence MRP1-mediated transport of [^{3}H]-LTC_4. Therefore, Baf was used in all further experiments.

Several reports have suggested an important role of GSH in MRP1-mediated drug efflux from cells with a multidrug resistant phenotype (O'Brien & Tew, 1996 and references therein). Experiments with MRP1-transfected cells showed that depletion of GSH results in a complete reversal of resistance to vinca alkaloids and anthracyclines (Zaman *et al*., 1995). Furthermore, MRP1 mediates vincristine transport only in the presence of GSH (Loe *et al*., 1996b, this study). Reducing agents such as 2-mercaptoethanol, dithiothreitol and L-cysteine, have been tested but none of these could increase uptake of [^{3}H]-vincristine in membrane vesicles from MRP1 transfected cells (Loe *et al*., 1996b) indicating that it is not the reducing capacity of GSH that is responsible for this effect. GSH itself seems to play an essential role in MRP1-mediated transport of hydrophobic drugs and our findings support this. In this study we show that in the presence of physiological concentrations of GSH, the hydrophobic basic drug daunorubicin is transported by MRP1 in an ATP-dependent manner. The rate of MRP1-mediated daunorubicin transport is dependent on the concentration of GSH. This implies that changes in the intracellular GSH concentration will have a marked effect on the MRP1-mediated daunorubicin transport from drug resistant cells.

The specificity of MRP1-mediated cation transport, in particular of daunorubicin, was demonstrated by the MRP1-specific mAb QCRL-3 (Loe *et al*., 1996b; 1997) and by MK571 inhibition. QCRL-3 inhibited MRP1-mediated transport of daunorubicin in the presence of GSH better than LTC_4 transport. One explanation for this effect may be a difference in binding sites for hydrophobic cations and for anions at MRP1. The competitive inhibitor MK571 inhibited MRP1-mediated GSH-dependent daunorubicin transport in a dose-dependent manner with an IC_{50} value of 0.4 μM. This is similar to what has been reported for LTC_4-inhibition (Leier *et al*., 1994). The difference between MRP1 inhibition by QCRL-3

and by MK571 can be explained by the difference in binding of each of these compounds to MRP1.

The mechanism by which GSH facilitates MRP1-mediated transport of hydrophobic cationic drugs has not yet been fully elucidated. There is no evidence for conjugation of GSH to drugs to which MRP1 confers resistance (O'Brien & Tew, 1996; Zaman *et al*., 1995). There are indications that MRP1 mediates GSH-transport (Rappa *et al*., 1997; Zaman *et al*., 1995), and may function as a co-transporter for GSH and the drug. Thus GSH may be a low affinity substrate for MRP1. From experiments using the vanadate-trapping technique it has been suggested that GSH as well as anticancer drugs directly interact with MRP1 (Taguchi *et al*., 1997). Transport of anionic MRP1-substrates such as GSH- and glucuronide-conjugates are inhibited by hydrophobic (cationic) vinca alkaloids (Loe *et al*., 1996b; Müller *et al*., 1994b) and anthracyclines (Loe *et al*., 1996b). One hypothesis explaining these results is that MRP1 may contain two binding sites: one for hydrophobic compounds and one for hydrophilic compounds. This would allow a similar binding of GSH and the hydrophobic drug as well as binding of hydrophobic compounds conjugated to GSH, glucuronate or sulphate. Further studies are needed to address this important issue.

In conclusion, we showed that in addition to vincristine MRP1-mediated transport of the unmodified anticancer drug daunorubicin is dependent on GSH. APDA is not a substrate for MRP1.

This study is supported by grant RUG 95-1007 from the Dutch Cancer Society. Hans Koning (University Hospital Groningen) is acknowledged for technical assistance. We thank Dr A.W. Ford-Hutchinson for providing MK571 and Dr G. Kurz for the gift of [^{3}H]-APDA. In addition we thank Drs R. Scheper, R. Oude Elferink and B.J. Kroesen for providing monoclonal antibodies and Dr H.W. van Veen (Dept. Microbiology, University of Groningen) for critical comments on the manuscript.

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(Received August 10, 1998
Revised November 11, 1998
Accepted November 13, 1998)