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Transport properties of the multidrug resistance-associated protein (MRP) in human tumour cells

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Abstract In this paper we demonstrate that the expression of the multidrug resistance-associated protein (MRP) in a variety of intact human tumour cells results in the ATP-dependent, mutually exclusive extrusion of both the acetoxymethyl ester and the free anion forms of the fluorescent dye calcein, as well as that of a fluorescent pyrenemaleimide–glutathione conjugate. The MRP-dependent transport of all these three model compounds closely correlates with the expression level of MRP and is cross-inhibited by hydrophobic anticancer drugs, by reversing agents for MDR1, and also by compounds not influencing MDR1, such as hydrophobic anions, alkylating agents, and inhibitors of organic anion transporters. Cellular glutathione depletion affects neither the MRP-dependent extrusion of calcein AM or free calcein, nor its modulation by most hydrophobic or anionic compounds, although eliminating the cross-inhibitory effect of glutathione conjugates. These results suggest that the outward pumping of both hydrophobic uncharged and water-soluble anionic compounds, including glutathione conjugates, is an inherent property of MRP, and offer sensitive methods for the functional diagnostics of this transport protein as well as for the rapid screening of drug-resistance modulating agents.

Key words: Multidrug resistance-associated protein; Multidrug-resistance protein; P-glycoprotein; Drug resistance; Calcein; Fluorometry; Immunoblotting; Transport assay

1. Introduction

A large number of clinically observed resistance of cancers to chemically unrelated cytotoxic compounds is caused by the overexpression of multidrug transporter proteins in the tumour cell membranes. The two major multidrug transporter gene products identified are MDR1 (P-glycoprotein, P-170), and MRP (multidrug resistance-associated protein, P-190), which are both members of the ATP binding cassette (ABC) transporter family, and perform an ATP-dependent extrusion of various cytotoxic agents. Many of the transported compounds are of natural origin, and in the case of MDR1 they are relatively large, hydrophobic, either uncharged or weakly basic molecules [1–4].

MRP has recently been cloned [4] and its function as a drug-extrusion pump established [5,6], but the nature of the transported drugs and other chemicals is still controversial. According to several reports, MRP effectively pumps out most substrates of MDR1, but may also interact with a wider range of chemicals, and seems to be specifically modulated by genistein and probenecid [6–14]. MRP was shown to bind and transport glutathione S (GS) conjugates, such as leukotriene C_4 [8–10], and other large anionic substances ([15]; P. Twentyman, personal communication), thus raising the possible similarity or identity of MRP and the multispecific organic anion transporter (MOAT; [16]). Moreover, a recent communication [17] suggested that the key mechanism of the cytotoxic drug resistance induced by MRP is the extrusion of intracellularly formed drug–GS conjugates.

Earlier we have shown [18] that hydrophobic acetoxymethyl ester (AM) derivatives, but not the free acid forms of various fluorescent indicators, are actively extruded by the P-glycoprotein (MDR1), and that the measurement of the cellular accumulation of such an indicator, e.g. calcein, can be effectively utilised for the diagnostics and the transport characterization of MDR1 [19,20]. Moreover, the rapid extrusion of calcein AM before its cleavage by cellular esterases suggested a drug-transport mechanism by MDR1 directly from the membrane phase. The experiments described in this paper demonstrate that calcein AM is also extruded by the human MRP, although in this case the hydrophilic free calcein anion, and an intracellularly formed fluorescent GS-pyrenemaleimide complex can also be exported. The studies presented here on the cross-inhibition of the transported compounds, as well as the effects of different drugs and of cellular glutathione depletion on the MRP-dependent transport, may significantly help our understanding of the molecular mechanism of this clinically important transport pathway.

2. Materials and methods

2.1. Cell culturing

Cell culturing was performed under standard conditions in RPMI or DMEM media, containing 10% fetal calf serum, 5 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. S1 (SW1573 cells), and S1MRP (MRP-transfected S1 cells re-selected and cloned in ADR-containing media), were originally prepared as described in [6]. GLC4, and GLC4 ADR (GLC4 cells selected in ADR and regularly re-selected with 1 μM ADR containing media) were described in [21]. These cell lines were kind gifts of Drs. P. Borst, A. Schinkel and G. Zaman, while HL60 ADR (ADR-selected HL60 cells) were gifts of Dr. M. Center (see [22]). K562 MDR1 (selected in ADR and regularly re-selected in 50 ng/ml ADR), were prepared in our laboratory. KB3 and KB-V1 (containing MDR1 and grown in media containing 50–500 ng/ml vincristine) human epidermoid carcinoma cells, NIH 3T3 fibroblasts and their human MDR1-transfected counterpart (NIH 3T3 MDR1 G185, see [23]) were kind gifts of Dr. M. M. Gottesman. Drug resistance was determined by cell counting after a

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Abbreviations: ADR, adriamycin (doxorubicin); AM, acetoxymethylester; CCCP, chlorocarbonyl cyanide phenylhydrazone; 2,4-DNP, 2,4-dinitrophenol; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GSH, reduced glutathione; MDR1, multidrug-resistance protein; MRP, multidrug resistance-associated protein; NEM, *N*-ethylmaleimide; NPM, *N*-pyrenemaleimide; PGA1, prostaglandin A_1; PVDF, polyvinylidene difluoride; VBL, vinblastine.

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72 h incubation of the cells in 24-well plates in the respective culture media, supplemented with various concentrations of adriamycin. Adherent cells (NIH 3T3, S1, GLC4, KB, and KBV1 control and drug-resistant counterparts) were detached by mild trypsinization (5 min, 1 mg/ml trypsin), then washed and resuspended in HPMI medium.

2.2. Calcein fluorescence

Calcein fluorescence was measured by incubating $2–5\times10^5$ cells/ml in HPMI medium [18,24] containing 0.25 μM calcein AM (Molecular Probes, Eugene, OR, USA), at 37°C with gentle stirring in a Hitachi F-4000 fluorescence spectrophotometer (excitation and emission wavelengths for calcein were 493 and 515 nm, respectively, with a band width of 5 nm). No self-quenching of free calcein was observed under the experimental conditions used in the present experiments (short incubation periods and low concentrations of calcein AM in the incubation medium). The multidrug-resistance activity factor (MAF, see [20]) was calculated according to the equation:

$$MAF = (F^*-F)/F^* \quad (1)$$

where F^* and F designate the dye accumulation rate in the presence and absence of an inhibitor of the multidrug transporter, respectively.

2.3. Inhibition of MRP-, or MDR1-dependent calcein AM extrusion

Inhibition of MRP-, or MDR1-dependent calcein AM extrusion by various compounds was determined by measuring the rate of increase in fluorescence of free calcein for 5 min periods, and the IC_{50} values were obtained by estimating the drug concentrations resulting in 50% of the maximum transport inhibition. Maximum inhibition was achieved in each experiment by the addition of 100 μM verapamil or 100 μM vinblastine, and the 100% range was calculated by subtracting the rate of fluorescence increase without any inhibitor from that at maximum inhibition. In each case the values represent the range obtained in at least 3 independent experiments with each cell line.

2.4. ATP depletion

ATP depletion was achieved by 1 h incubation of the cells at 37°C in HPMI media containing no glucose but 5 mM 2-deoxyglucose and 5 mM NaN_3, producing cellular ATP levels below 10% of the original [15].

2.5. GSH depletion

GSH depletion was achieved by 24 h preincubation of the cells in the culture media supplemented with 50 μM butathione sulfoximine (BSO), or with a 5 min preincubation with 50 μM diamide, followed by a washing in HPMI medium. Cellular GSH levels were examined by NPM fluorescence measurements (see below), and in both methods the non-protein SH levels were reduced to less than 10% of the original (see also [12–14]).

2.6. Quantitative immunoblotting

Quantitative immunoblotting was performed by washing the cultured cells twice in a protein-free (HPMI) medium followed by dissolution and sonication in a disaggregation buffer [24]. Detection of human MRP with the R1 (rat) monoclonal anti-MRP antibody, kindly provided by Dr. R. Schaper, was carried out as described in [25], while detection of MDR1 was performed with the 4077 anti-MDR1 polyclonal antibody on the same blots [18]. The second antibodies were obtained from Boehringer (anti-rat, peroxidase conjugated IgG, 1000× dilution), and from Jackson Immunoresearch (anti-rabbit, peroxidase conjugated goat IgG, 20000× dilution). HRP-dependent luminescence on the blots (ECL, Amersham) was determined by autoradiography and by excising the respective bands from the PVDF membrane and measuring their luminescence in a liquid scintillation counter (Beckman LS 6000, Single Photon Monitor mode).

2.7. NPM labelling

NPM labelling of the cells was performed by incubating 5×10^6 cells in 1 ml HPMI medium in the presence of 0.5 μM NPM for 5 min at 4°C. The cell suspension was spun in an Eppendorf microcentrifuge (20 s, 10000×*g*) and the pellet rapidly diluted in 5 ml of HPMI, prewarmed to 37°C. The concentration of unconjugated NPM (the fluorescence of which is less than 10% of the S-conjugated molecule) after the loading procedure was determined by the addition of free glutathione to the loading medium, and was found to be negligible. Non-protein SH was determined by NPM titration of the supernatant of trichloroacetic acid-precipitated cells.

2.8. NPM-S conjugate efflux measurements

In the NPM-S conjugate efflux measurements, samples of the cell suspension, taken at the times indicated, were rapidly spun as above, and the supernatants were collected. The amount of total releasable NPM-S conjugate was determined by the addition of 1 mg/ml digitonin to the cell suspension and by preparing a cell-free supernatant by rapid centrifugation, as described above (this was not significantly different from the amount of non-protein NPM-S conjugate, determined after trichloroacetic acid precipitation of the cells). The fluorescence of NPM-S conjugate was determined at excitation and emission wavelengths of 337 and 375 nm, respectively, with a band width of 20 nm, in samples diluted 5 times with distilled water. The above fluorescence measurement procedure minimised the possible effects of energy transfer due to the changing environment of the fluorophore.

3. Results and discussion

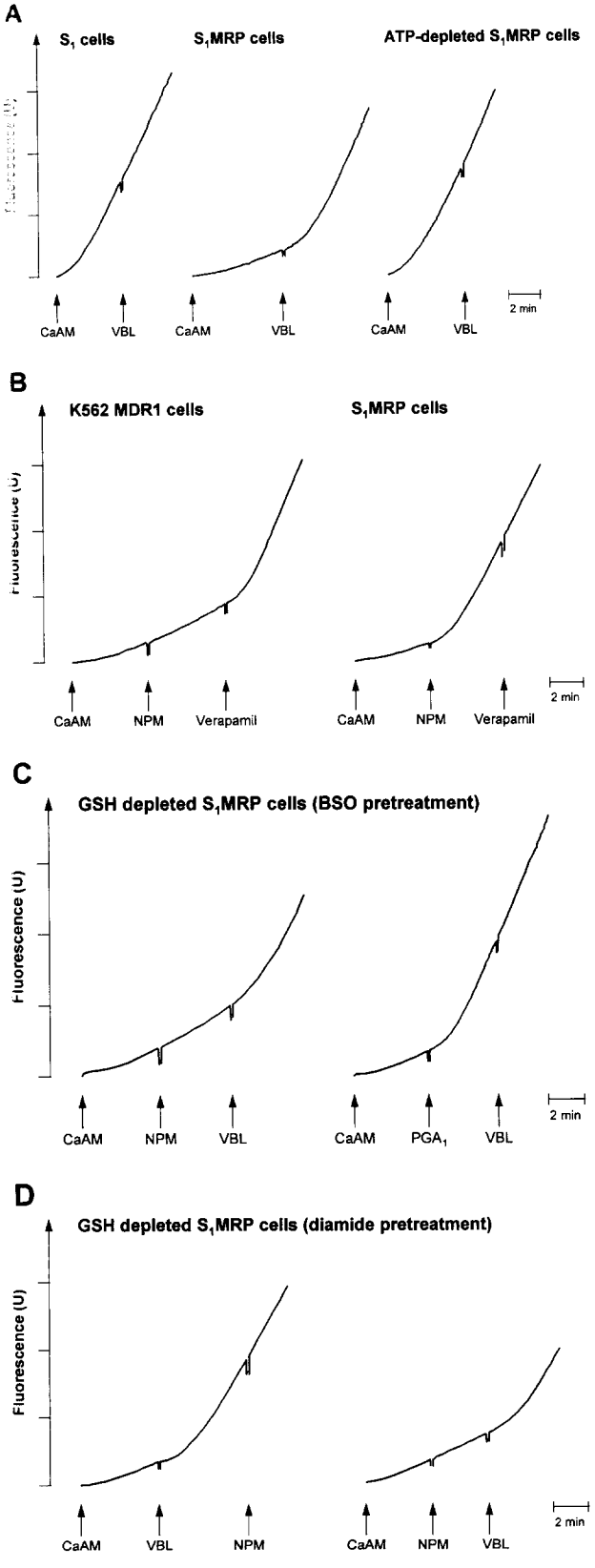
Calcein AM is non-fluorescent, and the fluorescence of free calcein, which is produced by esterases and then trapped in the cytoplasm of most living cells, is essentially insensitive to changes of pH, calcium, or magnesium concentrations [26]. As shown in Fig. 1A, when tumour cells were incubated in the presence of low concentrations (e.g. 0.25 μM) of calcein AM, and the increase of free calcein concentration was followed by fluorometry in a stirred cuvette at 37°C, this increase was much slower in MRP-transfected SW1573/S1 lung cancer cells

Table 1
Inhibitors of the MRP-and/or MDR1-mediated calcein AM extrusion

Compound	IC_{50} (μM)	
	MRP	MDR1
Vinblastine	2–5	2–3
Tamoxifen	3–6	2–5
Cyclosporine A	2–4	0.5–1
Verapamil	4–8	2–5
Econazole	3–10	1–3
Quinine	50–100	20–30
Oligomycin	1–2	1–2
CCCP	10–30	>200[a]
2,4-DNP	30–50	>200[a]
Probenecid	500–800	>2000[a]
Sulfinpyrazone	300–500	>2000[a]
Benzbromarone	5–10	>500[a,b]
Indomethacin	10–20	>800[a,b]
Na-cholate	200–300	>1000[b]
Bromosulfophthalein	100–150	>1000[b]
Ethacrynic acid	20–30	>800[b]
Merthiolate	10–20	>200[b]
NEM	0.2–0.5	>50[b]
NPM	0.1–0.2	>20[b]
Genistein	150–200[b]	>1000[a,b]
Chlorambucil	30–50	>500[b]
Prostaglandin A_1	3–5	>100[a]

Drug effects on MRP were estimated from calcein accumulation measurements in S1MRP and in HL60 ADR cells, while the effects on MDR1 were deduced from similar measurements in 3T3 MDR and K562 MDR cells. In each case the values represent the range obtained in at least 3 independent experiments with each cell line. > indicates that 50% inhibition could not be achieved in the measurable concentration range, and either a solubility problem ([a]), or a strong esterase inhibition ([b]) was experienced at higher concentrations.

than in untransfected control cells. Since in the MRP-transfected cells the rate of calcein production was greatly increased (up to the rates seen in the control cells) by ATP depletion, or by the addition of vinblastine or verapamil, the slower calcein production was not due to reduced cellular esterase activity, but to active, MRP-dependent elimination of calcein AM from tumour cells. A similar strong reduction of free calcein production, and its reversal by ATP depletion, verapamil or vinblastine was observed in HL60 ADR and in GLC4 ADR multidrug-resistant tumour cells, all of which express MRP but not MDR1 (see below).



In these fluorometry experiments the conversion of calcein AM to free calcein was measured, thus MRP most probably extrudes the hydrophobic calcein AM before its esterase cleavage. These findings indicate a similar drug transport mechanism to that seen in the case of MDR1 [18]. In order to clarify the possible role of MDR1 in the observed phenomena, the cell extracts were subjected to immunoblotting with specific monoclonal antibodies. As shown in Fig. 2, MRP-expressing cell lines had no detectable levels of MDR1, and MDR1-expressing cells did not show increased MRP expression (a very low level of MRP could be detected even in most of the parental cell lines as well). Moreover, the differences in calcein accumulation rates in the absence and presence of vinblastine, respectively (shown as the multidrug-resistance activity factor, MAF), as well as the drug resistance of these different cell lines, closely correlated with the relative amounts of the respective multidrug-resistance proteins (Fig. 2).

In the following experiments we have compared the modulation of the MRP-dependent and MDR1-dependent calcein AM extrusion, respectively, by several pharmacologically active compounds. Acceleration of cellular calcein production to the control level, i.e. inhibition of calcein AM extrusion, was produced in the S1MRP and HL60 ADR cells by several unrelated compounds, acting with the respective apparent IC_{50} values as listed in Table 1. The inhibitory effects of all these compounds on calcein extrusion were found to be similar in GLC4 ADR cells as well, although in this case the respective IC_{50} values were generally about 50% higher than in the two other MRP-expressing cell types. As shown in Table 1, some of these compounds (e.g. vinblastine, tamoxifen, verapamil, cyclosporin A, quinine, or econazole) inhibited calcein AM extrusion from MRP or MDR1-expressing cells with almost the same efficiency. However, several molecules, such as the hydrophobic anions CCCP, 2,4-DNP; weak acids modulating uric acid or bile acid transport (e.g. probenecid, sulfinpyrazone, benzbromarone, bromosulfophthalein, ethacrynic acid, indomethacin, cholic acid); alkylating agents (e.g. chlorambucil), cell-permeant mercury or maleimide derivatives (e.g. merthiolate, NEM and NPM; see Fig. 1B), as well as prostaglandin A_1, but not E_1 or $F_{2\alpha}$, selectively inhibited MRP-dependent dye extrusion. Although some of these compounds during longer incubations may induce cellular ATP depletion or a non-specific increase in membrane permeability, during the measurement periods of 10–15 min the applied concentrations had no such effects, as shown by

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Fig. 1. Production of free calcein in drug-sensitive and drug-resistant cells: effects of glutathione depletion. Drug-sensitive (S1) and drug-resistant (S1MRP and K562 MDR1) cells were incubated in the presence of 0.25 μM calcein-AM, and at the times indicated by the arrows the respective agents were added to the media. The plots show calcein fluorescence (in arbitrary units) against time. (A) S1, S1MRP and ATP-depleted S1MRP cells; (B) K562 MDR1 and S1MRP cells; (C) S1MRP cells depleted from GSH by 24 h BSO pretreatment; (D) S1MRP cells depleted from GSH by pretreatment with diamide. Additions: CaAM = 0.25 μM calcein AM; VBL = 10 μM vinblastine, verapamil = 100 μM; NPM = 1 μM *N*-pyrenemaleimide.

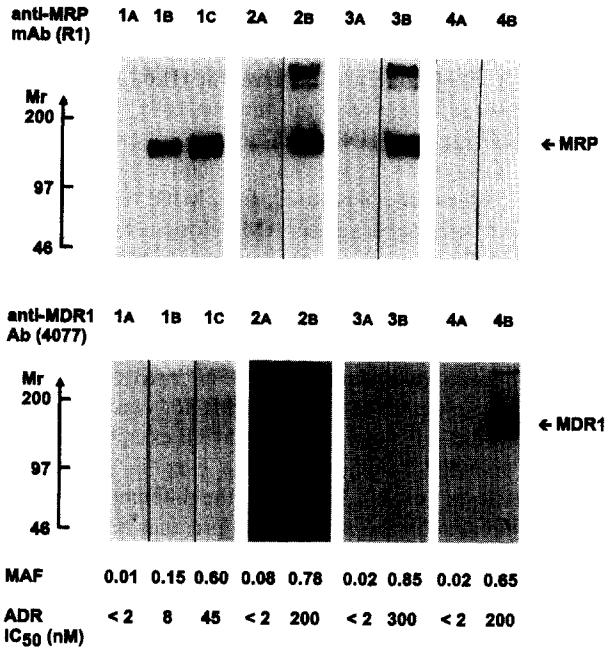

Fig. 2. Estimation of MRP and MDR1 expression by immunoblotting in various cell lines, and characteristics of their calcein extrusion rate and ADR resistance. (A) Immunoblot detection of human MRP by the R1 anti-MRP monoclonal antibody; (B) detection of MDR1 protein by the polyclonal antibody, 4077. Each lane contained 20 μg cellular protein. Lanes: 1A, S1 control cells; 1B, S1MRP cells; 1C, S1MRP cells with ADR selection; 2A, HL60 control cells; 2B, HL60 ADR cells; 3A, GLC4 control cells; 3B, GLC4 ADR cells; 4A, K562 control cells; 4B, K562 MDR1 cells. For the calculation of the multidrug-transporter activity factor (MAF), and the measurement of adriamycin resistance see section 2.

the lack of modulation of calcein AM extrusion in MDR1-expressing cells (Fig. 1B).

Since the formation of drug-GS conjugates was suggested as the key mechanism for MRP-dependent drug extrusion [11–15,27], in the following experiments we have examined the effects of cellular glutathione (GSH) depletion on MRP-dependent calcein AM efflux. In S1MRP or HL60 ADR cells GSH depletion, achieved either in a 24 h preincubation with 50 μM BSO (a specific inhibitor of glutathione synthetase, see [11–15,27]), as shown in Fig. 1C, or in a 5 min preincubation with 50 μM diamide (a selective glutathione oxidising agent; see [28]), followed by washing of the cells in a medium without amino acids (Fig. 1D), did not significantly affect calcein AM extrusion, or its inhibition by vinblastine. This was true for many other agents, e.g. verapamil, cyclosporine A, PGA1 (see Fig. 1C), or probenecid. In contrast, the inhibition of the MRP-dependent calcein AM extrusion by micromolar concentrations of the hydrophobic SH-reactive agents, *N*-ethylmaleimide (NEM) and *N*-pyrenemaleimide (NPM; see Table 1) was eliminated by GSH depletion (Fig. 1C,D). It should be mentioned that the addition of 50 μM BSO or diamide to the calcein AM uptake assay induced a slight increase in free calcein production, thus these compounds may (partially) directly inhibit MRP-dependent drug transport.

In the calcein AM transport experiments we noted that NEM or NPM inhibition of free calcein formation was fully reversible upon 5–10 min incubation of the cells at 37°C, in spite of the irreversible reaction of these agents with SH groups. The S-conjugates of NPM show a characteristic fluorescence emission, thus the complex formation and transport of this molecule in the tumour cells could be directly examined. The NPM fluorescence measurements (see section 2) showed that this maleimide derivative, when added in submicromolar concentrations to the medium, rapidly diffused into the cells and formed predominantly non-protein S-, thus mostly GS-conjugates. As shown in Fig. 3 for HL60 cells, in contrast to the parental non-resistant cells, MRP-containing tumour cells exhibited rapid extrusion of the intracellularly formed, water-soluble GS-NPM complex, and this extrusion was inhibited by low concentrations of calcein AM, vinblastine, or PGA1. The same results were obtained with S1 and S1MRP cells, respectively, i.e. rapid GS-NPM efflux was only observed in the MRP-expressing cell, while the MDR1-expressing K562 cells did not show such GS-NPM extrusion. In both HL60 MRP and S1MRP cells the rapid phase of GS-NPM efflux was inhibited by verapamil, quinine, or probenecid, and also eliminated by ATP depletion (data not shown).

The above experiments indicate that intracellular GS conjugates are transported substrates of MRP, although, as shown above, the interaction of MRP with several transported molecules does not seem to involve GS-conjugate formation. Still, GS conjugates inhibit calcein AM extrusion and calcein AM inhibits GS-NPM conjugate transport, while both of these transports are similarly affected by all the different MRP-modulating compounds.

In most living cells, including MDR1-expressing tumour cells, free calcein is trapped and is not significantly released [18,26]. However, a recent report indicated relatively rapid, ATP-dependent efflux of the accumulated free calcein from MRP-expressing cells [15]. Indeed, when measuring the efflux of free calcein from the S1 MRP and HL60 ADR cells, in contrast to that seen in the MDR1-expressing fibroblasts or K562 tumour cells, we also observed increased calcein efflux, blocked by low concentrations of verapamil, vinblastine, prostaglandin A_1, NEM, NPM, and by all the agents inhibiting calcein AM extrusion (as listed in Table 1). In accordance with the results in [15], in our experiments GSH depletion by BSO did not modify the MRP-dependent free calcein efflux either. The rates of free calcein extrusion in the different cell types correlated well with the expressed amounts of MRP: the time required for a 50% decrease of cellular free calcein (at an

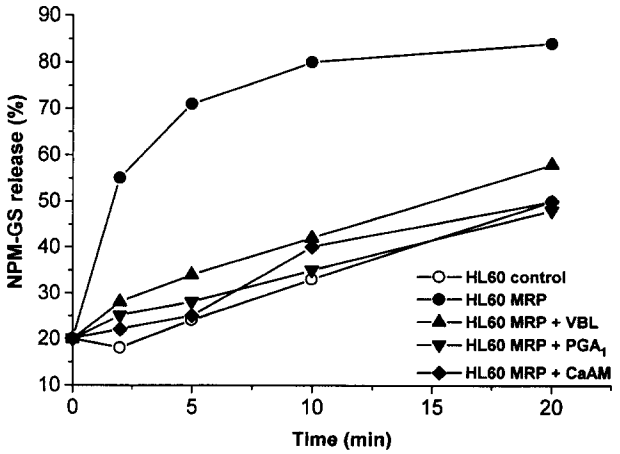

Fig. 3. Efflux of water-soluble NPM-S conjugate from HL60 tumour cells. (○) HL60 control cells; (●) HL60 ADR cells; (▲) HL60 ADR cells+100 μM vinblastine; (▼) HL60 ADR cells + 10 μM PGA1; (◆) HL60 ADR cells + 0.5 μM calcein AM.

initial value of about 1 mM) was more than 60 min in S1MRP cells, 25 min in ADR-selected S1MRP cells and 20 min in HL 60 ADR cells. An important point in these experiments is that relatively high intracellular free calcein concentrations (0.5–2 mM, achieved by preincubating the cells for 10 min in media containing 1–5 μM calcein AM) were required for a full stimulation of the free dye efflux by MRP, while submicromolar concentrations of calcein AM already produced efficient MRP-dependent extrusion (see Fig. 1). These observations render it unlikely that the MRP-dependent transport of any non-fluorescent, partially hydrolysed (thus negatively charged) form of calcein AM may significantly contribute to the observed calcein AM extrusion.

Based on the above experimental data, we suggest that MRP has an inherent transport activity for a variety of chemically and pharmacologically unrelated compounds, e.g. uncharged hydrophobic molecules, lipid-soluble anions, and hydrophilic anions, including glutathione conjugates with multiple and fully dissociating negative charges. The hydrophobic substrates most probably do not have to be converted into GS conjugates, but seem to be recognised by MRP near to or in the hydrophobic membrane phase, as suggested for MDR1. This versatility makes MRP a highly efficient xenobiotic efflux pump, although the large differences in the apparent IC_{50} values for the different chemicals still indicate a limited substrate acceptance. The specificity of the transporter is clearly shown by the relatively small transport effect of the intracellularly formed GS-SG complex (obtained in millimolar levels during GSH depletion by diamide), or the selective inhibition of the MRP-dependent calcein movements by PGA1, and not by several other closely related prostaglandins. An earlier report [29], by studying multidrug resistance in L1210 mouse leukaemia cell line, demonstrated a pattern of shared substrates and inhibitors closely resembling that shown in the present paper (including verapamil, quinidine, cholate, and prostaglandin A_1).

An interesting observation in these experiments is the close correlation of the MRP-inhibitory effects with the pharmacological effectivity of several agents (probenecid, sulfinpyrazone and benzbromarone) in blocking the active reabsorption of uric acid in the human kidney [30]. This finding and the effects of other agents, e.g. indomethacin and bromosulfophthalein, on the MRP-dependent drug transport further support the possible molecular similarity or identity of MRP with the multispecific organic anion transporter (MOAT; see [16]), present in various secretory epithelia.

In this report we demonstrate that the measurement of the formation of free calcein from calcein AM in the tumour cells is especially suitable for characterizing the transport activity of MRP (in flow cytometry the additive effects of calcein AM and free calcein extrusion may occur), and by using appropriate inhibitors, the function of MDR1 and MRP can be differentiated. This method has several advantages over other fluorescent dye (e.g. rhodamine 123 [31], or free calcein [15]) efflux measurements, due to the steady-state conditions for calcein AM transport, and the high-level accumulation of free calcein (see [20]). Both the calcein accumulation and fluorescent GS-NPM conjugate transport measurements are also applicable for screening the MRP-inhibitory effects of various compounds, as well as for determining the involvement of intracellular glutathione conjugation in the MRP-dependent transport of a given molecule.

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