ATP-dependent efflux of calcein by the multidrug resistance protein (MRP): no inhibition by intracellular glutathione depletion

N. Feller, H.J. Broxterman*, D.C.R. Währer, H.M. Pinedo

Department of Medical Oncology, BR 232, Free University Hospital, PO Box 7057, 1007 MB Amsterdam, The Netherlands

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Abstract In this study we report that the multidrug resistance protein (MRP) transports calcein from the cytoplasmic compartment of tumor cells, in contrast to P-glycoprotein which transports calcein acetoxymethyl ester from the plasmamembrane. The transport of calcein by MRP is ATP-dependent and is inhibited by probenecid and vincristine. Intracellular glutathione (GSH) depletion which occurred when cells were exposed to buthionine sulfoximine had no effect on the efflux of calcein, whereas it reversed the daunorubicin accumulation deficit in MRP overexpressing tumor cells. In conclusion, ATP-dependent transport of calcein and possibly other organic anions by MRP is not inhibited by a large decrease of the intracellular GSH concentration, that inhibits daunorubicin efflux by MRP.

Key words: Multidrug resistance protein; P-glycoprotein; Calcein; Glutathione

1. Introduction

The development of resistance of tumor cells to a wide range of natural anticancer agents, such as certain anthracyclines, actinomycin D, vinca alkaloids and etoposide generally is called multidrug resistance (MDR) [1,2]. One type of MDR is due to the overexpression of plasma membrane drug transporters, which act as ATP-dependent drug efflux pumps, resulting in lower intracellular drug concentrations and, hence, in drug resistance [1,3]. Hitherto, two different plasma membrane drug transporters have been cloned, namely the *MDR*1 encoded P-glycoprotein (Pgp) [4] and the recently discovered multidrug resistance protein (MRP) [5]. Both Pgp and MRP belong to the ATP-binding cassette (ABC) superfamily of membrane transport proteins [6]. However, the mechanism of drug transport by MRP differs considerably from that by Pgp. First of all, MRP and Pgp do not result in exactly the same spectrum of drug resistance. Pgp mediated MDR shows cross-resistance to taxol and mitoxantrone, whereas MRP causes very little cross-resistance to taxol and mitoxantrone [7,8]. MRP, but not Pgp, causes cross-resistance to some heavy metal ions such as arsenite and antimonials [8]. Furthermore, Pgp mediated MDR, in contrast to MRP mediated MDR, is readily reversed by drugs such as verapamil, cyclosporin A and the cyclosporin analogue PSC833 [9]. A potent specific inhibitor of MRP mediated daunorubicin (DNR) transport is the isoflavonoid genistein [9].

Another important difference between the two transporters seems to be the role of glutathione (GSH). GSH depletion by exposing of cells to buthionine sulfoximine (BSO), a potent inhibitor of GSH synthesis, increases the accumulation of daunorubicin (DNR), vincristine (VCR) and rhodamine in several MRP, but not in Pgp MDR cells [10,11]. Since it is described that GSH *S*-conjugates and leukotriene C_4 (LCT_4) are transported by MRP [12] it is suggested that MRP is either identical to the GSH-*S*-conjugate export carrier (GS-X pump) or is able to activate an endogenous GS-X pump activity [13].

In the present study the mechanism of transport of MRP was further characterized using the fluorescent dye calcein. Previously it was shown that Pgp overexpressing cells have an accumulation defect for calcein when they are loaded with calcein acetoxy-methyl ester (calcein-AM) [14]. Calcein-AM is a lipophilic, non-fluorescent compound, which rapidly permeates the plasma membrane of cells and upon cleavage of the ester bonds by intracellular esterases is transformed into the highly fluorescent organic anion calcein. It is of interest to note that due to the different fluorescent properties of calcein-AM and calcein, information can be obtained about the compartment (cytoplasm or plasma membrane) from which this dye is extruded by a drug transporter. In addition, it is suggested that Pgp transports calcein-AM directly from the cell plasma membrane compartment since calcein-AM, and not calcein, stimulates the vanadate-sensitive ATP-ase activity in isolated cell membranes of Pgp overexpressing insect cells [15]. Here we provide evidence that MRP actively transports calcein from the cytoplasm.

2. Materials and methods

2.1. Cell lines

The human epidermoid carcinoma Pgp MDR cell line KB8-5 was obtained from ATCC, Rockville, MD. The non-small lung cancer cell line SW-1573 and its Pgp MDR subline SW-1573/2R160 have been described previously [16]. SW-1573 (S1) cells stably transfected with the *MDR*1 gene or the *MRP* gene were obtained from Dr. F. Baas and Dr. G.J.R. Zaman (Neth. Cancer Inst.) [7]. These cell lines were cultured in Dulbecco's MEM (Flow labs, Irvine, Scotland) supplemented with 7.5% heat-inactivated fetal calf serum (FCS) (Gibco Europe, Paisley, Scotland). The human acute myeloblastic leukemia cell line HL60 and its MRP overexpressing subline HL60/ADR were obtained from Dr. M. Center [17]. The human small cell lung cancer cell line GLC_4 and its MRP overexpressing subline GLC_4/ADR have been characterized before [18]. The GLC_4 and HL60 and their sublines were cultured in RPMI 1640 (Flow labs, Irvine, Scotland) supplemented with 10% FCS. All resistant cells were cultured in the presence of a selecting drug (SW-1573(MRP) with G418) until 2–10 days before experiments were performed. The cell lines were mycoplasma-free as tested regularly with the Mycoplasma T.C. kit (Gen-Probe Inc., San Diego, CA, USA).

*Corresponding author. Fax: (31) (20) 4443 844.

Abbreviations: BSO, DL-buthionine (*S,R*)-sulfoximine; calcein-AM, calcein acetoxy-methyl ester; DNR, daunorubicin; GSH, glutathione; GS-X pump, GSH-*S*-conjugate export carrier; LTC_4, leukotriene C_4; MDR, multidrug resistance; MOAT, multispecific organic anion transporter; MRP, multidrug resistance protein; Pgp, P-glycoprotein; VCR, vincristine; Vp, verapamil.

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2.2. Calcein efflux and DNR accumulation

Parental and MRP MDR ($0.3–0.7 \times 10^6$) cells were loaded with 0.5 μM calcein-AM and Pgp MDR cells with 0.5 μM calcein-AM and 25 μM verapamil (Vp) for 10 min at 37°C (Molecular Probes, Inc, Eugene, USA) in medium A (growth medium without phenol red and bicarbonate buffer, but with 20 mM HEPES) + 10% FCS. After the incubation cells were washed in ice-cold medium A + 10% FCS and incubated in drug-free medium + 10% FCS with and without the relevant modulator. As a modulator 100 μM VCR (Sigma Chemical Company, St. Louis, MO, USA) [19] or probenecid in the concentration of 0.5 and 1 mM (Sigma) was used for parental and MRP MDR cells and 25 μM Vp was used for Pgp MDR cells. The efflux was stopped by centrifugation of the cells and by adding ice-cold medium.

To measure DNR accumulation ($0.3–0.7 \times 10^6$) cells were incubated for 60 min at 37°C with 2 μM DNR (Sigma) with or without a modulator in medium A + 10% FCS. After the incubation cells were washed and resuspended in ice-cold medium A + 10% FCS. Non-specific binding of calcein and DNR was measured by adding ice-cold drug containing medium to the cells and washing them immediately. The fluorescence of these dyes was analyzed with a FACScan flow cytometer (Becton Dickinson Medical Systems, Sharon, MA, USA) equipped with a laser excitation wavelength of 488 nm. The fluorescence of calcein was logarithmically measured with a 530 nm band-pass filter and the fluorescence of DNR was measured with a 575 nm filter.

2.3. GSH and ATP depletion

In order to assess if calcein was effluxed in an ATP-dependent way, we decreased the cellular ATP concentrations by pre-incubating of cells for 15 min at 37°C in medium A without glucose but containing 10 mM sodium azide and 5 mM deoxyglucose (medium C). In this medium the ATP concentration is decreased to 10–15% of the initial concentration [20]. After loading the cells with calcein-AM in medium C, the efflux of calcein was measured in medium A and C.

In order to examine the effect of glutathione depletion by BSO on calcein efflux and DNR accumulation, cells were cultured in the presence of 25 μM BSO for 20 h. Intracellular non-protein thiol (largely GSH) was measured with 5,5′-dithiobis(-2-nitrobenzoic acid) (Ellman's reagent; Boehringer Mannheim) in the supernatant of TCA precipitated cells [21,22].

3. Results

In order to test whether calcein was effluxed by MRP we have loaded parental and MRP overexpressing cells with calcein-AM, which is converted in the cytoplasm of the cell into the fluorescent dye calcein and we measured the efflux of calcein from the cells. Fig. 1a and 1b show that in the parental cell lines HL60 and GLC_4, which have a low endogenous MRP level, little efflux of calcein was detected ($t_{1/2} > 90$ min). The efflux of calcein was much faster in the MRP overexpressing cell lines HL60/ADR and the GLC_4/ADR compared to the corresponding parental cell lines ($t_{1/2} < 20$ min). Furthermore, these figures show that the efflux of calcein was completely blocked in glucose-free medium with 10 mM sodium azide and 5 mM deoxyglucose, which leads to cellular ATP depletion. In the strongly Pgp overexpressing cell line SW-1573/2R160 [16] we did not find an increased ATP-dependent calcein efflux compared to the parental cell line SW-1573 (not shown), which is in accordance with the finding that calcein did not stimulate vanadate-sensitive ATPase activity in isolated Pgp containing cell membranes [15]. The slight effect of ATP depletion in parental and Pgp MDR cells is not due to the effect of a lower cytoplasmic pH after ATP depletion, since calcein transport is pH independent between pH 7.0 and 7.8 [14]. These results strongly suggest that calcein is actively transported by MRP from the cytoplasmic compartment after it has been formed from calcein-AM by cytoplasmic esterases.

The fact that we found that calcein, which has a net negative charge, was transported from the cytoplasmic compartment by MRP overexpressing cells might be in line with the hypothesis that positively charged drugs, such as anthracyclines, have to be conjugated with GSH in order to be transported by MRP. To gain more insight into the role of GSH in relation to drug transport by MRP, we examined the effects of GSH depletion on the efflux of calcein in several parental, and in Pgp and MRP overexpressing MDR cells. We decreased the GSH concentration by culturing cells during 20 h with 25 μM BSO [21]. The concentration of GSH was decreased to 28% in GLC_4, 18% in GLC_4/ADR, 20% in HL60, 16% HL60/ADR cells (percentage of controls; means of at least 2 exp). As a control for the effectiveness of GSH depletion, we measured its effect on DNR accumulation since it was reported that the accumulation defect of DNR in MRP MDR cells can be reversed by GSH depletion [21]. In addition, we compared the effect of GSH depletion with the effect of transport modulators on the DNR accumulation and calcein efflux. As shown in Table 1, we found that BSO

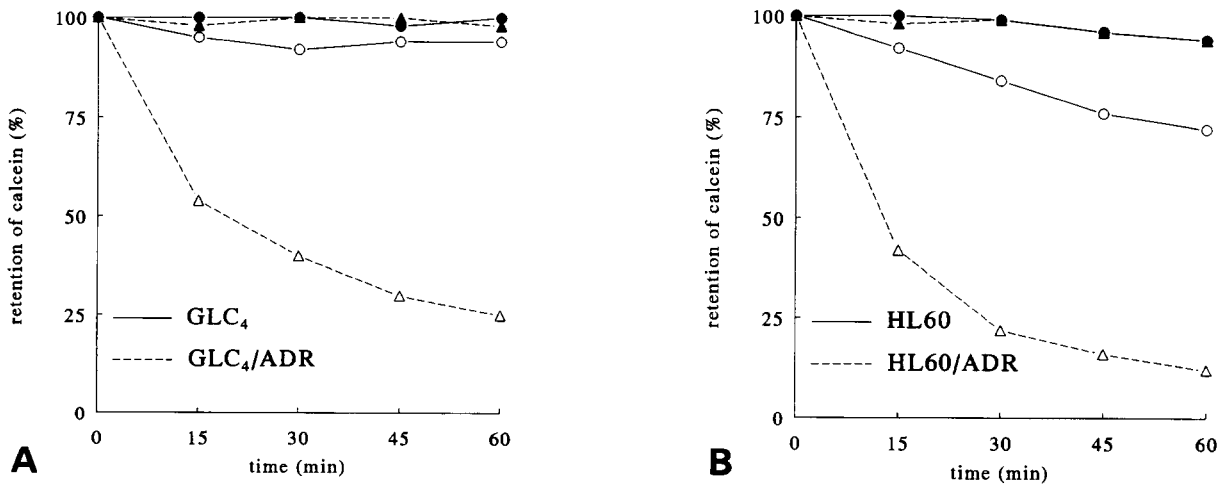

Fig. 1. Efflux of calcein and the effect of energy depletion on the efflux in various parental (*circles*) and MRP (*triangles*). Cells were pre-incubated for 15 min in medium C and then loaded for 10 min with 0.5 μM calcein-AM in medium C at 37°C. Efflux of calcein was measured after suspending cells in calcein-AM-free medium at 37°C in medium A (*open symbols*) and medium C (*closed symbols*). Data are means of two independent experiments.

Table 1
Effect of GSH depletion and modulators on the accumulation of DNR

	DNR accumulation	
	Effect of modulator	Effect of BSO
Parental cell lines		
SW-1573	1.05 ± 0.19	1.17 ± 0.10
HL60	0.93 ± 0.03	0.94 ± 0.03
GLC_4	1.05 ± 0.09	0.93 ± 0.16
Pgp MDR cell lines		
KB8-5	2.07 ± 0.54	1.05 ± 0.06
SW-1573/2R160	11.88 ± 2.36	1.07 ± 0.07
MRP MDR cell lines		
HL60/ADR	2.51 ± 0.27	2.42 ± 0.26
GLC_4/ADR	2.89 ± 0.40	3.42 ± 1.91

Data are means ± S.D. of at least three independent experiments. GSH depletion was achieved by culturing cells during 20 h with 25 μM BSO. 100 μM VCR was used as a modulator for parental and MRP MDR cells and 25 μM Vp was used for Pgp MDR cells. The effect of modulator or GSH depletion is the ratio of DNR accumulation with modulator or in GSH-depleted cells divided by DNR accumulation in control cells.

Table 2
Effect of GSH depletion and modulators on the efflux of calcein

	Calcein efflux		
	Effect of probenecid	Effect of vincristine	Effect of BSO
Parental cell lines			
SW-1573	1.18 ± 0.03	1.10 ± 0.12	0.93 ± 0.10
HL60	1.30 ± 0.08	1.20 ± 0.09	0.94 ± 0.02
GLC_4	1.10 ± 0.03	1.10 ± 0.05	1.01 ± 0.04
Pgp MDR cell lines			
KB8-5	n.d.	1.07 ± 0.03	1.01 ± 0.06
SW-1573/2R160	n.d.	1.06 ± 0.06[1]	1.05 ± 0.07
SW-1573 (MDR1)	1.14 ± 0.02	1.24 ± 0.14	n.d.
MRP MDR cell lines			
SW-1573(MRP)	3.70 ± 0.80	1.90 ± 0.30	1.07 ± 0.07
HL60/ADR	3.70 ± 0.28	3.96 ± 0.49	1.25 ± 0.07
GLC_4/ADR	3.05 ± 0.39	2.18 ± 0.13	0.93 ± 0.09

Data are means ± S.D. of at least three independent experiments; n.d. is not determined. GSH depletion was achieved by culturing cells during 20 h with 25 μM BSO. The effect of modulator (1 mM probenecid, 100 μM VCR or 25 μM verapamil for 2R160 cells[1]) or GSH depletion is the ratio of the retention of calcein with modulator or in GSH-depleted cells divided by the retention of calcein in control cells after 60 min of calcein efflux.

could reverse the DNR accumulation to the same extent as the modulator VCR in MRP MDR cells, showing its effectiveness in modulating DNR transport by MRP. In parental and Pgp MDR cells no effect of GSH depletion by BSO was found on the DNR accumulation. However, Table 2 shows that GSH depletion had no effect on the amount of calcein effluxed from the MRP MDR cell line GLC_4/ADR (ratio of calcein retention after BSO divided by control was 0.93) and had a slight effect in HL60/ADR (a ratio of 1.25) whereas the modulator VCR had a large inhibitory effect on the efflux of calcein (a ratio of 2.18 and 3.96 in GLC_4/ADR and HL60/ADR respectively). Thus, these results obtained with the highly MRP overexpressing GLC_4/ADR and HL60/ADR cells suggest that the ATP-dependent transport of calcein in these cell lines is not dependent on intracellular GSH concentrations.

To obtain more evidence for the role of MRP in calcein efflux we have performed experiments using SW-1573 cells, stably transfected with the *MRP* gene and tested the organic anion probenecid as blocker of the calcein efflux in these transfected cells as well as in the *MRP* overexpressing GLC_4/ADR and HL60/ADR cells. Since the resistance of the *MRP* transfected cells is small compared to the other cell lines [7], as a control for these experiments the SW-1573(MDR1) transfected cells were used, which have resistance factors similar to the *MRP* transfected cells. Table 3 shows that the efflux of calcein was much faster in the SW-1573(MRP) cells, which results in a lower retention in these *MRP* transfected cells, compared to the parental SW-1573 and Pgp MDR SW-1573(MDR1) cells. In addition, Table 2 shows that 1 mM probenecid has a large inhibitory effect on calcein efflux in GLC_4/ADR, HL60/ADR and SW-1573(MRP) cells, whereas it has a slight effect in the parental and in the SW-1573(MDR1) cells. Notably, 1 mM probenecid inhibited the efflux of calcein for almost 100% in the MRP overexpressing cells. A lower concentration of probenecid (0.5 mM) had an effect comparable to that of 100 μM VCR (not shown). Also, in contrast to the effect of probenecid and VCR, GSH depletion by BSO exposure (to 34% of control) had no effect on the efflux of calcein in the *MRP* transfected SW-1573(MRP) cells. These results show that the MRP mediated efflux of calcein can be inhibited by VCR and the organic anion transport inhibitor probenecid, but is not sensitive to a large decrease in intracellular GSH concentration.

4. Discussion

This study gives evidence that the organic anionic dye calcein is actively transported from the cytoplasmic compartment of cells by MRP leading to a decreased accumulation of calcein in MRP MDR cells. However, the decreased accumulation of calcein in Pgp MDR cells as shown before [14], is not caused by the transport of calcein but by the transport of the lipophilic calcein-AM ester directly from the cellular plasma membrane. Probably, calcein-AM is transported by Pgp due to the relatively hydrophobic tails of the ester groups, which contain a basic nitrogen atom and an extended side chain, a combination which is often present in Pgp modulatory compounds. Thus, the accumulation defect of calcein in Pgp and MRP MDR cells is largely the result of different pharmacological characteristics of the Pgp and MRP transporters. In addition, it could still be that part of the accumulation defect of calcein in MRP overexpressing cells is also due to the transport of calcein-AM by MRP.

Since calcein is a strongly negatively charged molecule (net charge is approximately −4 at pH 7.0), the transport of calcein by MRP is in accordance with the recent finding that MRP acts

Table 3
Retention of calcein in SW-1573 and transfected cells

	Calcein retention	
	After 30 min (%)	After 60 min (%)
SW-1573	85 ± 10	70 ± 5
SW-1573(MDR1)	84 ± 4	73 ± 5
SW-1573(MRP)	29 ± 2	18 ± 4

Cells were loaded for 10 min with 0.5 μM calcein-AM after which calcein efflux was measured. Data are means ± S.D. of three independent experiments.

as a multispecific organic anion transporter (MOAT) [13]. Also, the inhibition of calcein transport by the organic anion probenecid would be consistent with that suggestion. In fact, Jedlitschky et al. [23] and Müller et al. [13] found that transport of LTC_4 and *S*-dinitrophenylglutathione was increased in membrane vesicles from the HL60/ADR and GLC_4/ADR cells compared to vesicles from the parental drug sensitive cells. Because of the identification of MRP as MOAT, it has been suggested that positively charged and neutral drugs such as DNR and etoposide, respectively, which are actively transported by MRP [20], might be transported after conjugation with GSH or other negatively charged groups [13,23]. However, this is an unlikely explanation since hitherto such conjugates of anthracyclines have not been found to be formed in tumor cell lines in any substantial amount [24]. Also, a rapid conversion of etoposide (VP-16) would have to take place if a VP-16 conjugate would have to account for the very rapid ATP-dependent transport of VP-16 from MRP overexpressing GLC_4/ADR cells [25].

Alternative explanations for the GSH requirement of the transport of positively charged or neutral drugs by MRP, derived from the results of GSH depletion experiments may be that GSH depletion has some other side effects. One possible effect of BSO exposure could be that the plasma membrane is damaged by oxidative stress due to a decreased protection by GSH, resulting in an increased drug influx and reversal of resistance. An endogenous inhibitor of MRP function might also be activated by GSH depletion. Our present results, which show that calcein transport by MRP is not inhibited by GSH depletion after BSO exposure, exclude above mentioned possible other effects of GSH depletion. Thus, the different effects of GSH depletion on transport of negatively charged (calcein) and positively charged molecules (DNR, VCR, rhodamine) strengthen the hypothesis that MRP transports negatively charged molecules. GSH could be needed as co-substrate or indirect stimulator for transport of positively charged drugs, but not or at a very low concentration for transport of negatively charged molecules by MRP.

In conclusion, this study demonstrates that calcein is transported from the cytoplasmic compartment of the cell by MRP, but not by Pgp, in an ATP-dependent way. The transport of calcein is inhibited by VCR and the organic anion transport inhibitor probenecid but not by a lowered intracellular GSH concentration, which is probably due to the fact that calcein is negatively charged. The relative importance of the charge of molecules for their transport by MRP has to be further investigated using molecules which differ in charge.

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