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The Role of Multidrug Resistance Protein 1 (MRP1) in Transport of Fluorescent Anions across the Human Erythrocyte Membrane

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Abstract. We employed human red blood cells as a model system to check the affinity of MRP1 (Multidrug Resistance-associated Protein 1) towards fluorescein and a set of its carboxyl derivatives: 5/6-carboxyfluorescein (CF), 2′,7′-bis-(2-carboxyethyl)-5/6-carboxyfluorescein (BCECF) and calcein (CAL). We found significant differences in the characteristics of transport of the dyes tested across the erythrocyte membrane. Fluorescein is transported mainly in a passive way, while active efflux systems at least partially contribute to the transport of the other compounds. Inside-out vesicle studies revealed that active transport of calcein is masked by another, ATP-independent, transport activity. Inhibitor profiles of CF and BCECF transport are typical for substrates of organic anion transporters. BCECF is transported mainly via MRP1, as proven by the use of QCRL3, a monoclonal antibody known to specifically inhibit MRP1-mediated transport. Lack of effect of QCRL3 on CF uptake excludes the possibility of MRP1 being a transporter of this dye. No inhibition of CF accumulation by cGMP, thioguanine and 6-mercaptopurine suggests also that this fluorescent marker is not a substrate for MRP5, another ABC transporter identified in the human erythrocyte membrane.

Key words: Erythrocyte — MRP1 — Fluorescein — Carboxyfluorescein — Calcein — BCECF

Introduction

Multidrug resistance, a major obstacle in the therapy of cancer and parasitic diseases, demonstrates as decreased susceptibility of target cells towards frequently structurally unrelated chemotherapeutics. Although several mechanisms may contribute to multidrug resistance, drug extrusion from malignant cells is one of the most thoroughly investigated. ABC (ATP Binding Cassette) superfamily proteins: MXR, MDR1, MRP1, MRP2 and MRP3 play a central role in generating multidrug resistance of cancer cells via phase III (elimination) reactions [25]. Their overexpression in tumor tissue correlates with the level of resistance towards chemotherapeutics, and their ability to actively remove various drugs from the cytoplasm to the extracellular milieu has been proven (for an extensive review *see* [32]).

Homolya et al. [22] measured reduced retention of 6 different fluorescent dyes, among them calcein and 2′,7′-bis-(2-carboxyethyl)-5/6-carboxyfluorescein (BCECF, both administered in acetoxymethyl ester forms), in MDR1-transfected NIH-3T3 murine fibroblasts, in comparison to wild-type cells. Further studies established a basis for the functional test of MDR1 activity using the acetoxymethyl ester of calcein as a substrate [20]. Two independent groups have shown that the free acid form of the dye is a substrate for MRP1 [17, 47]. Calcein efflux was inhibited by probenecid and vincristine (both compounds known as modulators of MRP1 activity) [17] and decrease in intracellular glutathione level did not influence the rate of transport [47]. The acetoxymethyl ester form of calcein turned out to be also transported by MRP1 [21] and MRP1 transport activity for both the ester and anionic form of the dye was specifically inhibited by a set of typical inhibitors of organic anion transport. No calcein transport was detected in cells overexpressing MRP5 [35].

BCECF is another fluorescent substrate of MRP1 [13]. BCECF efflux from MRP1-overexpressing cells is not dependent on the glutathione level but is sensitive to metabolic depletion, vincristine [13] and indomethacin [14]. Diminished retention of BCECF was also observed in MRP5-overexpressing cells [35].

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MRP1 inhibitors indomethacin and probenecid increased fluorescein accumulation in brain microvessel endothelial cells [23]. Accumulation of the dye was markedly diminished in MRPI-transfected MDCKII cells and this effect could be reversed by probenecid, MK571 and LY402913 (the latter two compounds are specific inhibitors of MRP-mediated transport) [42]. A significantly lower level of fluorescence retention was also observed in the MRP5-transfected HEKc10 cell line when compared to mock-transfected HEKc5 cells [35]. 5-Carboxyfluorescein efflux from $HT29^{col}$ cells selected for resistance to colchicine and overexpressing MRP1 was inhibited by MK571, a leukotriene D_4 analogue, an inhibitor of MRP1 activity [28].

The clinical significance of the multidrug resistance phenomenon gives rise to a need for rapid and reliable diagnostic tests. It has been shown that functional tests based on fluorescent marker efflux, measuring the actual activity of a given multidrug resistance protein, may be of greater clinical value than measurement of protein expression [5, 30, 46]. This creates the need for more detailed characterization of transport of fluorescent markers that are used for activity measurements of multidrug resistance-related proteins.

The aim of the study was to kinetically characterize the transport of carboxyl derivatives of fluorescein by MRP1. As a research model we have chosen the human erythrocyte, a cell easy to obtain, a rich source of pure plasma membranes (due to lack of internal compartmentation), which makes possible large-scale investigations of inside-out vesicles. The expression of MRP1 and MRP5 was reported in human red blood cells [26, 36]. The presence of MDR1 in the erythrocyte membrane has been claimed [1], however, the actual expression of other multidrug resistance-related proteins in human red blood cells remains obscure. As a "native" cell, i.e., not drug-selected or otherwise artificially forced to overproduce multidrug resistance proteins, the erythrocyte ensures a proper membrane environment for activity of the transporters to be investigated.

Materials and Methods

Cells

Fresh human venous blood was obtained from healthy volunteers. The plasma and leukocyte buffy coat were carefully removed after centrifugation (2000× *g*, 10 min, 4°C). The erythrocytes were resuspended and washed with phosphate-buffered saline. The residual leukocytes and platelets were removed by passing the erythrocyte suspension through the cellulose column [7]. The cells were retained for transport experiments or were used as a source of membrane vesicles (*see* below).

HL60, human promyelocytic leukemia cells and the drug-resistant sublines HL60ADR (adriamycin-resistant, MRP1-overexpressing) and HL60VINC (vincristine-resistant, MDR1-overexpressing), kindly provided by Dr. Melvin Center, Kansas University, were cultured in RPMI1640 medium supplemented with 10% fetal calf serum and 100 U/ml penicillin and 100 μg/ml streptomycin, in the presence of selection factors for resistant clones (doxorubicin and vincristine, respectively). V79, Chinese hamster lung fibroblasts and a V79MRP1 subline transfected with human MRP1-gene, kindly provided by Prof. Brian Burchell, Dundee University, were grown in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum and standard concentrations of antibiotics (for more information about these cell lines, *see* [11]).

Chemicals

All cell culture media and additives were from Gibco™, Invitrogen (Carlsbad, CA). Acetoxymethyl esters of calcein (CAL-AM), 2′,7′-bis-(2-carboxyethyl)-5/6-carboxyfluorescein (BCECF-AM), free acid forms of calcein (CAL) and 2′,7′-bis-(2-carboxyethyl)-5/6-carboxyfluorescein (BCECF) were from Molecular Probes (Eugene, OR). Diacetyl esters of fluorescein (FDA) and 5/6-carboxyfluorescein (CFDA) as well as free acid forms of fluorescein (F) and 5/6-carboxyfluorescein (CF) were from Sigma (Poznań, Poland). C219, QCRL1, QCRL3, MRPr1, M2II-12, M3II-9 and M5II-54 antibodies were from Alexis (Läufelfingen, Switzerland). [^{3}H]-labelled glutathione was obtained form DuPont NEN (Boston, USA). [^{3}H]-labelled 2,4-dinitrophenyl-S-glutathione was synthesized and purified according to Akerboom and Sies [3]. All other reagents used were of analytical grade.

Cell Transport Studies

The cells were suspended in an ice-cold transport buffer (in mM: 138 NaCl, 5 KCl, 1 $MgCl_2$, 5.6 glucose buffered with 10 phosphate, pH 7.4) at a 5% hematocrit (about 500 million erythrocytes/ml) or final concentration of 2 million cells/ml (for cultured cells). The suspension was mixed with an equal volume of 2 μM solution of fluorescent compound ester in the same buffer and cells were allowed to load for 10 minutes on ice. The samples were then incubated at 37°C, and aliquots were drawn at given time points. Then the cells were spun down (18,000× *g*, 5 min) and the fluorescence of the supernatant was measured (excitation/emission wavelengths: 490 nm/515 nm for F, 492 nm/517 nm for CF, 494 nm/517 nm for CAL and 439 nm/535 nm for BCECF). For inhibitor studies, the cells were preincubated for 15 minutes at room temperature with a given concentration of inhibitor before the ester of the fluorescent marker was added. Control samples were incubated with the proper volume of the solvent added (dimethylsulphoxide for most compounds, the concentration of the solvent was kept always below 0.5%). Care was taken to maintain the level of hemolysis in the samples comparable to that in control (below 1%), as strong quenching effect of hemoglobin on dye fluorescence was observed.

2,4-Dinitrophenyl-S-glutathione export from intact erythrocytes and metabolic depletion and reconstitution of the ATP level in erythrocytes were performed according to Board [9].

Membrane Preparation

The erythrocyte membranes and inside-out vesicles were isolated by a modified procedure of Steck and Kant [41]. Briefly, the erythrocytes were lysed in 15 volumes of ice-cold 20 mM TrisHCl, 1 mM $EDTANa_2$, 575 μM PMSF, pH 7.4 at 4°C. After three subsequent

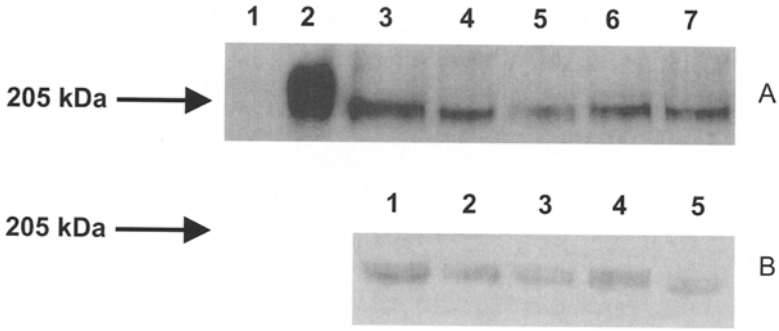

Fig. 1. Immunodetection of MRP1 (panel *A*) and MRP5 (panel *B*) proteins in human erythrocyte membranes. (*A*) Lane *1*: 7 μg of V79 membrane preparation, lane *2*: 7 μg of V79MRP1 membrane preparation; lanes *3–7*: 30 μg of erythrocyte plasma preparation. Each lane corresponds to a different donor. (*B*) Lanes 1–5: 30 μg of erythrocyte plasma preparation, each lane corresponds to a different donor. Arrow points to the location of a 205 kDa molecular mass marker.

washes (60,000× *g*, 15 minutes, 4°C) with the buffer, the pellet of white ghosts was suspended in 50 volumes of ice-cold vesiculation buffer (VB, 250 mM sucrose, 0.1 mM $EDTANa_2$ buffered with 0.5 mM TrisHCl, pH 8.0 at 4°C) and incubated overnight on ice. Then the suspension was centrifuged (60,000× *g*, 15 minutes, 4°C) and the vesicle pellet was resuspended in VB to a final concentration of approximately 4 mg protein/ml. The degree of sidedness of the vesicles was determined by comparison of accessible and total activity of acetylcholinesterase by a method of Ellman et al. [15]. The average inside-out vesicle content was 39 ± 4%. The vesicles were snap-frozen in liquid nitrogen and stored at −75°C until used in transport experiments. No significant change in transport activity of the vesicles was observed within 12 months of storage in such conditions.

The plasma membranes of the nuclear cells were isolated according to the procedure of Keppler et al. [27].

The protein content in membrane preparations was determined according to Lowry et al. [34] with bovine serum albumin as a protein standard.

Inside-out Vesicle Transport Studies

The accumulation of fluorescent anions inside the vesicles was measured in transport medium containing in mM: 2.5 ATP (or AMP for control samples), 250 sucrose, 10 $MgCl_2$, 0.66 ouabain, 10 phosphocreatine, 1 U/ml phosphocreatine kinase, buffered with 10 TrisHCl, pH 7.4 at 37°C. Standard concentration of a fluorescent anion was 25 μM. Final concentration of membrane vesicles was 0.5–1.0 mg of protein/ml. Final sample volume was 500 μl. The samples were incubated at 37°C. The transport was stopped by adding 1.5 ml of ice-cold VB and subsequent centrifugation (18,000× *g*, 5 minutes, 4°C). The supernatant was carefully removed and the vesicle pellet was washed twice with 1 ml of VB. The final pellet was dissolved in 1 ml of 1% Triton X-100 buffered with 10 mM Tris, pH 10.0, at room temperature and the fluorescence was measured as mentioned above.

The transport of [^{3}H]-labelled 2,4-dinitrophenyl-S-glutathione in the same transport medium was determined by a rapid filtration method according to Ishikawa [24]. The final volume of the sample was 50 μl.

The active transport of a substrate was determined as the difference between accumulation in presence of ATP and AMP.

SDS-PAGE and Western Blotting

The erythrocyte plasma membrane samples (protein amount 30 μg) were subjected to SDS-polyacrylamide gel electrophoresis in 7.5% gels according to Laemmli [29]. Western blot analysis was performed according to Towbin et al. [45]. Immunoblots were developed with the enhanced chemiluminescence system [31].

Abbreviations

ABC, ATP-Binding Cassette; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; BCECF, 2′,7′-bis-(2-carboxyethyl)-5/6-carboxyfluorescein; BCECF-AM, acetoxymethyl ester of BCECF; CAL, calcein; CAL-AM, acetoxymethyl ester of CAL; CDNB, 1-chloro-2,4-dinitrobenzene; CF, 5/6-carboxyfluorescein; CFDA, 5/6-carboxyfluorescein diacetate; cGMP, cyclic guanosine 3′,5′-monophosphate; DNPSG, 2,4-dinitrophenyl-S-glutathione; F, fluorescein; FDA, fluorescein diacetate; GSX, glutathione S-conjugate; HA, high affinity/low capacity system of glutathione S-conjugate transport; K_m, Michaelis constant; LA, low affinity/high capacity system of glutathione S-conjugate transport; MDR, multidrug resistance protein (P-glycoprotein); MRP, multidrug resistance protein (multidrug resistance-associated protein); MXR, mitoxantrone resistance protein; PMSF, phenylmethylsulphonyl fluoride; SDS, sodium dodecyl sulphate; V_{max}, maximal velocity.

Results

Immunodetection of Multidrug Resistance Proteins in Human Erythrocyte Membranes

The presence of MRP1 and MRP5 proteins was detected in human erythrocyte membrane preparations by immunoblotting with specific monoclonal antibodies: MRPr1 and M_5II-54, respectively (Fig. 1). No reactivity against C219 (anti-MDR), M_2II-12 (anti-hMRP2) nor M_3II-9 (anti-hMRP3) antibodies was detected in human erythrocyte membranes (*not shown*).

Export of Fluorescent Anions from Intact Cells

Although most of the widely used methods for determination of fluorescent anion transport are based on the measurement of the rate of fluorescence decrease in the cytoplasm of the analyzed cell (*see* [16, 21]), this approach could not be used in the present study due to the presence of hemoglobin, which quenches the fluorescence of studied compounds. That is why the increase in fluorescence of the extracellular medium was chosen as a measure of transport. The ester forms of fluorescent compounds rapidly disappeared from the extracellular medium

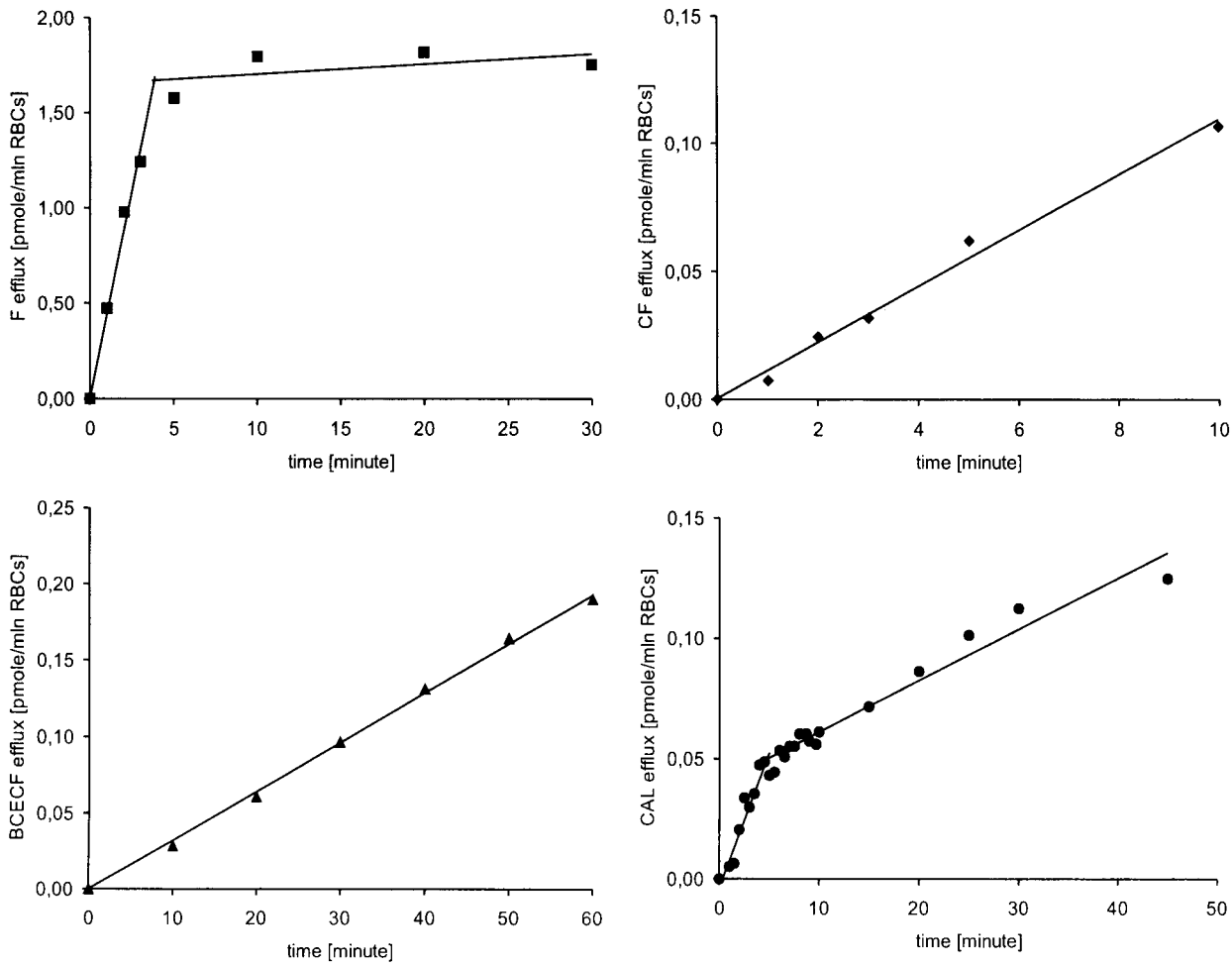

Fig. 2. Efflux of fluorescent anions from intact erythrocytes. All data presented are the results of a single representative experiment performed in triplicate.

(the half time was about 1 minute for FDA and about 4 minutes for CAL-AM, *not shown*), which suggests that their preferential location is inside the cells (or plasma membrane). Incubation of unloaded erythrocytes in the presence of anionic forms of the compounds studied produced no decrease in the fluorescence of the medium, which suggests that there is no backward transport into the cytoplasm (*not shown*). No significant ester hydrolysis was detected in the absence of cells (*not shown*). All those results confirm the suitability of the experimental setup.

Although all the compounds studied are derivatives of fluorescein and as such could be expected to show a similar pattern of transport, significant differences were observed, not only in terms of export rate but also in inhibition profile among them. The highest rate of transport across erythrocyte membrane was observed for fluorescein. After 3–4 minutes of incubation, virtually all the substrate was exported from the erythroplasm. Increase in fluorescence of the external medium was linear up to at least 10 minutes for CF and up to 60 minutes for BCECF. A significantly different pattern was observed for CAL: its efflux was linear up to 5 minutes, then the rate of fluorescence increase in the external medium dropped down to 20% of the initial value but stayed linear up to at least 60 minutes (Fig. 2). For further studies, the initial rate of the dye efflux was measured in the following periods: 0 to 4.5 minutes for F and CF, 0 to 30 minutes for BCECF and 0 to 4.5 minutes and 10 to 60 minutes for CAL. For comparison, efflux rate of the compounds studied was determined for cell lines overexpressing MRP1 or MDR1 (Table 1). Transport rate of all dyes (except for CF efflux from HL60 and derivative cells) was significantly higher in both MRP1-overexpressing cell lines V79MRP1 and HL60ADR, which suggests involvement of this protein in the transport of various fluorescein derivatives. MDR1 seems not to play an important role in the export of free acids of any dyes except for F. The observed significant differences in the transport rates between erythrocytes and other cells used may reflect not only the size difference between the cell types but also the density of transporter distribution over the cell surface.

Table 1. Initial rates of dye efflux for erythrocytes and cultured cell lines

	F	CF	BCECF	CAL
Erythrocyte	0.570 ± 0.140	0.050 ± 0.021	0.009 ± 0.002	0.003 ± 0.001
HL60	61 ± 10	13.6 ± 3.6	2.6 ± 0.3	2.2 ± 0.6
HL60ADR	204 ± 28	4.5 ± 2.5	16.1 ± 0.8	7.2 ± 2.4
HL60VINC	116 ± 23	3.7 ± 0.0	2.0 ± 0.3	1.1 ± 0.2
V79	170 ± 47	8.7 ± 1.7	5.5 ± 2.1	2.9 ± 1.4
V79MRP1	308 ± 79	15.3 ± 5.5	15.5 ± 8.4	7.9 ± 2.8

Data are expressed as pmole of dye/(10^6 cells × min) and presented as average calculated from at least three independent determinations ± SD.

Table 2. Effect of selected inhibitors and metabolic depletion on dye efflux rate from intact erythrocytes

	F	CF	BCECF	CAL (0–4.5 min)	CAL (10–60 min)
Non-specific inhibitors of active transport					
Fluoride	20%	13.2 ± 5.0	2.1 ± 0.1	30%	3.3 ± 3.2
Orthovanadate	10%	20%	5.1 ± 1.9	n.d.	n.d.
Inhibitors of organic anion transport					
Benzbromarone	0%	20%	2.4 ± 1.0	20%	35 ± 26
Indomethacin	0%	40%	40%	50%	3.0 ± 3.4
Probenecid	0%	30%	150 ± 50	580 ± 400	240 ± 170
Verapamil	0%	stimulation	20%	20%	30%
CDNB[1]	97 ± 6	72 ± 18	48 ± 3	80 ± 13	82 ± 7
Metabolic depletion[1]					
Metabolic depletion	116 ± 29	55 ± 10	3 ± 4	68 ± 26	46 ± 10
ATP-level reconstitution	99 ± 14	95 ± 6	83 ± 14	93 ± 22	106 ± 7

[1] Effect given as percent of control.

For calcein (CAL), data are presented both for short (0–4.5 min) and longer (10–60 min) times of incubation. Whenever possible, IC50 values (mean ± SD) are presented. Maximal inhibition (%) is given when the effect of the inhibitor was too weak to calculate the IC50 value. All data are averaged from at least three independent determinations.

Concentration range of inhibitors tested: fluoride: 0.5–20 mM, orthovanadate: 2.5–100 μM, benzbromarone: 1–100 μM, indomethacin: 2–25 μM, probenecid: 5–1000 μM, verapamil: 5–100 μM. For determination of influence of glutathione-S conjugates formed during incubation with 1-chloro-2,4-dinitrobenzene (CDNB), on dye efflux, erythrocytes prior to fluorescent marker loading were incubated with 1 mM CDNB for 15 min at 37°C as described in [9]. Effect of orthovanadate on calcein efflux was not determined due to quenching of CAL fluorescence in presence of orthovanadate.

Further experiments involving metabolic depletion of cells and use of a set of typical organic anion transport inhibitors revealed a complicated picture of transport of fluorescein derivatives in red blood cells (Table 2). Virtually all the dyes used were transported in a different way. F efflux was not influenced by metabolic depletion and was insensitive to any of the inhibitors tested. However, a low value of activation energy of its transport suggests the participation of a protein carrier facilitating its efflux (*see* below). CF transport was influenced by metabolic depletion although its inhibition was not complete. Among inhibitors of organic anion transport only fluoride was able to decrease the dye efflux rate down to 50% of control in the range of concentrations used. The effects of orthovanadate, benzbromarone, indomethacin and probenecid were significant but not profound. An astonishing effect was observed for verapamil, a calcium ion channel blocker known also to inhibit both MDR and MRP proteins: a dose-dependent stimulation of CF efflux was observed in the range from 5 to 100 μM of verapamil (Fig. 3). Transport of BCECF and CAL followed the pattern expected for a model MRP1 substrate: it was significantly diminished by fluoride, orthovanadate (measured only for BCECF, as fluorescence of CAL is quenched by orthovanadate), benzbromarone, probenecid and indomethacin. BCECF and CAL exhibited different sensitivity to metabolic depletion: BCECF efflux was practically stopped, while transport of CAL was inhibited only by about 50%. Activation energy values measured for the temperature range of 32–44°C were comparable for all dyes (F: 52 ± 16 kJ/mole, CF: 68 ± 6 kJ/mole, BCECF: 57 ± 25 kJ/mole, CAL: 65 ± 11 kJ/mole) and speak in favour of the hypothesis that the observed dye efflux is a result of activity of protein carriers.

Activity of the so-called glutathione S-conjugate pump (GSX-pump) [25] is now ascribed to MRP.

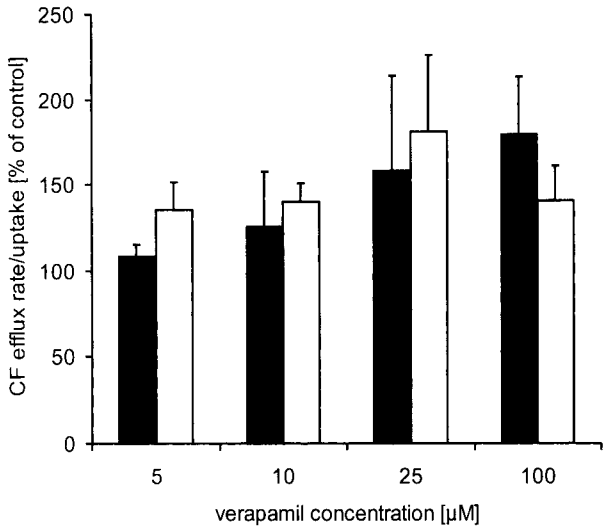

Fig. 3. Effect of verapamil on CF efflux from intact erythrocytes (*black bars*) and ATP-dependent accumulation of the dye in inside-out vesicles (*white bars*). Averaged results of three independent experiments. Differences are statistically significant (variance analysis, $P < 0.05$).

Preincubation of erythrocytes with 1-chloro-2,4-dinitrobenzene results in the formation of large quantities of 2,4-dinitrophenyl-S-glutathione (DNPSG), a model GSX-pump substrate, transport of which can be followed spectrophotometrically [9]. None of the dyes studied interfered with export of DNPSG from human erythrocytes (measured at 10% hematocrit and 4 μM of dye ester, *not shown*), but the effect of DNPSG on transport of BCECF and CF was clearly visible (*see* Table 2). Only mild inhibition of CAL efflux was observed (80 ± 7% of control value for incubation times longer than 10 minutes) and there was no effect on F export.

Accumulation of Fluorescent Anions inside Membrane Vesicles

To further investigate the role of erythrocyte membrane carrier systems in the transport of fluorescein derivatives, inside-out vesicle experiments were conducted (Fig. 4). No active transport was observed for F, which is in full agreement with previous intact-cell experiments. Surprisingly, similar results were observed for CAL: no clear ATP-dependent accumulation of the dye was measured for concentrations up to 1 mM of CAL (*not shown*). Recently, a negative effect of glutathione depletion on CAL efflux was reported [4]. However, addition of glutathione (at ratios in a range of between 1- and 40-fold with respect to calcein) had no effect on CAL uptake (*not shown*). Apparent ATP-dependence of accumulation inside vesicles was visible for both CF and BCECF. Transport of both dyes was also osmotically sensitive (*not shown*) and concentration dependent (*see* Table 3). Benzbromarone, indomethacin and probenecid significantly inhibited accumulation of both substrates (Fig. 5). No such effect was observable for cyclic GMP, a model MRP5 substrate [26], and 6-mercaptopurine and thioguanine, compounds for which MRP5 confers resistance [49]. Verapamil was able to inhibit BCECF accumulation down to about 40% of control at 5 μM concentration (*not shown*) but its effect on CF transport was again stimulatory (*compare* Fig. 3). These results suggest involvement of two different systems in the transport of BCECF and CF, which was further confirmed by antibody inhibition study. It has been known that QCRL3, a monoclonal antibody specific against a conformational epitope of MRP1, is able to inhibit transport of any substrate mediated by this protein [19]. QCRL1 antibody, specific against a linear epitope of MRP1, and not able to influence the transport, was chosen as a negative control. The results (Fig. 6) identified MRP1 as a protein transporting BCECF and excluded such a possibility for CF.

Two systems of glutathione S-conjugate transport exist in human erythrocytes, differing mainly in affinity towards DNPSG and sensitivity to inhibition by some organic anions including glutathione disulphide and aromatic compound glucuronates [2, 6, 39]. The interaction between DNPSG- and dye-transport systems was studied. 1 μM DNPSG was able to non-competitively inhibit transport of both CF and BCECF (Table 3), while the high-affinity component of DNPSG transport in erythrocytes, ascribed to MRP1 [48], was inhibited only by BCECF (also non-competitively). The low-affinity component of DNPSG transport, whose identity remains obscure, was inhibited by both dyes in a non-competitive manner (Table 4).

Discussion

Expression of MRP1 and MRP5 in human erythrocyte plasma membrane is well documented [18, 26, 36], however, there are no convincing data about the expression of other multidrug resistance proteins. Our results confirm lack of immunochemically measurable expression of MRP2, MRP3 and MDR proteins (P-glycoproteins) in the human erythrocyte membrane. Absence of detectable MDR expression is in contradiction to some other reports [1]. A low level of expression of Mdr1a and Mdr1b (orthologues of human MDR1) was observed in murine erythrocytes (A.J. Smit, unpublished, after [12]). This discrepancy may be explained by i) interspecies difference between human and murine tissues, ii) contamination of erythrocyte preparation with white blood cells highly expressing MDR. In our study, care was taken to prevent erythrocytes from contamination with other blood cell populations.

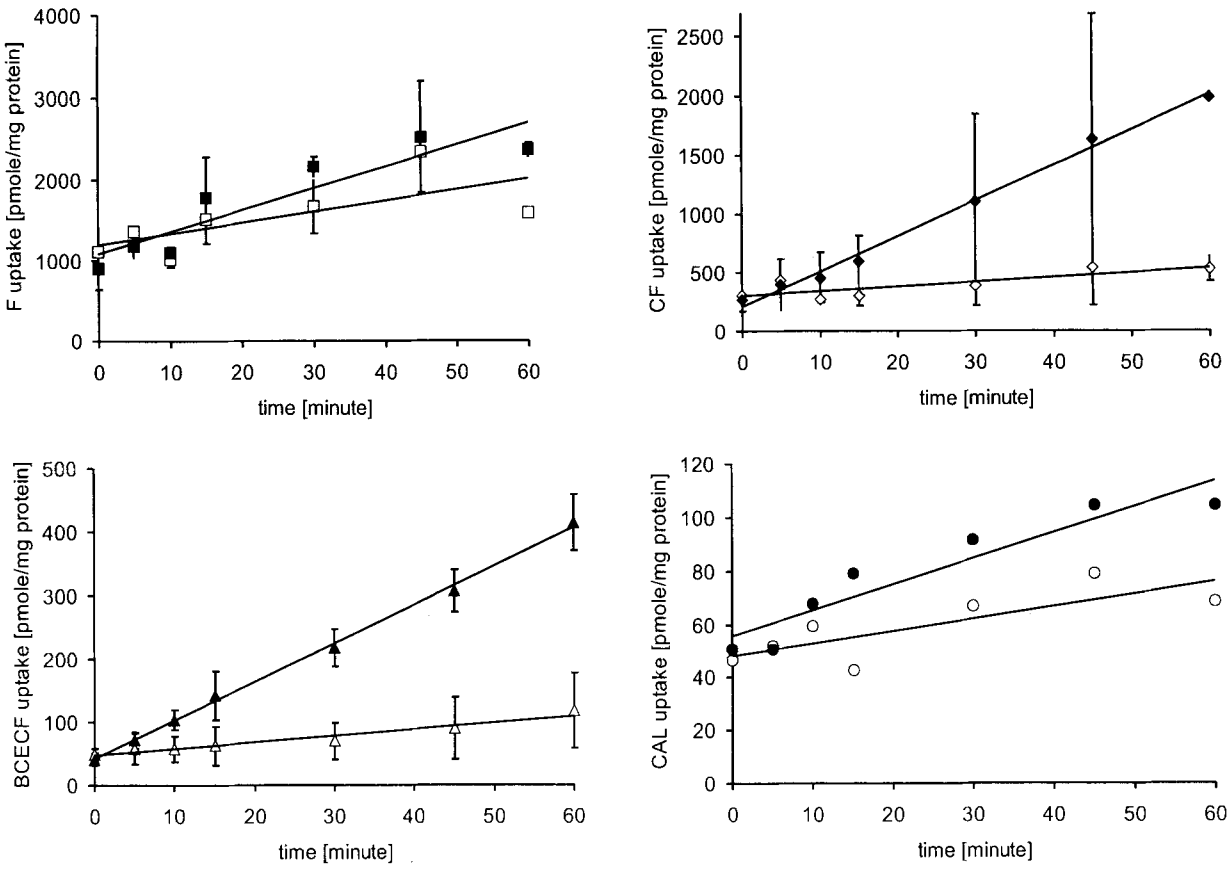

Fig. 4. Time-dependent accumulation of fluorescent anions in erythrocyte plasma membrane inside-out vesicles; dye concentration 25 μM. Closed symbols, uptake in presence of ATP, open symbols, uptake in presence of AMP. Averaged data ± SD from two independent determinations except for CAL where a representative experiment is presented.

Table 3. Effect of 2,4-dinitrophenyl-S-glutathione (DNPSG) on kinetic properties of CF and BCECF uptake by inside-out vesicles

	CF	BCECF
Control		
Apparent K_m [μM]	14.3 ± 3.7	10.6 ± 1.8
V_{max} [pmole/(mg protein × min)]	4.3 ± 1.0	4.7 ± 0.7
DNPSG 1 μM		
Apparent K_m [μM]	14.2 ± 4.0	11.0 ± 3.3
V_{max} [pmole/(mg protein × min)]	3.4 ± 0.9	2.1 ± 0.6

Data were averaged from three independent experiments.

Comparable and relatively low activation energy values for efflux of all dyes tested speak in favor of protein carrier engagement in dye transport. One should be aware, however, that due to the complexity of the processes observed (the efflux is a result of ester internalization within the cell, its cleavage by intracellular esterases and transport of free acid across the membrane), activation energy values do not reflect only the transport phase and thus should be interpreted with care. Significant differences in the rate of dye efflux from erythrocytes and their divergent vulnerability to metabolic depletion suggest involvement of a different carrier system for each marker.

Fluorescein seems to be transported mainly in a passive way. Its efflux was not inhibited by metabolic depletion nor by any of the organic anion transport modulators used. However, HL60ADR and V79MRP1 cells extruded fluorescein at least 2-fold faster than their parental counterparts, which is generally in line with other observations [42]. A high rate of F extrusion from human erythrocytes and lack of accumulation of this dye into inside-out vesicles suggest the involvement of a passive carrier (a monocarboxylate carrier?), activity of which possibly masks the activity of multidrug resistance proteins.

Transport of CF and CAL was inhibited by about 50% by metabolic depletion, which indicates at least partial contribution of an active transport system. MRP1 activity modulators exerted a rather weak effect on CF efflux from intact erythrocytes. Contrary to these results, inside-out vesicle studies show clear ATP-dependent (i.e., active) accumulation of CF that is vulnerable to a typical set of organic anion transport inhibitors. However, MRP1 is not involved in the transport of carboxyfluorescein, as dye accumulation was insensitive to preincubation with QCRL3 antibody, a highly specific inhibitor of MRP1 transport activity. Also MRP1-overexpressing

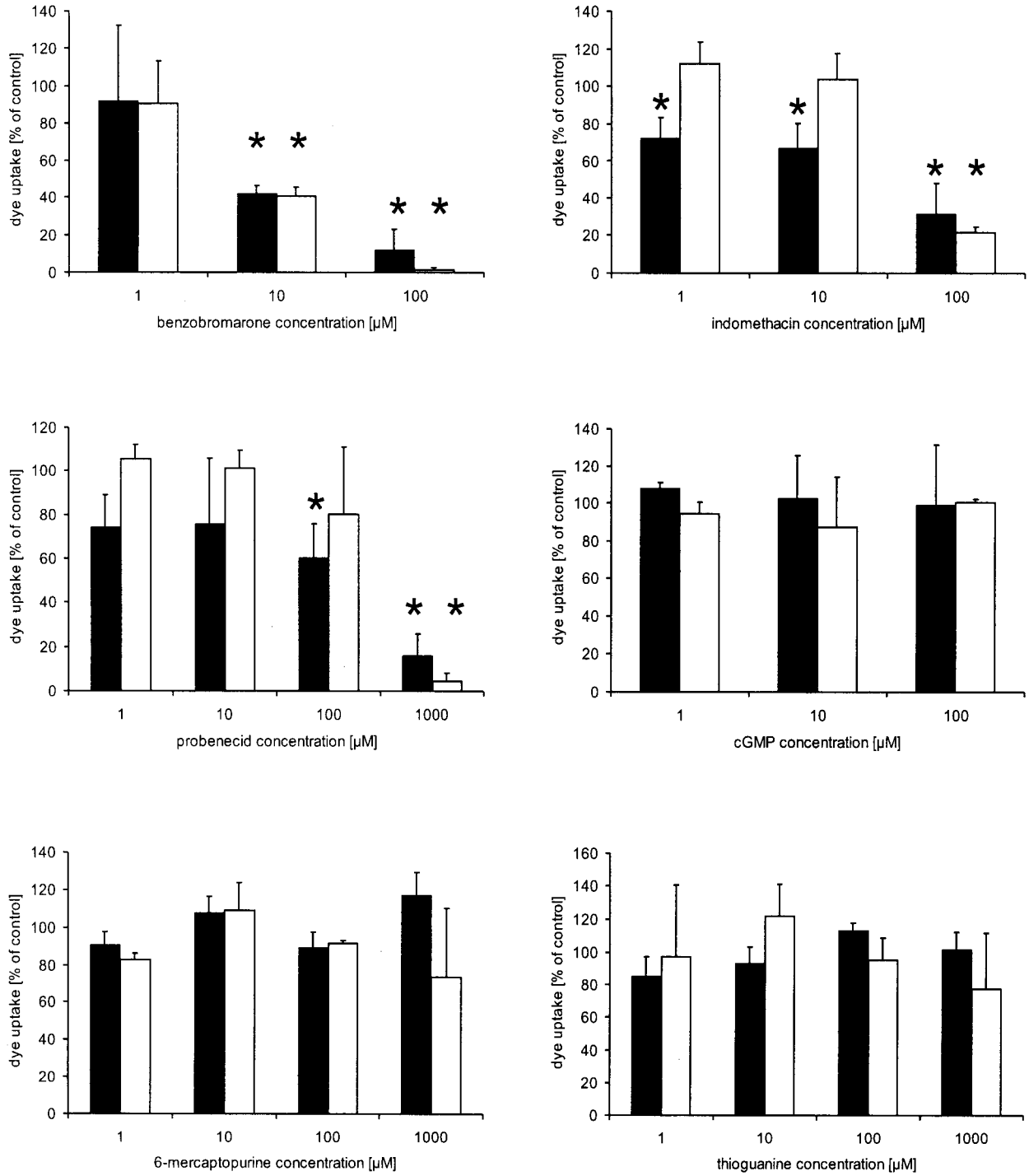

Fig. 5. Effect of selected inhibitors on CF (*black bars*) and BCECF (*white bars*). ATP-dependent uptake in inside out vesicles. Averaged data ± SD from two independent experiments, each performed in triplicate. Asterisks mark statistically significant effects.

cells do not extrude CF significantly faster than parental cell lines.

Efflux of CF was diminished in erythrocytes preincubated with 1-chloro-2,4-dinitrobenzene, which suggests that it interacts with at least one of the systems of glutathione S-conjugate transport in human erythrocytes. Two such systems, discerned by affinity towards glutathione S-conjugates, exist in the human erythrocyte membrane [2, 6] (for review, *see* [37]). The high-affinity/low-capacity system was ascribed to MRP1 [48], while the identity of the low-affinity/high-capacity system remains obscure. It may be due to the activity of another MRP identified so far in human erythrocyte membrane, viz. MRP5, and some results seem to support this hypothesis. Uptake of cyclic GMP, a model substrate of MRP5, by inside-out vesicles derived from human red blood cells has been relatively well described by the group of

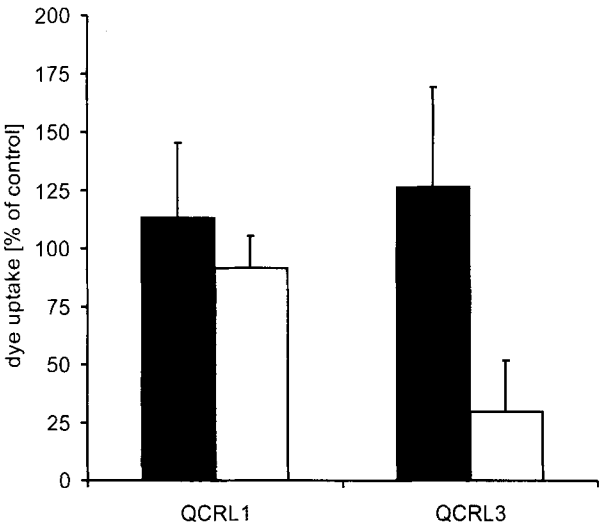

Fig. 6. Influence of monoclonal antibodies on CF (*black bars*) and BCECF (*white bars*). ATP-dependent accumulation in inside-out vesicles. Membrane vesicles were preincubated with monoclonal antibodies at protein ratio 1000:1 for one hour at room temperature. Prior to experiments, a commercial antibody solution was dialyzed against 250 mM sucrose, 0.1 mM $EDTANa_2$, 0.5 mM TrisHCl, pH 8.0, to diminish the ionic strength that might interfere with membrane experiments. Averaged data ± SD from two independent experiments, each performed in triplicate. Effect of QCRL3 on BCECF uptake is statistically significant (variance analysis, $P < 0.05$).

Sager [38, 40]. It exhibits an inhibition profile resembling that of MRP5 [44]. This uptake is inhibited by high (hundred micromolar) concentrations of glutathione S-conjugates [43], and some of its other characteristics (pH optimum, affinity towards ATP [8, 43]) may suggest similarity of this transport system with the low-affinity/high-capacity system of glutathione S-conjugate transport.

CF accumulation in inside-out vesicles was non-competitively inhibited by 1 μM 2,4-dinitro-S-glutathione, but no interaction between CF and the high-affinity/low-capacity transport system of glutathione S-conjugate transport in erythrocytes was detected. This supports the hypothesis that MRP1 is not involved in carboxyfluorescein transport in human red blood cells. Weak non-competitive inhibition of the low-affinity/high-capacity transport system may suggest some interaction of CF with the carrier responsible for that phenomenon. However, the participation of MRP5 in CF uptake is also questionable, as cGMP as well as 6-mercaptopurine and thioguanine did not interfere with CF accumulation.

Verapamil exerted an interesting stimulatory effect on both CF efflux from intact erythrocytes and on its accumulation in inside-out vesicles. This compound, although itself not a substrate for MRP1, stimulates in a concentration-dependent manner glutathione transport by this protein. Its inhibitory effect on the transport of some MRP1 substrates such as leukotriene C_4, 17-β-estradiol, 17-β-D-glucuronate and vincristine, is visible only in presence of glutathione or its S-methyl derivative [33]. Verapamil probably binds to a pocket in the transporter molecule, which triggers the transport cycle but to which glutathione (and/or 5-carboxyfluorescein) is not able to bind. This hypothesis explains also an inhibitory effect of verapamil on the transport of other drugs by competition for this key site in the transporter molecule. However, it is unlikely that the stimulatory effect of verapamil may be attributed to triggering the activity of MRP1 towards CF, because no verapamil-dependent stimulation of CF efflux from HL60ADR cells was observed (Rychlik, unpublished).

Results obtained in this study for BCECF confirm the thesis that this dye is a substrate for MRP1. Metabolic depletion practically abolished BCECF efflux from intact cells. All MRP1 modulators tested exerted a strong inhibitory effect on the BCECF transport. The inhibition profile of BCECF efflux from intact erythrocytes resembled that reported for another fluorescein derivative: 2′,7′-bis-(2-carboxypropyl)-5/6-carboxyfluorescein [10].

BCECF uptake by inside-out vesicles was clearly ATP- and concentration-dependent. Preincubation with QCRL3 inhibited BCECF accumulation by about 70%. Mutual non-competitive inhibition of BCECF uptake by 1 μM 2,4-dinitrophenyl-S-glutathione and of the high-affinity/low-capacity glutathione S-conjugate transport system by 10 μM BCECF further supports this hypothesis. The lack of an effect of cGMP, thioguanine or 6-mercaptopurine on BCECF uptake excludes participation of MRP5 in the dye transport. However, significant non-competitive inhibition of the low-affinity/high-capacity system of glutathione S-conjugates suggests interaction of BCECF with a yet unidentified organic anion transport system of human red blood cell.

The most intriguing results were obtained for calcein. Transport of this fluorescent dye by multidrug resistance-related proteins has been intensively studied. However, multidrug resistance proteins probably do not play an important role in the transport of CAL across the human erythrocyte membrane. Its efflux from intact red blood cells was biphasic. The initial rapid phase was almost insensitive to metabolic depletion and MRP1 inhibitors. After about five minutes the efflux rate was markedly diminished but became vulnerable to the transport inhibitors tested. The coincidence of transport rate depression time point with the half-life for calcein acetoxymethyl ester in the extracellular milieu may suggest that the effect observed was due to significant inhibition of the free acid efflux by the ester form that accumulates in cells and that is a better substrate for MRP1 [16]. This is, however, unlikely, due to the fact that washing the cells after ester loading had no effect

Table 4. Effect of 25 μM CF and 10 μM BCECF on glutathione S-conjugate transport system in human erythrocyte membrane

	HA[1]	HA + CF	LA[2]	LA + CF
Apparent K_m [μM]		*	620 ± 396	614 ± 211
V_{max} [nmole/(mg protein × min)]		*	1.41 ± 0.86	1.00 ± 0.33
	HA	HA + BCECF	LA	LA + BCECF
Apparent K_m [μM]	0.78 ± 0.12	0.79 ± 0.04	658 ± 226	736 ± 671
V_{max}[nmole/(mg protein × min)]	0.45 ± 0.04	0.38 ± 0.01	3.2 ± 1.1	1.1 ± 1.0

[1] HA: high-affinity/low-capacity system; [2] LA: low-affinity/high-capacity system. Averaged data from two independent experiments.
* No effect of CF on the high-affinity system of glutathione S-conjugate transport was observed for a CF concentration range from 5 up to 100 μM.

on the shape of the time curve of CAL efflux (*not shown*). It is probable that the initial phase of the calcein efflux from human erythrocytes is mediated by a carrier whose contribution to the net efflux diminishes after achievement of a certain dye concentration in the extracellular medium. If calcein was transported via an active system, one would expect ATP-dependent accumulation of the dye in inside-out vesicles. However, we were not able to reproducibly find such an effect for 25 μM CAL within 60 minutes. As some reports suggest a low affinity of MRP1 towards CAL (apparent Michaelis constant of about 270 μM [16]), we decided to increase the CAL concentration up to 1 mM. No clear ATP-dependence was observed in these conditions. We were not able to observe enhanced CAL uptake into inside-out vesicles even after co-administration of an up to 40-fold excess of glutathione.

We expected to find more similarities between transport characteristics of fluorescein and its carboxyl derivatives in our system. There are reports concerning the ability of MRP1 to transport all of the investigated dyes [13, 17, 42, 46, 47]. However, most of the studies employed MRP1-overexpressing cells, which could mask activity of other transport systems present in the plasma membrane. Human erythrocytes, although highly specialized in terms of cytoskeleton and cytoplasm organization, ensure a membrane composition that may be typical for a normal (i.e., not genetically engineered) cell. Thus, they may serve as a model suitable for determination of whether an investigated drug is a predominant substrate of MRP1 (or MRP5) or whether other transport systems are able to interfere with the results. Our results emphasize the importance of highly specific inhibitors of organic anion transporters, such as monoclonal antibodies, that are able to discern between different carrier proteins exhibiting similar pharmacological profiles. Care should be taken when discriminating the ability of a protein to transport a specific drug or marker on the basis of inhibition profile. Our study clearly shows the possibility that even tumor cells that do not express multidrug resistance-related proteins may give false positive results in tests employing carboxyfluorescein or calcein, which may have severe clinical consequences.

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