FEBS Letters 391 (1996) 126–130 FEBS 17327

Transport of the glutathione conjugate of ethacrynic acid by the human multidrug resistance protein MRP

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Received 20 June 1996

Abstract The multidrug resistance protein MRP has been shown to mediate the transport of glutathione *S*-conjugates across membranes. In this study we demonstrate that the glutathione *S*-conjugate of the diuretic drug ethacrynic acid, which is an efficient inhibitor of glutathione *S*-transferases, is a high-affinity substrate and inhibitor of the glutathione *S*-conjugate pump associated with MRP. This implies that ethacrynic acid may modulate drug resistance of tumor cells not only by inhibiting glutathione *S*-transferase activity, but also by inhibiting the export of drug conjugates from the cell by MRP.

Key words: Multidrug resistance; Glutathione *S*-transferase; Alkylating drug; Ethacrynic acid

1. Introduction

The human multidrug resistance-associated protein MRP confers resistance to natural product drugs by lowering their intracellular concentration [1–3]. Although the resistance spectrum of MRP resembles that of *MDR1* P-glycoprotein (Pgp), there are several indications that the mechanism of drug transport by MRP is fundamentally different from that of Pgp. (i) Increased expression of *MRP* is associated with increased ATP-dependent transport of glutathione *S*-conjugates (GS-X) into isolated membrane vesicles [4–6] and from living cells [7]. (ii) MRP requires GSH to export anti-cancer drugs and arsenite out of the cell [8]. (iii) Drug transport by MRP is relatively insensitive to classic reversal agents of Pgp-mediated multidrug resistance (MDR), such as verapamil and cyclosporin (analogues) [2,3], but is modulated by inhibitors of organic anion transport, such as probenecid and sulfinpyrazone [6,7,9,10]. These data suggest that MRP is a GS-X pump [11] and link MRP-mediated MDR to drug resistance associated with GSH and GSH *S*-transferases (GSTs) [5,8,12].

GSTs are a family of isoenzymes with broad substrate specificity that catalyze the conjugation of the tripeptide GSH to many xenobiotics [13]. Conjugation to GSH improves the solubility of these substrates and may facilitate their removal from the cell by GS-X pumps. In addition, GSTs have been suggested to serve as intracellular sinks and carrier proteins for certain hydrophobic and electrophilic molecules [13–15]. Increased cellular amounts of GST isoenzymes are associated with resistance against alkylating agents and doxorubicin [12]. The diuretic drug ethacrynic acid (EA) is a potent inhibitor of the activity of GSTs and has been used to study the role of GSTs in drug resistance in cell lines [16], in colon tumor xenografts [17] and in a phase I clinical trial to improve the efficacy of the alkylating drug thiotepa [18]. Moreover, EA inhibited the GST-catalyzed conjugation of GSH with the clinically important alkylating drug chlorambucil [19]. EA can modulate drug resistance in several ways. (i) EA decreases intracellular GSH levels. (ii) Both EA and its conjugate (EA-SG) are reversible inhibitors of GSTs of the α, μ and π subclasses [20,21]. (iii) EA can also bind covalently to GSTπ [20,21].

Here we show that GS-EA is an efficient substrate of the GS-X pump associated with *MRP*-expression and that it can inhibit dinitrophenylglutathione (DNP-SG) uptake in membrane vesicles containing MRP. This implies that EA may modulate drug resistance in tumor cells not only by decreasing GST activity, but also by inhibiting the export of drug conjugates from the cell by MRP.

2. Materials and methods

2.1. Chemicals and enzymes

Dinitrophenyl[*glycine*-2-^{3}H]glutathione (DNP-[^{3}H]SG) with specific activity of 370 GBq/mmol was a gift from Dr. R.P.J. Oude Elferink (Academic Medical Center, University of Amsterdam). The radioactive conjugate of EA [2,3-dichloro-4-(2-methylene-1-oxobutyl)phenoxy]acetic acid was chemically synthesized from [^{14}C]EA (0.555 GBq/mmol; Amersham, Bucks, UK) and GSH (Sigma Chemical Co., St. Louis, MO) and purified by high-performance liquid chromatography (HPLC) as described previously [20,21]. 4-Glutathionyl cyclophosphamide was synthesized by adding 2 mmol 4-hydroperoxycyclophosphamide (kind gift from Dr. J. Pohl, Asta Medica, Frankfurt am Main, Germany) to 20 mmol GSH in 400 ml of 70 mM phosphate buffer, pH 7.0. After incubation for 2 h at 25°C the 4-glutathionyl cyclophosphamide was purified from the mixture by HPLC as described [22]. GST isoenzymes were purified from rat liver (GST1-1 and GST3-3) and rat kidney (GST 7-7) by affinity chromatography with *S*-hexylglutathione-Sepharose 6B and chromatofocussing [23]. Purity was confirmed by SDS-polyacrylamide gel electrophoresis, isoelectric focussing and HPLC [23,24].

2.2. Cell lines and yeast strains

S1(MRP) is a subline of the human non-small cell lung cancer cell line SW-1573/S1 that overexpresses *MRP* 15-fold [3,8]. This cell line was obtained by transfection of S1 cells with an expression vector containing *MRP* cDNA and a neomycin-resistance gene and selection with Geneticin (G418) [3]. GLC4/ADR is a subline of the human small cell lung cancer cell line GLC4 that overexpresses *MRP* 50–100-fold [3,5]. This cell line was isolated by a multistep selection of GLC4 cells up to 1152 nM doxorubicin [3,5]. The yeast strain DTY168 (*Matα, ura3-52, ycf1Δ::his, leu2-3, -112, his6*) [25] was used for heterologous expression of the MRP cDNA [6].

2.3. Preparation of membrane vesicles

Membrane vesicles from human lung cancer cells were prepared as described by Müller et al. [5]. Cell monolayers of S1 and S1(MRP)

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PII S0014-5793(96)00718-1

(10^9 cells) were washed twice, scraped into ice-cold phosphate-buffered saline (PBS) and collected by centrifugation at 1000×*g* for 10 min at 4°C. GLC4 and GLC4/ADR cells were harvested by centrifugation and washed twice with PBS. The cell pellets were resuspended in 50 ml of a hypotonic buffer (1 mM Tris-HCl, pH 7.0) and stirred gently for 2 h on ice in the presence of 400 units of recombinant *Serratia marcescens* nuclease (Benzonase, grade I, protease-free; Merck Darmstadt, Germany) and the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), leupeptide (2 μg/ml), pepstatin (1 μg/ml), and aprotinin (2 μg/ml). The cell lysate was centrifuged at 100 000×*g* for 30 min at 4°C, and the resulting pellet was suspended in 10 ml of isotonic TS buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4) and homogenized with a Dounce B homogenizer (glass/glass, tight pestle, 30 strokes) in the presence of Benzonase (800 units) and protease inhibitors. The crude membrane fraction was layered on top of a 38% (w/v) sucrose solution in 5 mM Tris-HEPES (pH 7.4) and centrifuged in a Beckman SW41 rotor at 280 000×*g* for 2 h at 4°C. The turbid layer at the interface was collected, diluted to 20 ml with TS buffer, and centrifuged at 100 000×*g* for 30 min at 4°C. The resulting pellet was suspended in 400 μl of TS buffer. Vesicles were formed by passing the suspension 30 times through a 25-gauge needle with a syringe. The membrane vesicles were finally frozen in liquid nitrogen and stored at −70°C until used in transport assays. Protein concentration was determined using a Bio-Rad protein assay (Bio-Rad, Richmond, CA).

Membrane vesicles from *S. cerevisiae* cells were prepared as described by Tommasini et al. [6]. In short, cells were grown overnight in SD medium (0.7% (w/v) yeast nitrogen base without amino acids (Difco), 0.5% (w/v) casamino acids (Difco), 2% (w/v) glucose) to an OD_{600} of approx. 10. The cells were harvested by centrifugation, washed with water, and resuspended in the original culture volume in 1.1 M sorbitol, 20 mM Tris-HCl (pH 7.6), 1 mM dithiotreitol, containing 57 units of lyticase (Sigma, St. Louis) per ml. After incubation for 90 min at 30°C, spheroplasts were collected by centrifugation. The spheroplasts were resuspended in homogenization buffer (1.1 M glycerol, 50 mM Tris-ascorbate (pH 7.4), 5 mM EDTA, 1.5% (w/v) polyvinylpyrrolidone K30 (Fluka, Buchs, Germany) and disrupted in a dounce homogenizer. Unbroken cells and cell debris were removed by centrifugation at 4000×*g* for 10 min. The supernatant was centrifuged at 150 000×*g* for 45 min. The pellet was resuspended at an OD_{600} of 4 in suspension buffer (1.1 M glycerol, 50 mM Tris-Mes (pH 7.4), 1 mM EDTA), frozen in liquid nitrogen, and stored at −70°C.

2.4. Vesicle uptake studies

Transport of DNP-[^{3}H]SG and [^{14}C]EA-SG was measured by a rapid filtration technique using nitrocellulose filters (0.45 μm pore size; Schleicher and Schuell) presoaked in TS buffer. Membrane vesicles from lung cancer cells were rapidly thawed and pre-incubated for 15 s at 25°C for DNP-[^{3}H]SG uptake experiments, or at 37°C for [^{14}C]EA-SG uptake experiments. The reaction was started by adding the labeled substrate. The reaction buffer was TS supplemented with 4 mM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate and 100 μg/ml creatine kinase (Boehringer Mannheim). The final volume was 110 μl. Samples of 20 μl (Fig. 1) or 33 μl (other experiments) were taken at the indicated time points and diluted in 1 ml of ice-cold TS buffer. This solution was applied to the pre-soaked filters and rinsed with 5 ml of TS buffer. In control experiments for DNP-[^{3}H]SG transport ATP was omitted; in control experiments for [^{14}C]EA-SG transport ATP was replaced by AMP-PNP (4 mM). Each filter was placed in liquid scintillation fluid, and radioactivity was measured by liquid scintillation counting.

Uptake of [^{14}C]EA-SG in yeast microsomal vesicles was measured essentially as described above with the following modifications. One part of the vesicles was mixed with six parts of transport solution (0.4 M glycerol, 100 mM KCl, 20 mM Tris-Mes, pH 7.4) and incubated at 25°C in the presence of 5 mM MgATP and 2 μM [^{14}C]EA-SG. Samples of 100 μl were taken at the indicated time points.

3. Results

We examined the ability of EA-SG and of a metabolite of the alkylating drug cyclophosphamide, i.e. 4-glutathionyl cyclophosphamide (4-GS-CP) [22], to inhibit the ATP-dependent transport of DNP-[^{3}H]SG into plasma membrane vesicles isolated from the *MRP*-overexpressing lung cancer cell line S1(MRP) [3]. The ATP-dependent uptake of DNP-[^{3}H]GS in vesicles of S1(MRP) cells was approx. 8-fold greater than in vesicles from parental S1 cells (i.e. 8.3 ± 3.6-fold; $n = 4$), in agreement with previous experiments with these cells [5]. Among the compounds tested, EA-SG was the most potent inhibitor of DNP-[^{3}H]SG transport (Table 1). EA-SG inhibited transport 2000-fold more effectively than probenecid and 400-fold more effectively than sulfinpyrazone. Inhibition of transport by 4-GS-CP also occurred, but only at relatively high concentrations (Table 1).

The transport of EA-SG was characterized using ^{14}C-labeled EA-SG. Vesicles of S1(MRP) cells showed time- and MgATP-dependent accumulation of [^{14}C]EA-SG (Fig. 1A). The transport rate correlated with the level of MRP. In the drug-selected lung cancer cell line GLC4/ADR, containing greater amounts of MRP than S1(MRP) [3], the transport rate was also higher (Fig. 1B). The transport rates in S1(MRP) and GLC4/ADR were 7- and 20-fold higher than

Table 1
Effect of inhibitors on ATP-dependent uptake of DNP-[^{3}H]SG (10 μM) into plasma membrane vesicles from S1(MRP) cells[a]

Inhibitor		Relative uptake (%)[c]
–		100
GSH	(1 mM)	83 ± 4
Probenecid	(10 mM)	−17 ± 13
	(1 mM)	55 ± 4
Sulfinpyrazone	(2 mM)	9 ± 5
	(400 μM)	56 ± 5
GSH *S*-conjugate of ethacrynic acid	(100 μM)	−17 ± 7
	(10 μM)	4 ± 30
	(1 μM)	61 ± 40
	(100 nM)	95 ± 12
	(10 nM)	120 ± 14
4-Glutathionyl cyclophosphamide	(1 mM)	44 ± 21
	(100 μM)	73 ± 11
AMP[b]	(4 mM)	−4 ± 7

[a]S1(MRP) membrane vesicles were incubated at 25°C for 3 min as described in Section 2.
[b]ATP was replaced by AMP.
[c]ATP-dependent uptake = $uptake_{ATP} - uptake_{-ATP}$. Values are means ± S.D. of 3 independent experiments, with each determination (+ and −ATP) performed in triplicate.

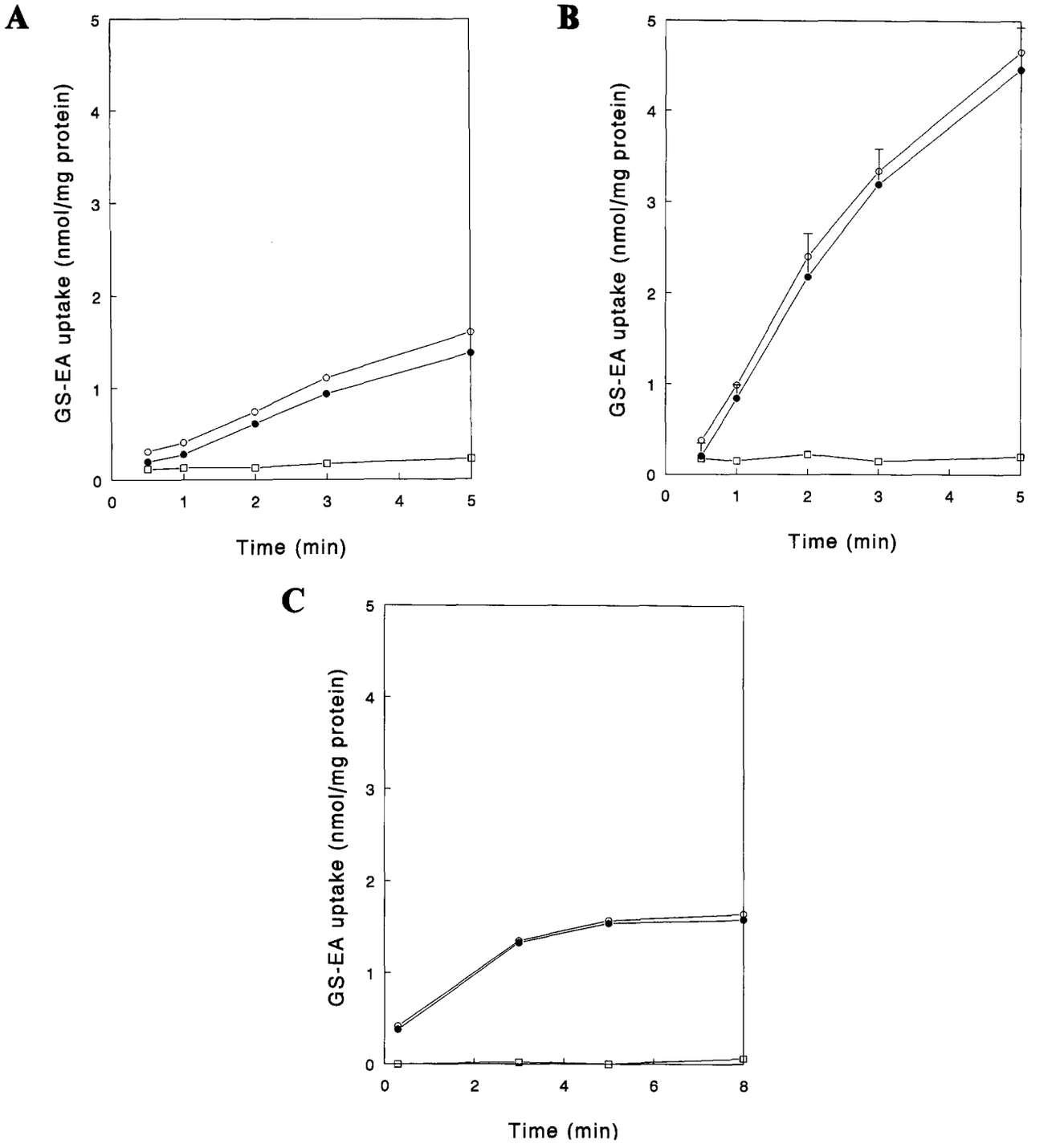

Fig. 1. Time course of uptake of [^{14}C]EA-SG into plasma membrane vesicles from human lung cancer cells overexpressing *MRP* (S1(MRP) and GLC4/ADR cells), and microsomal vesicles from yeast cells expressing *MRP* (DTY168(MRP) cells). Uptake of 10 μM [^{14}C]EA-SG into S1(MRP) (A), GLC4/ADR (B), or of 2 μM [^{14}C]EA-SG into DTY168(MRP) (C) vesicles in the presence of MgATP (○) or of MgAMP (□). ATP-dependent transport (●) = $\text{values}_{ATP} - \text{values}_{AMP}$. Means of three independent experiments are shown, with each determination performed in duplicate.

in the respective parental cell lines S1 and GLC4 (results from parental cell lines not shown). The K_m value calculated for [^{14}C]EA-SG transport in membrane vesicles from S1(MRP) cells was 28 ± 4 μM, with a V_{max} of 505 ± 68 pmol mg protein^{-1} min^{-1} (Figs. 2 and 3). These values are similar to those for DNP-[^{3}H]SG [5,6], suggesting that EA-SG and DNP-SG are transported with equal efficiencies by MRP. Unmodified EA (10 μM) was taken up into membrane vesicles of lung cancer cells, but uptake was independent of MgATP and was not increased in the *MRP*-overexpressing cell lines, indicating that unmodified EA is not a substrate of MRP (results not shown).

We recently showed that expression of human *MRP* cDNA in a *S. cerevisiae* mutant strain with a disrupted YCF1 gene

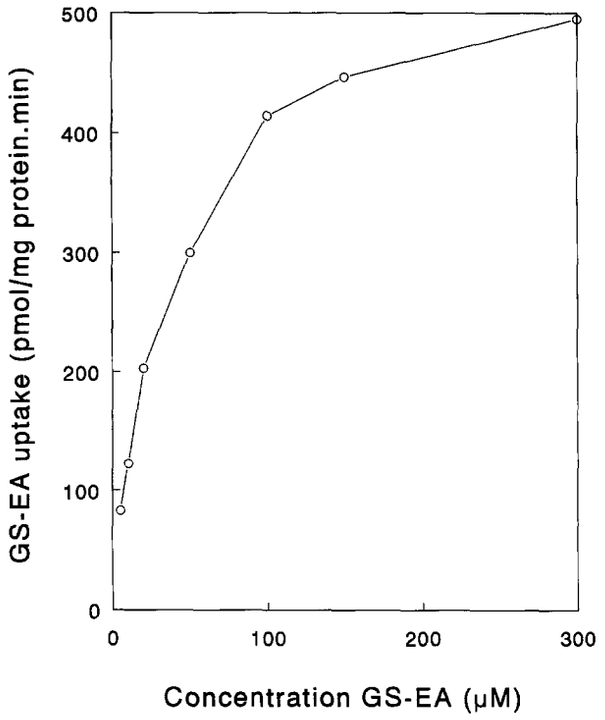

Fig. 2. Concentration dependence of ATP-dependent uptake of [^{14}C]EA-SG into membrane vesicles from S1(MRP) cells. S1(MRP) vesicles were incubated for 3 min at 37°C. ATP-dependent transport (○) = values$_{ATP}$ − values$_{AMP}$. Means of 3 independent experiments, with each determination (+ATP and +AMP) performed in duplicate.

(strain DTY168 [25]) was able to restore the uptake of DNP-SG in microsomal vesicles isolated from DTY168 [6]. Vesicles from DTY 168 cells expressing *MRP* cDNA (strain DTY168(MRP) [6]) also showed time- and MgATP-dependent uptake of [^{14}C]EA-SG (Fig. 1C). The transport rate was 11-fold higher than in DTY168 cells (results not shown). The apparent K_m value was 5 μM for EA-SG in microsomal vesicles from DTY168(MRP) cells, with a V_{max} of 1250 pmol mg protein^{-1} min^{-1}.

The uptake of [^{14}C]EA-SG into S1(MRP) membrane vesicles was inhibited by probenecid, sulfinpyrazone and oxidized glutathione (GSSG), but not by reduced glutathione (GSH) (Table 2). EA-SG has been shown to inhibit GST-catalyzed conjugation reactions [15–21]. However, addition of purified rat GST isoenzymes 1-1, 3-3 and 7-7 (10 μM) had no effect on the time-dependent uptake of [^{14}C]EA-SG into membrane vesicles (results not shown). We conclude that the binding of EA-SG to GSTs [20,21] does not influence the transport of this conjugate by MRP.

Table 2
Effect of inhibitors on ATP-dependent uptake of [^{14}C]EA-SG (10 μM) into plasma membrane vesicles from S1(MRP) cells[a]

Inhibitor		Relative uptake (%)[b]
–		100
GSH	(1 mM)	95
GSSG	(1 mM)	60
Probenecid	(1 mM)	53
Sulfinpyrazone	(400 μM)	44

[a]S1(MRP) membrane vesicles were incubated at 37°C for 3 min as described in Section 2.
[b]ATP-dependent uptake = uptake$_{ATP}$ − uptake$_{AMP}$. Values are means of 2 independent experiments, with each determination performed in triplicate.

4. Discussion

Our data show that EA-SG is a substrate and a high-affinity inhibitor of the GS-X pump associated with *MRP* expression. This implies that EA may modulate cellular drug resistance not only by decreasing GST activity, but also by inhibiting the export of drug conjugates from cells by MRP. Consistent with this, EA increased the accumulation of the fluorescent dye calcein acetoxy methyl ester in tumor cells containing increased amounts of MRP [10]. Furthermore, resistance against EA in a colon cancer cell line has been associated with increased GSH, GST and *MRP* mRNA levels, suggesting that all three components may act together in EA resistance [26].

The K_m value for EA-SG transport in vesicles from lung cancer cells or from yeast cells expressing *MRP* is in the same range as for DNP-SG (i.e. 5–30 μM). Also, our inhibition experiments with DNP-[^{3}H]SG suggest that EA-SG and DNP-SG are transported with equal efficiencies by MRP. However, [^{14}C]EA-SG gave almost no background binding to nitrocellulose filters, which is a problem with DNP-[^{3}H]SG, as well as with another model substrate of GS-X pumps, LTC_4. [^{14}C]EA-SG may therefore be the most suitable substrate for routine screening of compounds for their capacity to inhibit transport by MRP.

There has been considerable speculation in the literature on the transport mechanism of ABC transporters, such as P-glycoprotein or MRP. One of the possibilities discussed is that these transporters affect drug distribution in an indirect fashion, e.g. by influencing another transporter or by affecting ion transport. The fact that MRP is able to transport EA-SG

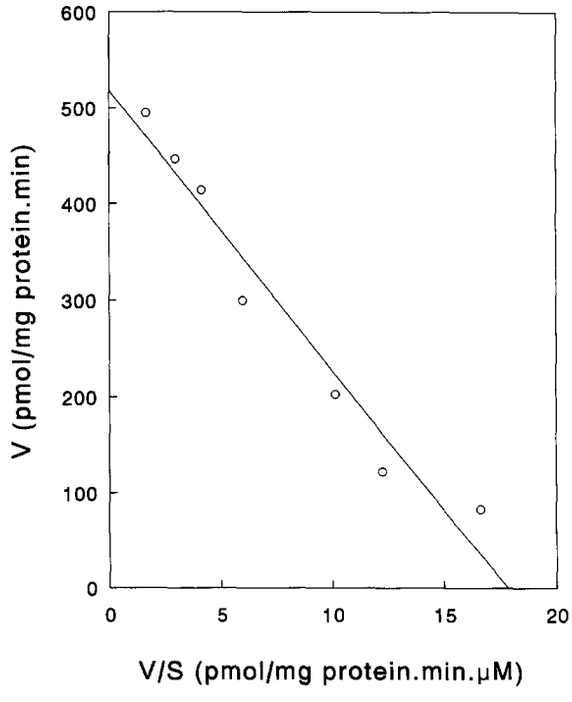

Fig. 3. Eadie-Hofstee plot of values of Fig. 2.

both in the physiological context of the mammalian membrane and in the unphysiological context of a yeast membrane provides support to the interpretation that MRP directly transports EA-SG over the membrane itself.

It is unclear whether, and to what extent, the inhibition of GS-X transport by GS-EA may have contributed to the modulatory effect of ethacrynic acid on drug resistance in cell lines and patients [16–19]. The biochemical effects of ethacrynic acid on cells are diverse, and also include depletion of GSH levels, inhibition/reaction with GSTs and probably other enzymes containing sulfhydryl groups [20,21]. However, since GS-EA is effective in inhibiting GS-X transport at concentrations that are well below those toxic to cancer cell lines in tissue culture [16,27], the inhibition of transport by GS-EA may be relevant in vivo.

4-GS-CP inhibited the GS-X pump activity associated with *MRP* expression only at relatively high concentrations. This conjugate is therefore unlikely to be a substrate of MRP under physiological conditions, but may be a substrate of other GS-X pumps, such as the canalicular multispecific organic anion transporter (cMOAT) [28]. cMOAT is a homologue of MRP that is mainly present in the canalicular membrane of hepatocytes where it mediates the excretion of organic anions into bile [28]. Some multidrug resistant tumor cell lines overexpress *cMOAT*, however, suggesting that cMOAT may also play a role in drug resistance (Kool, M., et al., unpublished data). Transfection experiments are in progress to test this possibility.

Acknowledgements: This work was supported in part by a collaborative project of the University of Amsterdam and The Netherlands Cancer Institute (to P.B.), by the Dutch Cancer Society (TNOV 92-93 to P.J.B., and NKI 92-41 to P.B.), and the European Union (ERB-CIPACT 930265 to P.B and A.H. Schinkel).

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