

## Inhibition of the Multidrug Resistance Protein 1 (MRP1) by Peptidomimetic Glutathione-Conjugate Analogs

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### ABSTRACT

Inhibition of multidrug resistance protein 1 (MRP1) mediated cytostatic drug efflux might be useful in the treatment of drug resistant tumors. Because the glutathione (GSH) conjugate of ethacrynic acid (EA), GS-EA, is a good substrate of MRP1, GS-EA derivatives are expected to be good inhibitors of MRP1. To study structure-activity relationships of MRP1 inhibition, a series of novel GS-EA analogs was synthesized in which peptide bonds of the GSH backbone were replaced by isosteric groups [*Bioorg Med Chem* **10**:195-205, 2002]. Several of these compounds were effective inhibitors of MRP1-mediated [<sup>3</sup>H]GS-EA and [<sup>3</sup>H]E<sub>2</sub>17βG transport, as studied in membrane vesicles prepared from MRP1-overproducing Sf9 cells. The modifications of the peptide backbone have distinct implica-

tions for recognition by MRP1: the γ-glutamyl-cysteine peptide bond is important for binding, whereas the cysteinyl-glycine amide does not seem essential. When the γ-glutamyl-cysteine peptide bond (C-CO-N) is replaced by a urethane isostere (O-CO-N), an effective competitive MRP1-inhibitor ( $K_i = 11 \mu\text{M}$ ) is obtained. After esterification of this compound to improve its cellular uptake, it inhibited MRP1-mediated efflux of calcein from 2008 ovarian carcinoma cells overexpressing MRP1. This compound also partially reversed the resistance of these cells to methotrexate. Because the urethane isostere is stable toward γ-glutamyl transpeptidase-mediated breakdown, it is an interesting lead-compound for the development of in vivo active MRP1 inhibitors.

Multidrug resistance (MDR) may hamper the efficacy of cytostatic drugs in cancer treatment. The resistance of tumor cells is often the result of the enhanced ability of these cells to impair efficacy of cytostatics through increased elimination by phase II and III metabolism [drug conjugation and drug efflux, respectively, mediated by, for instance, P-glycoprotein and multidrug resistance protein 1 (MRP1)] (Saves and Masson, 1998; Litman et al., 2001; Borst and Oude Elferink, 2002).

MRP1 is a member of the ATP-binding cassette transporter proteins (Ishikawa et al., 2000); it transports a broad range of substrates across cellular membranes (Jedlitschky et al., 1994; Leier et al., 1994; Muller et al., 1994). The preferred substrates of MRP1 are anionic products of phase II metabolism, such as sulfate-, glucuronide- and glutathione (GSH)-conjugates (for reviews, see Hipfner et al., 1999; König

et al., 1999; Borst et al., 2000). MRP1 can mediate efflux of several unconjugated hydrophobic drugs, such as vincristine, by cotransport with GSH or in a GSH-stimulated fashion (Loe et al., 1998; Renes et al., 1999). Leukotriene C<sub>4</sub> (LTC<sub>4</sub>), a GSH-conjugate, is the substrate with the highest affinity for MRP1 (Leier et al., 1994; Hipfner et al., 1999; König et al., 1999), and murine MRP1 has a physiological role as transporter of LTC<sub>4</sub> and drugs in vivo (Muller et al., 1994; Wijnholds et al., 1997, 2000; Johnson et al., 2001).

The structural elements that contribute to the affinity of a molecule for MRP1 are not clearly defined, but recognition is partly determined by the number and spatial distribution of anionic residues (Seelig et al., 2000). The presence of positively charged arginine and lysine residues in the membrane-spanning domains of MRP1 (Seelig et al., 2000; Ito et al., 2001b) may aid in transmembrane transport of the charged substrates. GSH conjugates have at least two carboxylate residues, which contribute to recognition by MRP1. Recently, new glutathione conjugates have been used as inhibitors of MRP1 in inside-out membrane vesicles (Furuta et al., 1999). After esterification to improve their cellular uptake, these compounds were potent MRP1 inhibitors in MRP1-overexpressing HL60 cells (Furuta et al., 1999; Ishikawa et al.,

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**ABBREVIATIONS:** MDR, multidrug resistance; MRP1, multidrug resistance (associated) protein; GSH, glutathione; γGT, γ-glutamyl transpeptidase; GST, glutathione S-transferase; EA, ethacrynic acid; MTX, methotrexate; AM, acetoxymethyl ester; E<sub>2</sub>17βG, estradiol 17-β-glucuronide; TS, tris-sucrose; PBS, phosphate-buffered saline; LTC<sub>4</sub>, leukotriene C<sub>4</sub>.

2000). A disadvantage of these compounds is their intrinsic sensitivity toward breakdown by  $\gamma$ -glutamyl transpeptidase ( $\gamma$ GT), an enzyme that is highly expressed in the kidney and in a variety of other cell types (Hanigan, 1998). In vivo use of these compounds, therefore, may be limited.

For several years, we have been involved in the development of GSH conjugate analogs to study substrate recognition and inhibition of glutathione *S*-transferase (GST) in vivo (Ouwerkerk and Mulder, 1998, and references therein). Recently we employed peptidomimetic strategies to obtain compounds that structurally resemble GSH but in which the peptide bonds were replaced by isosteric groups (Burg et al., 2002). Thus, a series of GSH analogs was obtained that differed only slightly from the parent compound (Fig. 1). These changes to the tripeptide backbone resulted in large differences in inhibition of rat liver cytosolic GSTs, and yielded three compounds that were stabilized toward  $\gamma$ GT (Fig. 1; **VI** and **VII** are completely stable, **IV** is slowly hydrolyzed) (Burg et al., 2002). We conjugated these compounds to ethacrynic acid (EA) to obtain potent inhibitors of GST. Be-

cause the GSH-conjugate of EA is a good substrate of MRP1 (Zaman et al., 1996), this series of new compounds could potentially also be good MRP1 substrates and competitive inhibitors.

Because of its instability (it can dissociate from the GSH-sulfhydryl by retro-Michael reaction) and lack of selectivity between MRP1 and other GSH-conjugate binding proteins, the EA moiety is not ideally suited as drug candidate. The aim of this study was therefore to test our panel of GS-EA analogs in MRP1 transport, with the objective of finding a suitable GSH analog as lead compound for the development of novel MRP1 inhibitors. Furthermore, this series of GS-EA analogs was used to probe the GSH-conjugate binding site in MRP1.

Using vesicular drug transport, we found that the peptide bond modifications have distinct consequences for substrate recognition by MRP1. Deviations from the parent compound in MRP1 inhibition clearly show the participation of the peptide bonds in GSH in the enzyme-substrate recognition. We selected one of the  $\gamma$ GT stable compounds, a urethane peptidomimetic, for inhibition studies in MRP1 expressing 2008 cells, because it showed MRP1-inhibition characteristics similar to those of GS-EA. After esterification to increase its cellular uptake, this compound inhibited MRP1 mediated calcein-efflux in intact cells. In addition, resistance of 2008/MRP1 cells toward methotrexate (MTX) could be partially reversed by coincubation with this compound.

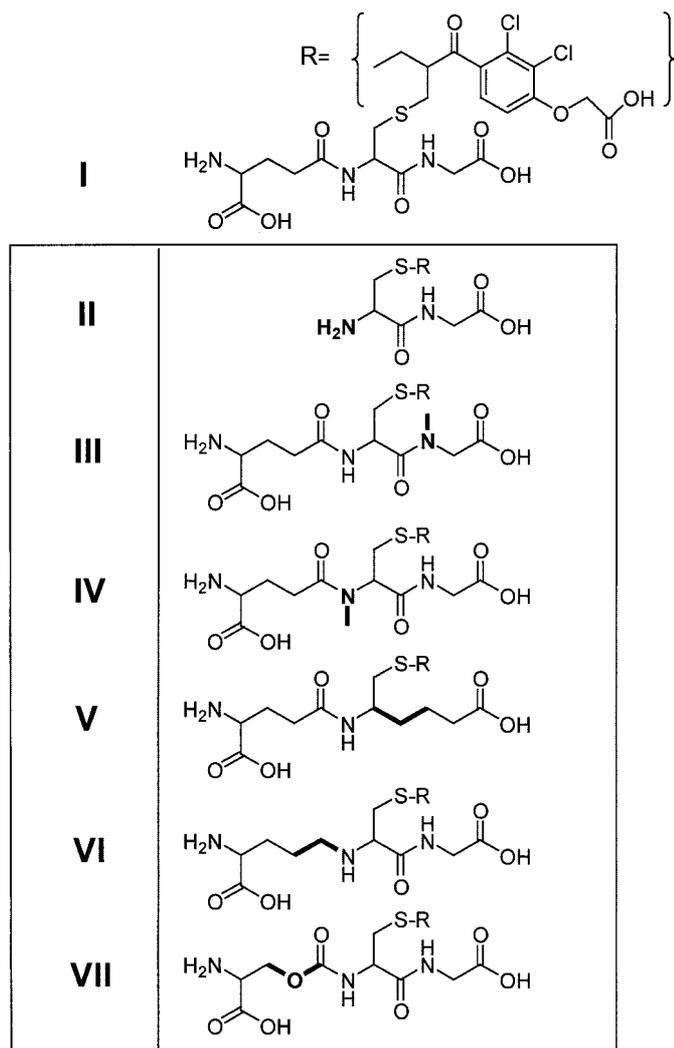
## Materials and Methods

**Materials.** All solvents were of analytical grade and were stored on molecular sieves when necessary. Ethacrynic acid was obtained from Sigma (St. Louis, MO). Methotrexate (L-amethopterin) and chlorotrimethylsilane were purchased from Acros Chemie (Beerse, Belgium). Calcein-acetoxymethyl ester (AM) was from Molecular Probes (Leiden, The Netherlands). [Gly2-<sup>3</sup>H]GSH and [<sup>3</sup>H]estradiol 17- $\beta$ -glucuronide ([<sup>3</sup>H]E<sub>2</sub>17 $\beta$ G), were obtained from NEN life science products (Boston, MA, USA). Cell culture media and supplements were from Invitrogen (Paisley, Scotland).

**Cell Culture.** The human ovarian carcinoma cell lines 2008/P (parental) and its stable MRP1 transfectant 2008/MRP1 were described previously (Hooijberg et al., 1999; Kool et al., 1999). Cells were cultured in RPMI 1640 medium containing 25 mM HEPES and 2 mM L-glutamine, supplemented with 10% heat-inactivated fetal calf serum and 100  $\mu$ g/ml penicillin/streptomycin. Cells were grown at 37°C in a humidified 5% CO<sub>2</sub> atmosphere.

**Synthesis.** We recently described the synthesis of the novel GS-EA mimics (Burg et al., 2002). All compounds were racemic at C <sub>$\alpha$</sub>  of the EA moiety and were more than 90% pure, as determined by high-performance liquid chromatography with mass-spectrometric analysis. For experiments with intact cells, a membrane-permeable analog of compound **VII** was prepared by esterification of the free carboxylic acid residues with chlorotrimethylsilane in dry methanol according to Brook and Chan (1983). The compound was purified by gel filtration on a Sephadex LH20 column, using methanol/water (9:1, v/v) as eluent. High-performance liquid chromatography with mass-spectrometric analysis showed that the product contained mainly the dimethyl ester (**VII**-dimethyl ester) and low amounts of monomethyl-esters, which may also be cell-permeable. Which of the three carboxylic acid residues were esterified could not be established.

[<sup>3</sup>H]GS-EA was prepared by conjugation of EA with [Gly2-<sup>3</sup>H]GSH; [<sup>3</sup>H]GSH (250  $\mu$ Ci, 0.12 nmol) was dissolved in 0.5 ml of acidified H<sub>2</sub>O, pH 3, and extracted with ethyl acetate to remove excess dithiothreitol. Ethacrynic acid (10 nmol), dissolved in 0.5 ml of



**Fig. 1.** Structures of GS-EA (**I**) and its peptide bond modified analogs. Sites of deviation from the parent compound GS-EA (**I**) are indicated in bold. Compounds **IV**, **VI**, and **VII** are stabilized toward  $\gamma$ GT mediated hydrolysis (Burg et al., 2002). In structure **I**, the *R*-group (ethacrynic acid) is indicated in brackets.

EtOH, was added and the pH adjusted to 7.5 with saturated aqueous NaHCO<sub>3</sub>. The solution was stirred for 4 h at 37°C. The product was separated from unreacted EA by preparative TLC on silica coated glass plates, using *n*-propanol/water (7:1, v/v) as eluent. After visualization by autoradiography, [<sup>3</sup>H]GS-EA was scraped from the plate and extracted from silica with water. Nontritiated GS-EA (compound I, Fig. 1) was added to obtain a 1 mM stock solution (verified by UV absorbency at 270 nm,  $\epsilon_{270} = 5.7 \text{ mM}^{-1}\text{cm}^{-1}$ ).

**Vesicular Transport.** *Spodoptera frugiperda* (Sf9) insect cells were infected with a baculovirus containing MRP1-cDNA according to Bakos et al. (1998). Preparation of inside-out membrane vesicles from these cells was performed according to Zelcer et al. (2001). MRP1-mediated [<sup>3</sup>H]GS-EA transport into the inside-out vesicles was determined by a rapid filtration technique using cellulose membrane filters (0.45- $\mu\text{m}$  pore size; Schleicher & Schüll, Dassel, Germany) presoaked in TS buffer (250 mM sucrose and 50 mM Tris/HCl, pH 7.4). The reaction mixture consisted of 4 mM ATP (or AMP), 10 mM MgCl<sub>2</sub>, 10 mM creatine phosphate, 100  $\mu\text{g/ml}$  creatine kinase, and 1.5  $\mu\text{M}$  [<sup>3</sup>H]GS-EA with or without inhibitor in TS buffer (total volume, 20  $\mu\text{l}$ ). After prewarming for 2 min at 37°C, the reaction was initiated by addition of the membrane vesicles (10  $\mu\text{g}$  of protein). After 2 min at 37°C, the reaction was stopped by addition of 1 ml of ice-cold TS and the mixture was subsequently applied to the TS presoaked membrane filters and washed twice with 3 ml of TS. The filters were dissolved in liquid scintillation fluid and <sup>3</sup>H content was determined by liquid scintillation counting. The concentration at which 50% transport-inhibition occurred (IC<sub>50</sub>) was determined by incubation of 1.5  $\mu\text{M}$  [<sup>3</sup>H]GS-EA with various amounts (1–25  $\mu\text{M}$ ) of the inhibitors. Identical inhibition experiments were performed with various concentrations of compound VII, using 1  $\mu\text{M}$  [<sup>3</sup>H]E<sub>2</sub>17 $\beta$ G as substrate.

Kinetic parameters for MRP1 inhibition by compound VII were determined by incubating the vesicles with various concentrations of [<sup>3</sup>H]GS-EA (2.5–200  $\mu\text{M}$ ) in the presence of different concentrations of inhibitor (1–25  $\mu\text{M}$ ). To determine ATP-dependent substrate uptake, results from identical experiments performed in the presence of ATP and AMP. The *K<sub>i</sub>* value was determined by nonlinear regression analysis (Prism; GraphPad Software, Inc., San Diego, CA) of the transport-velocity (*V*) at the different substrate concentrations (*S*), via the *K<sub>m,app</sub>* method described by Kakkar et al. (1999).

**Calcein Efflux from Cells.** Confluent monolayers of 2008/P and 2008/MRP1 cells in six-well polyethylene culture dishes were loaded with 1 mM calcein-AM for 30 min at 37°C in 1.5 ml of incubation buffer (136 mM NaCl, 5.3 mM KCl, 1.1 mM KH<sub>2</sub>PO<sub>4</sub>, 0.8 mM MgSO<sub>4</sub>, 1.8 mM CaCl<sub>2</sub>, 11 mM D-glucose, and 10 mM HEPES, pH 7.4). The calcein-containing incubation buffer was then replaced by 1.5 ml of buffer, containing various concentrations of VII-dimethyl ester. After 90 min, calcein content of the incubation buffer was determined by spectrofluorometry using an HTS7000 Bioassay reader (excitation, 485 nm; emission, 535 nm; PerkinElmer Life Sciences, Boston, MA). After completion of the experiment, cells were detached by trypsinization, lysed by ultrasonication, and protein content was determined by the Bradford protein assay.

Time course experiments were performed in a similar way; after 30 min of preincubation with calcein-AM, the incubation buffer was replaced by buffer (1.5 ml) containing 30  $\mu\text{M}$  VII-dimethyl ester or 1 mM probenecid, a well known inhibitor of MRP1. Samples (100  $\mu\text{l}$ ) of the incubation medium were taken at indicated times and stored immediately on ice. After completion of the experiment, cells were washed with ice-cold PBS. Cell-viability was above 85%, as measured by Trypan-blue exclusion.

**Growth Inhibition Assays.** Cells were seeded at 10<sup>4</sup> cells/well in 24-well polyethylene culture dishes (Greiner Bio-One GmbH, Frickenhausen, Germany). After overnight attachment, culture medium was replaced by serum-free medium containing VII-dimethyl ester or probenecid (final concentrations, 25 and 500  $\mu\text{M}$ , respectively) and the indicated concentrations of methotrexate (MTX) were added to the cells. After 4 h at 37°C, cells were washed twice with PBS and

TABLE 1

Inhibition of MRP1 mediated [<sup>3</sup>H]GS-EA transport by GS-EA analogs. IC<sub>50</sub> values ( $\pm$ S.D.) were determined for inhibition of MRP1-mediated, ATP-dependent, vesicular [<sup>3</sup>H]GS-EA transport by the GS-EA analogs. Membrane vesicles (10  $\mu\text{g}$  of protein) were incubated with 1.5  $\mu\text{M}$  [<sup>3</sup>H]GS-EA in the presence of various concentrations of inhibitor. IC<sub>50</sub> values indicate the inhibitor concentration at which 50% reduction of [<sup>3</sup>H]GS-EA transport was obtained.

Inhibitor	IC <sub>50</sub>
	$\mu\text{M}$
I	1.1 $\pm$ 0.2
II	— <sup>a</sup>
III	1.7 $\pm$ 0.4
IV	22 $\pm$ 5
V	3.7 $\pm$ 0.7
VI	27 $\pm$ 5
VII	1.6 $\pm$ 0.5

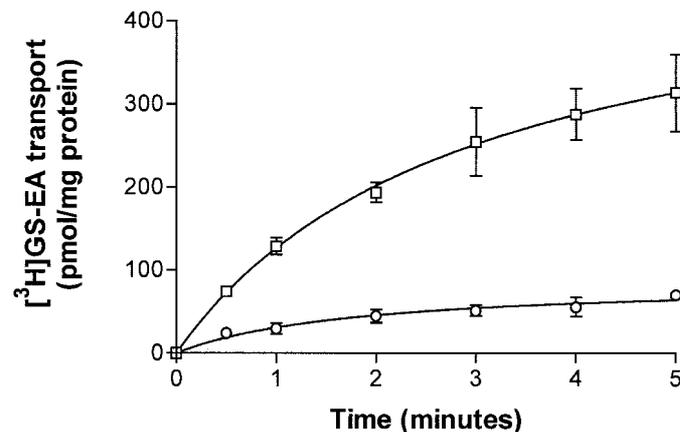
<sup>a</sup> No inhibition (highest concentration tested was 200  $\mu\text{M}$ ).

subsequently incubated for 72 h in normal culture medium. Cell proliferation was measured by DNA content, using Hoechst 33258 staining according to Rago et al. (1990). In short, the cells were rinsed once with PBS and lysed by repeated freeze-thaw cycles in 200  $\mu\text{l}$  of water, followed by homogenization on a rotary shaker. Hoechst 33258 (50  $\mu\text{l}$  of 20  $\mu\text{g/ml}$  in 10 mM Tris, 1 mM EDTA, 0.2 M NaCl, pH 7.4) was then added to 50  $\mu\text{l}$  of lysate. Stained DNA was measured by spectrofluorometry (excitation, 360 nm; emission, 465 nm). A calibration curve of calf thymus DNA was used to determine total DNA quantities.

## Results

**Inhibition of MRP1-Mediated [<sup>3</sup>H]GS-EA Transport in Sf9-Membrane Vesicles.** To establish [<sup>3</sup>H]GS-EA as substrate for MRP1 in the Sf9/MRP1 vesicular transport system, the time-dependent uptake of this substrate into inside-out membrane vesicles was investigated in the presence of ATP or AMP (Fig. 2). ATP strongly increased [<sup>3</sup>H]GS-EA transport, indicative of ABC-transporter dependent glutathione-conjugate transport. ATP-dependent [<sup>3</sup>H]GS-EA transport in vesicles prepared from wild-type Sf9 cells was less than 10% of the transport found for Sf9/MRP1 vesicles (data not shown). The *K<sub>m</sub>* value for [<sup>3</sup>H]GS-EA transport was 13  $\pm$  2  $\mu\text{M}$ , which was comparable with values found by others (5–28  $\mu\text{M}$ ) (Zaman et al., 1996).

IC<sub>50</sub> values (Table 1) were determined to compare the inhibitory potency of the different GS-EA analogs (depicted

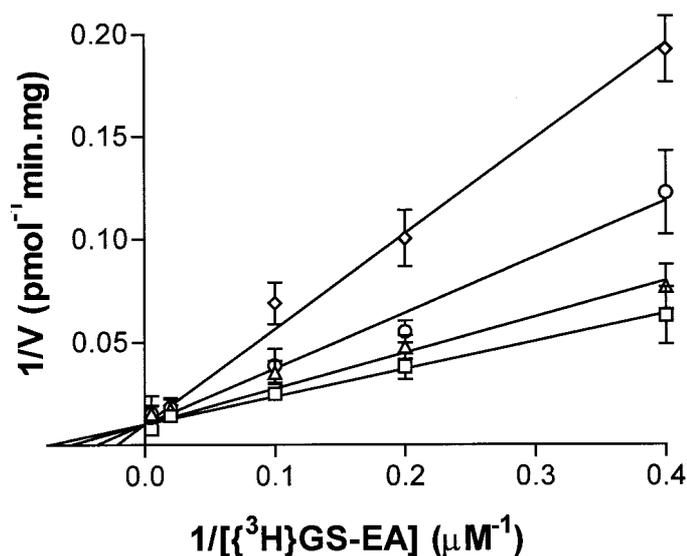


**Fig. 2.** ATP-dependent uptake of [<sup>3</sup>H]GS-EA in Sf9/MRP1 membrane vesicles. Vesicles were incubated with 1.5  $\mu\text{M}$  [<sup>3</sup>H]GS-EA at 37°C in the presence of ATP ( $\square$ ) or AMP ( $\circ$ ). Experiments were performed in triplicate with duplicate measurements. Shown are the mean values ( $\pm$  S.E.M.).

in Fig. 1). The compounds **III** and **VII** were as active as the native glutathione-ethacrynic acid conjugate (**I**). The EA-conjugated Cys-Gly dipeptide **II** showed no inhibition at the highest concentration tested (200  $\mu\text{M}$ ). The methylation of the Cys-Gly amide nitrogen in compound **III** had little effect, because it is still a good MRP1 inhibitor. In contrast, when the  $\gamma$ -glutamyl amide nitrogen was methylated (compound **IV**), much of the inhibitory potency is lost; its  $\text{IC}_{50}$  value was 20-fold higher than that of **I**. The  $\text{IC}_{50}$  of **V** is three-fold higher than **I**, indicating that omission of the Cys-Gly peptide-bond (**V**) affected the affinity for MRP1. Removal of the carbonyl-function of the  $\gamma$ -Glu-Cys amide, yielding a reduced isostere (**VI**), led to an even larger decrease of inhibition, because the  $\text{IC}_{50}$  value increased approximately 25-fold. Replacement of the glutamate C $\gamma$  by an oxygen atom (**VII**) resulted in a urethane-linkage; this biomimetic seemed to be a good MRP1 inhibitor, with an  $\text{IC}_{50}$  value similar to that of GS-EA, but **VII** has the advantage of being  $\gamma\text{GT}$  stable (Burg et al., 2002).

**Kinetic Analysis of Inhibition of MRP1 Transport.** **VII** was one of the most efficient MRP1 inhibitors in this series of novel GS-EA analogs and is also stable toward  $\gamma\text{GT}$ -mediated breakdown (Burg et al., 2002). Therefore, we selected this compound for further evaluation. To determine the inhibition characteristics of **VII** toward MRP1, we tested the inhibitor in uptake experiments with inside-out vesicles. The Lineweaver-Burk plot (Fig. 3) shows, as expected, that **VII** is a competitive inhibitor of MRP1-mediated [ $^3\text{H}$ ]GS-EA transport. The  $K_i$  value determined for **VII** ( $K_i = 11 \pm 1.5 \mu\text{M}$ ) was similar to the  $K_m$  value determined for GS-EA (13  $\mu\text{M}$ ). The urethane peptidomimetic **VII** therefore has equal affinity for MRP1 as GS-EA.

**Inhibition of MRP1-Mediated [ $^3\text{H}$ ]Estradiol-Glucuronide Transport by **VII**.** MRP1 may possess more than one substrate binding-site. It is possible, therefore, that the novel GSH-conjugate analog **VII** inhibits only the binding of



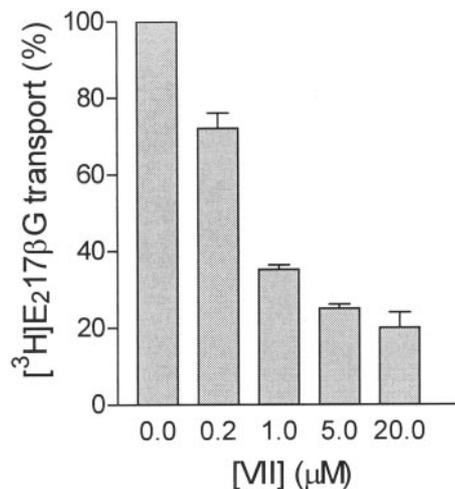
**Fig. 3.** Lineweaver-Burk plot of MRP1 inhibition by compound **VII**. Vesicles were incubated with the indicated concentrations of [ $^3\text{H}$ ]GS-EA in the presence of various concentrations of inhibitor **VII**: 0 ( $\square$ ), 5 ( $\triangle$ ), 10 ( $\circ$ ), and 25  $\mu\text{M}$  ( $\diamond$ ). [ $^3\text{H}$ ]GS-EA uptake velocities in Sf9/MRP1 membrane vesicles were determined in the presence of ATP or AMP. Shown are ATP-dependent (ATP-AMP) uptake values. Experiments were performed three times in duplicate; depicted are the mean values ( $\pm$  S.E.M.).

structurally related compounds and not of other substrates, such as glucuronides. We, therefore, also evaluated the inhibitory potency of compound **VII** on vesicular transport of an alternative substrate: [ $^3\text{H}$ ]Estradiol 17- $\beta$ -glucuronide (see Fig. 4). **VII** also proved to be a potent ( $\text{IC}_{50} = 0.3 \pm 0.04 \mu\text{M}$ ) inhibitor of MRP1-mediated transport of estradiol 17- $\beta$ -glucuronide (Fig. 4).

**Inhibition of Calcein Transport in Intact Cells.** Fluorescent calcein is formed intracellularly after esterase-mediated hydrolysis of nonfluorescent calcein-AM and subsequently exported out of the cytosol by MRP1 (Hollo et al., 1994). Compared with the 2008/P cell line, which has a low (but detectable) amount of MRP1, 2008/MRP1 cells show a strongly increased level of the transporter protein (Kool et al., 1997; Hooijberg et al., 1999). To determine MRP1 inhibition by **VII** in intact cells, a membrane-permeable analog was prepared by esterification and was tested for inhibition of MRP1-mediated calcein transport. This **VII**-dimethyl ester caused a concentration-dependent inhibition of calcein efflux from both 2008 cell lines (Fig. 5). The  $\text{IC}_{50}$  value for inhibition of calcein transport from 2008/MRP1 cells by **VII**-dimethyl ester was  $36 \pm 5 \mu\text{M}$  (mean  $\pm$  S.D.).

To further evaluate MRP1 inhibition, the efflux of calcein in time was studied in the absence of inhibitor, or in the presence of **VII**-dimethyl ester or probenecid (as positive control). The efflux of calcein in the absence of inhibitor was much higher in 2008/MRP1 cells than in the parental 2008/P cell line (Fig. 6). Addition of 30  $\mu\text{M}$  **VII**-dimethyl ester strongly reduced the calcein efflux to the level of parental cells (Fig. 6,  $\circ$ ). Addition of 1 mM probenecid reduced the calcein transport in the 2008/MRP1 cells even further (Fig. 6,  $\times$ ). Both **VII**-dimethyl ester and probenecid decreased the calcein-efflux in 2008/P cells to background levels, because of inhibition of endogenous MRP1.

**Modulation of MTX Cytotoxicity by MRP1 Inhibition.** The 2008/MRP1 cells used in this study are highly resistant toward short-term exposure to MTX (Hooijberg et al., 1999; Kool et al., 1999) (Fig. 7). Coincubation with **VII**-



**Fig. 4.** Inhibition of [ $^3\text{H}$ ]estradiol 17- $\beta$ -glucuronide transport by compound **VII**. Vesicles were incubated with 1  $\mu\text{M}$  [ $^3\text{H}$ ]E $_2$ 17 $\beta$ G and the indicated concentrations of **VII** at 37°C and collected by filtration. Experiments were performed in triplicate with duplicate measurements in the presence of ATP or AMP. Shown are the mean values ( $\pm$  S.D.) of the ATP-dependent transport (corrected for transport in the presence of AMP).

TABLE 2

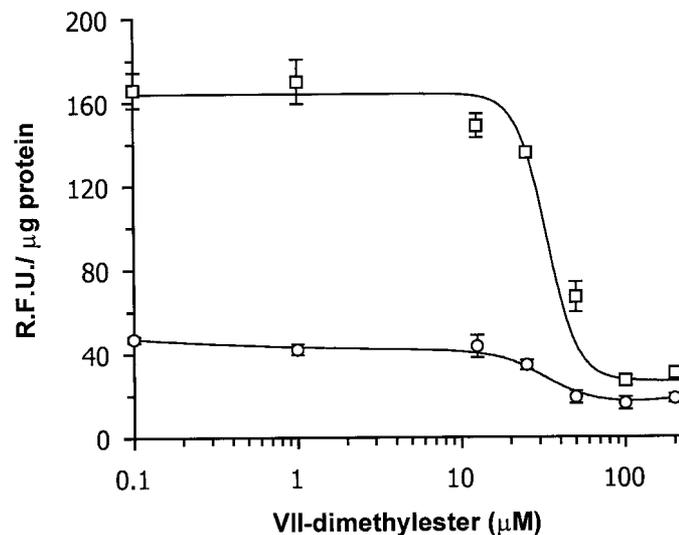
Enhanced methotrexate induced growth inhibition through MRP1 inhibition.

2008/P and 2008/MRP1 cells were exposed to different concentrations of MTX in the presence of VII-dimethyl ester (25  $\mu$ M) or probenecid (500  $\mu$ M). Cell proliferation was determined after 72 h. IC<sub>50</sub> ( $\pm$ S.D.) was determined by graphical analysis of the obtained growth curves (see Fig. 7).

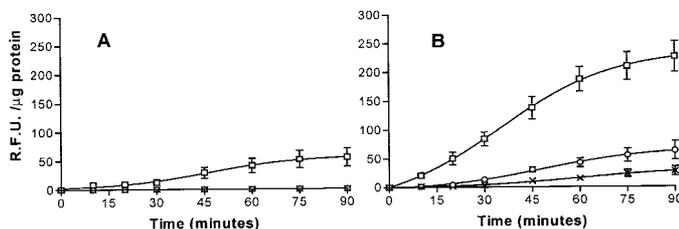
	IC <sub>50</sub>		RF <sup>a</sup>
	2008/P	2008/MRP1	
No Inhibitor	36 $\pm$ 3	612 $\pm$ 68	17
VII-dimethyl ester	28 $\pm$ 3	116 $\pm$ 7	4
Probenecid	28 $\pm$ 2	53 $\pm$ 36	2

<sup>a</sup> Relative resistance factor; ratio of IC<sub>50</sub> values of 2008/MRP1 and 2008/P.

dimethyl ester (25  $\mu$ M) or probenecid (0.5 mM) during the 4 h of MTX exposure partially reversed the drug-resistant phenotype, as indicated by the decrease in IC<sub>50</sub> values and relative resistance factors (Table 2). Both VII-dimethyl ester and probenecid strongly sensitized 2008/MRP1 cells toward MTX, whereas 2008/p cells were only slightly more responsive. The concentration of VII-dimethyl ester used was not



**Fig. 5.** Inhibition of calcein efflux by VII-dimethyl ester. 2008/P (○) and 2008/MRP1 (□) cells were loaded with 1 mM calcein-AM for 30 min at 37°C. Medium was then replaced by incubation buffer without calcein-AM, containing VII-dimethyl ester. After 90 min, calcein fluorescence of the incubation medium was measured by spectrofluorometry. Calcein fluorescence is given in arbitrary fluorescence units per microgram of total cellular protein. Shown are mean values ( $\pm$  S.E.M.) of three separate experiments.



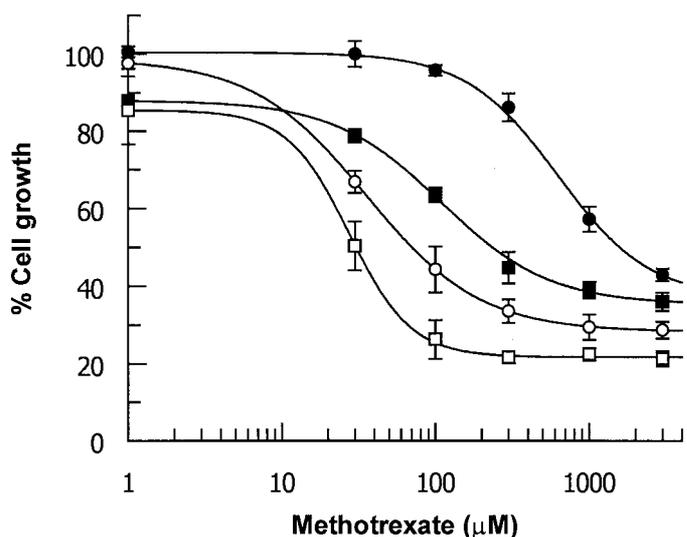
**Fig. 6.** Time course of calcein efflux from intact 2008 cells. 2008/P (A) and 2008/MRP1 (B) cells were loaded with 1 mM calcein-AM. Medium was then replaced by buffer without inhibitor (□), with 30  $\mu$ M VII-dimethyl ester (○), or with 1 mM probenecid (×). At indicated time-points, samples of the incubation medium were taken and analyzed by spectrofluorometry. Data are given as arbitrary fluorescence units per microgram of total protein. Shown are mean values ( $\pm$  S.E.M.) from three separate experiments.

sufficient to completely overcome MRP1 mediated MTX resistance. Concentrations higher than 25  $\mu$ M could not be used, because at a concentration of 50  $\mu$ M, the VII-dimethyl ester alone was cytotoxic; cell survival of both cell lines (in the absence of MTX) was only 40% (data not shown).

## Discussion

Although MRP1 has striking effects on drug resistance in vitro, its relevance in clinical MDR remains to be defined (Litman et al., 2001; Borst and Oude Elferink, 2002). Many compounds inhibit MRP1 in vitro but they are not clinically applicable. We therefore evaluated a recently synthesized series of GS-EA analogs as MRP1 inhibitors, some of which are stabilized toward  $\gamma$ -glutamyl transpeptidase and thus may be stable in circulation.

MRP1 mediated transport of unconjugated and conjugated compounds, such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol-*O*-glucuronide and vincristine is facilitated by GSH (Loe et al., 1998; Leslie et al., 2001b). *S*-Methyl-GSH and ophthalmic acid, a non-thiol-containing GSH analog, can replace GSH as transport-enhancer (Leslie et al., 2001b). This elegantly emphasizes that anionic moieties, rather than the reducing capacity of the sulfhydryl, are required for the transport-modulating properties of GSH. MRP1 transports many anionic phase II metabolites (Leslie et al., 2001a), which suggests that the substrate binding-site can accommodate a variety of negatively charged structures (Seelig et al., 2000). It has recently been stated that each substrate might have its own individual binding mode within the multipartite binding pocket of the protein (Leslie et al., 2001b). Our data, however, show that recognition of GSH-conjugates by MRP1 is more restricted. Slight changes in the tripeptide backbone result in pronounced differences in inhibition, which may indicate that the recognition of the GSH-conjugates requires a certain bioactive conformation of the tripeptide. This would also imply that the protein forms a defined binding site for GSH-conjugates. Within this binding site, electrostatic inter-



**Fig. 7.** Growth of 2008 cells after treatment with MTX. 2008/P (open symbols) and 2008/MRP1 (closed symbols) cells were exposed during 4 h to various concentrations of methotrexate, in the absence of inhibitor (circles), or in the presence of 25  $\mu$ M VII-dimethyl ester (squares). After 72 h, cell proliferation was determined by DNA content. Experiments were performed in triplicate ( $\pm$  S.E.M.).

actions of the anionic groups and the  $\gamma$ -glutamyl-cysteine peptide bond with the protein are the main contributors to substrate recognition. It is unlikely that the lipophilic thioether moiety of the substrate contributes to binding affinity; it has been shown that it is not required for the transport-modulating interaction of GSH with MRP1 (Leslie et al., 2001b). GSH itself is a poor substrate of MRP1, which implies that the lipophilic thioether, although not crucial for binding, is necessary to facilitate the transmembrane efflux of the GSH-conjugate. For example, the capacity of MRP1 to transport *S*-alkyl-GSH analogs correlates well with the length of the alkyl-chain (Ishikawa, 1989; Ishikawa et al., 1989). The lipophilic portion of GSH-conjugates presumably forms hydrophobic interactions (van der Waals and  $\pi$ -orbital stacking interactions) with hydrophobic residues in MRP1 and thereby influences the transport characteristics (Seelig et al., 2000; Ito et al., 2001a).

The importance of the  $\gamma$ -glutamyl moiety of GSH for recognition by MRP1 is shown by compound **II**, which did not inhibit MRP1 at all. Although this dipeptide still has two carboxylic acid groups, it is not recognized by MRP1. This is remarkable, because the cysteinyl-glycine conjugate LTD<sub>4</sub>, obtained after  $\gamma$ GT-mediated cleavage of LTC<sub>4</sub>, is transported by MRP1, albeit 4-fold less efficiently than the parent compound (Leier et al., 1994).

As was seen for dipeptide **II**, the  $\gamma$ -glutamyl portion of GSH is crucial for inhibition. Although the C <sub>$\beta$</sub> →O replacement (**VII**) is accepted by MRP1, methylation of the  $\gamma$ -Glu-Cys amide N (**IV**) results in a large decrease in inhibition. The introduction of the methyl group rigidifies the conformation of the peptide, which is usually beneficial because it reduces entropic contributions. However, the spatial shape of the tripeptide may not be optimal for MRP1 binding. This amide nitrogen may also act as an H-bond donor in enzyme-substrate interactions. Similarly, the reduced amide isostere **VI** is a poor MRP1 inhibitor. At physiological pH, the "reduced" amide nitrogen is protonated, thereby carrying a positive charge, which may result in impaired recognition by MRP1. Removal of the H-bond-accepting function of the  $\gamma$ -Glu-Cys amide carbonyl may also lead to disturbed recognition.

The role of the Cys-Gly peptide bond in enzyme-substrate interactions is shown by compounds **III** and **V**. The increased steric bulk and the resulting conformational change of the tripeptide, caused by the methyl-group in **III**, can be accommodated in the active site. Because **III** has lost only little of its inhibitory potency, the H-bonds of the Cys-Gly amide nitrogen are probably not important for substrate recognition. The complete omission of the Cys-Gly peptide bond in compound **V** is also accepted by MRP1, although it is a less efficient inhibitor than **I**. This may be the result of the increased flexibility of the ethylene moiety, which is unfavorable because of increased entropic effects. Disturbance of electrostatic enzyme-substrate interactions can also cause the decrease in inhibition. The inhibition characteristics of **V** indicate that the contribution of the Cys-Gly amide oxygen is also minor. Overall, the Cys-Gly peptide bond may be involved only in maintaining the GSH tripeptide in an optimal bioactive conformation.

Although the  $\gamma$ -glutamyl moiety is an important structural requirement for recognition, small changes in its structure are accepted by MRP1. Compound **VII** is a good inhibitor, as indicated by its relatively low  $K_i$  value (11  $\mu$ M). The intro-

duction of an additional oxygen atom in the  $\gamma$ -glutamyl moiety results in a urethane-type peptide bond. This linkage displays increased rigidity because of the participation of the oxygen atom in mesomeric effects with the directly bonded CO-NH. The bond angle of the urethanic O-C-N moiety is about 7° less than the C-C-N bond. Furthermore, the urethane group is somewhat less planar than the amide and has distinctly different H-bonding properties (Benedetti et al., 1980). Although its physicochemical characteristics are different from the normal peptide, compound **VII** is very well accepted by MRP1. Previous work (Burg et al., 2002) already showed that **VII** is stable toward  $\gamma$ GT, which is a crucial property for a MRP1 inhibitor to be used in vivo. Hence, of all the peptidomimetic GS-EA analogs used in this study, **VII** was the most promising. The urethane isostere proved to be a competitive inhibitor of MRP1 toward GS-EA; it may therefore be an MRP1-substrate itself.

Because MRP1 may contain more than one substrate binding site, it is possible that the novel GSH conjugate analogs do not inhibit MRP1-mediated transport of other substrates. We therefore also evaluated the effect of **VII** toward the structurally unrelated MRP1 substrate estradiol 17- $\beta$ -glucuronide. The inhibitor also potently reduced MRP1 mediated transport of this substrate (Fig. 4).

To test whether **VII** inhibited MRP1 in intact cells, we prepared a membrane-permeable dimethyl ester derivative of **VII**. This **VII**-dimethyl ester inhibited calcein efflux in MRP1-transfected 2008/MRP1 cells and in parental 2008/P cells, which are known to contain some MRP1 (Fig. 5 and 6) (Kool et al., 1997). In addition, the **VII**-dimethyl ester also partially reverted MTX resistance of 2008/MRP1 cells (Fig. 7, Table 2).

These preliminary experiments show that this membrane-permeable version of **VII** can be used to inhibit MRP1 in intact cells. Yet because concentrations higher than 30  $\mu$ M could not be used without toxicity, the compound was not as effective as 1 mM probenecid (Fig. 6B), a known inhibitor of MRP1. In short-term experiments, high concentrations (100–200  $\mu$ M) of **VII**-dimethyl ester were able to completely reverse MRP1 transport (Fig. 5). In long-term experiments, it was impossible to use concentrations above 50  $\mu$ M without inhibiting cell proliferation because of the cytotoxicity of **VII**-dimethyl ester. Because there are no apparent reactive centers in the EA conjugate, no direct explanation for this observed cytotoxicity can be given. The EA group can dissociate from the GSH-sulfhydryl, thereby regaining its  $\alpha,\beta$ -unsaturated ketone moiety, which may modify cysteine residues in proteins. In addition, the **VII**-dimethyl ester might induce apoptosis through inhibition of GST $\pi$ , as seen with other GST $\pi$  inhibitors in various systems (Morgan et al., 1996; Asakura et al., 2001; Ruscoe et al., 2001). Because compound **VII** is also an effective GST inhibitor (D. Burg, R. Hermanns, I. M. C. M. Rietjens, P. van Bladeren, G. van der Marel, G. J. Mulder, submitted for publication), the observed cell death may be caused by such GST inhibition.

In conclusion, our study has shed more light on the mechanism of recognition of GSH-conjugates by MRP1. We found that modification of the GSH backbone could alter its MRP1-binding characteristics. The  $\gamma$ -glutamyl moiety is of great importance for MRP1 recognition, because changes to this group have the strongest effects on inhibition. Of the seven compounds, the urethane peptidomimetic, **VII**, seems an in-

teresting lead compound for the development of new MRP1 inhibitors. The ethacrynic acid moiety used in this study is not ideally suited for *in vivo* inhibition, because it gradually dissociates (by a retro-Michael reaction) from the GSH-sulfidryl. Therefore, it may be beneficial to conjugate the thiol function to a more stable lipophilic moiety. This may also improve selectivity and potency toward MRP1, as has been shown by Furuta et al. (1999). Therefore, we think that the urethane isostere of GSH may be used as scaffold for the development of a new generation of MRP1 inhibitors.

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#### References

- Asakura T, Hashizume Y, Tashiro K, Searashi Y, Ohkawa K, Nishihira J, Sakai M, and Shibasaki T (2001) Suppression of GST-P by treatment with glutathione-doxorubicin conjugate induces potent apoptosis in rat hepatoma cells. *Int J Cancer* **94**:171–177.
- Bakos E, Evers R, Szakacs G, Tusnady GE, Welker E, Szabo K, de Haas M, van Deemter L, Borst P, Varadi A, et al. (1998) Functional multidrug resistance protein (MRP1) lacking the N-terminal transmembrane domain. *J Biol Chem* **273**:32167–32175.
- Benedetti E, Pedone C, Tonolio C, Nemethy G, Pottle MS, and Scheraga HA (1980) Preferred conformation of the tert-butylloxycarbonyl amino group in peptides. *Int J Pept Protein Res* **16**:156–172.
- Borst P, Evers R, Kool M, and Wijnholds J (2000) A family of drug transporters: the multidrug resistance-associated proteins. *J Natl Cancer Inst* **92**:1295–1302.
- Borst P and Elferink RO (2002) Mammalian ABC transporters in health and disease. *Annu Rev Biochem* **71**:537–592.
- Brook MA and Chan TH (1983) A simple procedure for the esterification of carboxylic acids. *Synthesis* **3**:201–203.
- Burg D, Filipov DV, Hermanns R, van der Marel GA, van Boom JH, and Mulder GJ (2002) Peptidomimetic glutathione analogues as novel  $\gamma$ GT stable GST inhibitors. *Bioorg Med Chem* **10**:195–205.
- Furuta K, Tomokiyo K, Tien Kuo M, Ishikawa T, and Suzuki M (1999) Molecular design of glutathione-derived biochemical probes targeting the GS-X pump. *Tetrahedron* **55**:7529–7540.
- Hanigan MH (1998) Gamma-glutamyl transpeptidase, a glutathionase: its expression and function in carcinogenesis. *Chem Biol Interact* **111–112**:333–342.
- Hipfner DR, Deeley RG, and Cole SP (1999) Structural, mechanistic and clinical aspects of MRP1. *Biochim Biophys Acta* **1461**:359–376.
- Hollo Z, Homolya L, Davis CW, and Sarkadi B (1994) Calcein accumulation as a fluorometric functional assay of the multidrug transporter. *Biochim Biophys Acta* **1191**:384–388.
- Hooijberg JH, Broxterman HJ, Kool M, Assaraf YG, Peters GJ, Noordhuis P, Scheper RJ, Borst P, Pinedo HM, and Jansen G (1999) Antifolate resistance mediated by the multidrug resistance proteins MRP1 and MRP2. *Cancer Res* **59**:2532–2535.
- Ishikawa T (1989) ATP/Mg<sup>2+</sup>-dependent cardiac transport system for glutathione S-conjugates. A study using rat heart sarcolemma vesicles. *J Biol Chem* **264**:17343–17348.
- Ishikawa T, Kobayashi K, Sogame Y, and Hayashi K (1989) Evidence for leukotriene C<sub>4</sub> transport mediated by an ATP-dependent glutathione S-conjugate carrier in rat heart and liver plasma membranes. *FEBS Lett* **29**:95–98.
- Ishikawa T, Tien Kuo M, Furuta K, and Suzuki M (2000) The human multidrug resistance-associated protein (MRP) gene family: from biological function to drug molecular design. *Clin Chem Lab Med* **38**:893–897.
- Ito K, Olsen SL, Qiu W, Deeley RG, and Cole SP (2001a) Mutation of a single conserved tryptophan in multidrug resistance protein 1 (MRP1/ABCC1) results in loss of drug resistance and selective loss of organic anion transport. *J Biol Chem* **276**:15616–15624.
- Ito K, Suzuki H, and Sugiyama Y (2001b) Single amino acid substitution of rat MRP2 results in acquired transport activity for taurocholate. *Am J Physiol* **81**:G1034–G1043.
- Jedlitschky G, Leier I, Buchholz U, Center M, and Keppler D (1994) ATP-dependent transport of glutathione S-conjugates by the multidrug resistance-associated protein. *Cancer Res* **54**:4833–4836.
- Johnson DR, Finch RA, Lin ZP, Zeiss CJ, and Sartorelli AC (2001) The pharmacological phenotype of combined multidrug-resistance Mdr1a/1b- and MRP1-deficient mice. *Cancer Res* **61**:1469–1476.
- Kakkar T, Boxenbaum H, and Mayersohn M (1999) Estimation of *K<sub>i</sub>* in a competitive enzyme-inhibition model: comparisons among three methods of data analysis. *Drug Metab Dispos* **27**:756–762.
- König J, Nies AT, Cui Y, Leier I, and Keppler D (1999) Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity and MRP2-mediated drug resistance. *Biochim Biophys Acta* **1461**:377–394.
- Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJ, Juijn JA, Baas F, and Borst P (1997) Analysis of expression of CMOAT (MRP2), MRP3, MRP4 and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Res* **57**:3537–3547.
- Kool M, van der Linden M, de Haas M, Scheffer GL, de Vree JM, Smith AJ, Jansen G, Peters GJ, Ponne N, Scheper RJ, et al. (1999) MRP3, an organic anion transporter able to transport anti-cancer drugs. *Proc Natl Acad Sci USA* **96**:6914–6919.
- Leier I, Jedlitschky G, Buchholz U, Cole SP, Deeley RG, and Keppler D (1994) The MRP gene encodes an ATP dependent export pump for leukotriene C<sub>4</sub> and structurally related conjugates. *J Biol Chem* **269**:27807–27810.
- Leslie EM, Deeley RG, and Cole SP (2001a) Toxicological relevance of the multidrug resistance protein 1, MRP1 (ABCC1) and related transporters. *Toxicology* **167**:3–23.
- Leslie EM, Ito K, Upadhyaya P, Hecht SS, Deeley RG, and Cole SPC (2001b) Transport of the  $\beta$ -O-glucuronide conjugate of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) by the multidrug resistance protein 1 (MRP1). *J Biol Chem* **276**:27846–27854.
- Litman T, Druley TE, Stein WD, and Bates SE (2001) From MDR to MXR: new understanding of multidrug resistance systems, their properties and clinical significance. *Cell Mol Life Sci* **58**:931–959.
- Loe DW, Deeley RG, and Cole SP (1998) Characterization of vincristine transport by the M<sub>1</sub> 190,000 multidrug resistance protein (MRP): evidence for co-transport with reduced glutathione. *Cancer Res* **58**:5130–5136.
- Morgan AS, Ciaccio PJ, Tew KD, and Kauvar LM (1996) Isozyme-specific glutathione S-transferase inhibitors potentiate drug sensitivity in cultured human tumor cell lines. *Cancer Chemother Pharmacol* **37**:363–370.
- Muller M, Meijer C, Zaman GJ, Borst P, Scheper RJ, Mulder NH, de Vries EG, and Jansen PL (1994) Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport. *Proc Natl Acad Sci USA* **91**:13033–13037.
- Ouwkerk MS and Mulder GJ (1998) Inhibition of glutathione conjugation in the rat *in vivo* by analogues of glutathione conjugates. *Chem Biol Interact* **111–112**:163–176.
- Rago R, Mitchen J, and Wilding G (1990) DNA fluorometric assay in 96-well tissue culture plates using hoechst 33258 after cell lysis by freezing in distilled water. *Anal Biochem* **191**:31–34.
- Renes J, de Vries EG, Nienhuis EF, Jansen PL, and Muller M (1999) ATP- and glutathione-dependent transport of chemotherapeutic drugs by the multidrug resistance protein MRP1. *Br J Pharmacol* **126**:681–688.
- Ruscoe JE, Rosario LA, Wang T, Gate L, Arifoglu P, Wolf CR, Henderson CJ, Ronai Z, and Tew KD (2001) Pharmacologic or genetic manipulation of glutathione S-transferase P1–1 (GSTpi) influences cell proliferation pathways. *J Pharmacol Exp Ther* **298**:229–345.
- Saves I and Masson JM (1998) Mechanisms of resistance to xenobiotics in human therapy. *Cell Mol Life Sci* **54**:405–426.
- Seelig A, Blatter XL, and Wohnsland F (2000) Substrate recognition by P-glycoprotein and the multidrug resistance-associated protein MRP1: a comparison. *Int J Clin Pharmacol Ther* **38**:111–121.
- Wijnholds J, deLange EC, Scheffer GL, van den Berg DJ, Mol CA, van der Valk M, Schinkel AH, Scheper RJ, Breimer DD, and Borst P (2000) Multidrug resistance protein 1 protects the choroid plexus epithelium and contributes to the blood-cerebrospinal fluid barrier. *J Clin Invest* **105**:279–285.
- Wijnholds J, Evers R, van Leusden MR, Mol CA, Zaman GJ, Mayer U, Beijnen JH, van der Valk M, Krimpenfort P, and Borst P (1997) Increased sensitivity to anticancer drugs and decreased inflammatory response in mice lacking the multidrug resistance-associated protein. *Nat Med* **3**:1275–1279.
- Zaman GJ, Cnubben NH, van Bladeren PJ, Evers R, and Borst P (1996) Transport of the glutathione conjugate of ethacrynic acid by the human multidrug resistance protein MRP. *FEBS Lett* **391**:126–130.
- Zelcer N, Saeki T, Reid G, Beijnen JH, and Borst P (2001) Characterization of drug transport by the human multidrug resistant protein 3 (ABCC3). *J Biol Chem* **276**:46400–46407.

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