Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 13033–13037, December 1994
Medical Sciences

Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione *S*-conjugate transport

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ABSTRACT **The multidrug resistance-associated protein (MRP) is a 180- to 195-kDa glycoprotein associated with multidrug resistance of human tumor cells. MRP is mainly located in the plasma membrane and it confers resistance by exporting natural product drugs out of the cell. Here we demonstrate that overexpression of the *MRP* gene in human cancer cells increases the ATP-dependent glutathione *S*-conjugate carrier activity in plasma membrane vesicles isolated from these cells. The glutathione *S*-conjugate export carrier is known to mediate excretion of bivalent anionic conjugates from mammalian cells and is thought to play a role in the elimination of conjugated xenobiotics. Our results suggest that MRP can cause multidrug resistance by promoting the export of drug modification products from cells and they shed light on the reported link between drug resistance and cellular glutathione and glutathione *S*-transferase levels.**

Cancer cells selected for resistance against a natural product drug, such as doxorubicin, are often cross-resistant to a range of other drugs with very different chemical structures or cellular targets (1–3). This type of resistance is known as multidrug resistance (MDR) and it is probably the most frequent form of resistance in cell lines exposed to natural product drugs *in vitro*. The best defined form of MDR in human cells is due to the P-glycoprotein (4) (Pgp) encoded by the human *MDR1* gene (3, 5) (standard gene symbol, *PGY1*). This protein is a member of the ABC superfamily of transporter proteins (6); it is located in the plasma membrane and can extrude a range of hydrophobic anticancer drugs from the cell against a concentration gradient (2–4, 7). Increased Pgp activity can lower cellular drug concentration and, hence, result in drug resistance. Gottesman (8) has estimated that Pgp is increased in as much as 50% of all human tumors at some stage of treatment with natural product drugs. For some tumors failure of therapy is clearly related to an increase in Pgp (9, 10).

Pgp is not the only cause of MDR, however. Many cells selected for resistance do not contain increased levels of Pgp but nevertheless are resistant to a broad range of natural product drugs (11–13). Several of these lines (14–18) contain raised levels of a second member of the ABC superfamily of transporter proteins, the MDR-associated protein (MRP), discovered by Cole *et al.* (14). Transfection of HeLa cells (19) or SW1573 lung carcinoma cells (20) with an expression vector containing *MRP* cDNA resulted in an increased level of resistance to natural product drugs. Like Pgp, MRP seems to be a drug pump that extrudes drugs from the cell (20). It is mainly present in the plasma membrane (20, 21) and is able to decrease cellular drug levels against a concentration gradient (20). However, MRP and Pgp do not result in exactly the same spectrum of drug resistance (20). Whereas taxol is an efficient substrate for Pgp, it is not for MRP (20); MDR caused by Pgp is readily reversed by verapamil and cyclosporin A (analogues), but MDR caused by MRP is not (20). These differences in resistance spectrum suggest differences in the detailed mechanism of drug transport by MRP and Pgp.

A third form of drug resistance that can affect several classes of drugs is associated with increased cellular levels of glutathione (GSH) and/or glutathione *S*-transferase (GST; EC 2.5.1.18) (22–29). Although it is firmly established by transfection experiments that increased levels of GST cause resistance to some alkylating agents (23–25, 27), it has been more difficult to prove that GSH and GST are directly involved in other forms of resistance—e.g., resistance to cisplatin (29) and anthracyclines (22, 26). Ishikawa and Ali-Osman (30) have shown, however, that this type of resistance may be complex, because it involves two steps: (*i*) formation of GSH *S*-conjugate and (*ii*) removal of the toxic conjugate from the cell by a GSH *S*-conjugate export carrier (GS-X pump) (31). Conjugation of cisplatin and GSH can occur nonenzymically under physiological conditions, but export from the cell requires the GS-X pump. In one cisplatin-resistant mutant the activity of this pump was increased (32). The GS-X pump is also known as the multispecific organic anion transporter (MOAT) (33, 34) or the leukotriene C_4 (LTC_4) transporter (35, 36) and is present in many mammalian cells, such as hepatocytes (33, 35, 37, 38), erythrocytes (34, 39), cardiac cells (40), leukemic cells (30), mast cells (36), and lung cells (41), and even in plants (42). The GS-X pump is inactive in the hepatocytes of a rat strain known as TR^-, allowing the identification of substrates that require the pump for their secretion into bile (33, 35, 37). The GS-X pump has a relatively broad substrate specificity. It transports substrates containing a hydrophobic (e.g., long-chain alkyl) part and at least two negative charges (31, 34, 40). Transport can be inhibited by orthovanadate and by competing anionic organic substrates, but not by many of the basic or neutral amphiphilic compounds that act as substrates for Pgp (3).

To test whether cisplatin-resistant cell lines other than the one studied by Ishikawa (32) also have increased activity of the GS-X pump, we analyzed our cisplatin-resistant lung cancer cell line (43) for this activity. No increase was

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Abbreviations: BSO, DL-buthionine (*S,R*)-sulfoximine; DNP-GS, *S*-dinitrophenylglutathione; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; GS-X pump, GSH *S*-conjugate export carrier; LTC_4, leukotriene C_4; MDR, multidrug resistance; MRP, MDR-associated protein; Pgp, P-glycoprotein; SCLC, small-cell lung cancer.

detected, but in a multidrug-resistant cell line, GLC_4/ADR (12), we noted a substantial increase of GS-X pump activity relative to its sensitive parent, the human small-cell lung cancer (SCLC) line GLC_4. Because the GLC_4/ADR line is known to contain a high level of MRP (15), we have investigated the relation between the GS-X pump and MRP in more detail, using a cell line transfected with a *MRP* cDNA expression vector (20). Our results show that MRP is either identical to the GS-X pump or able to activate an endogenous GS-X pump activity.

EXPERIMENTAL PROCEDURES

Materials. The multidrug-resistant cell line GLC_4/ADR was isolated from the human SCLC cell line GLC_4 by a multistep selection up to 1152 nM doxorubicin (12). GLC_4/ADR_{pr} is a partial revertant of GLC_4/ADR obtained after culturing of GLC_4/ADR in drug-free medium for 6 months (44). S1(MRP) is a subline of the non-SCLC cell line SW1573/S1, which stably overexpresses *MRP* (20). This cell line was obtained by transfection of SW1573/S1 cells with an expression vector containing *MRP* cDNA and a neomycin-resistance (*neo*) gene (pRc/RSV-MRP) and selection with Geneticin (G418) (20). S1*neo* was obtained after transfection of SW1573/S1 cells with the same expression vector without *MRP* cDNA insert (pRc/RSV; Invitrogen), followed by Geneticin selection.

[14,15,19,20-^{3}H(N)]LTC_4 with specific radioactivity of 6401 Gbq/mmol was from DuPont/NEN. Dinitrophenyl-[*glycine*-2-^{3}H]glutathione (DNP-[^{3}H]GS) with specific activity 0.325 Gbq/mmol was a gift from Folkert Kuipers (Department of Pediatrics, University of Groningen).

Procedures. Cells were harvested by centrifugation at 180 × *g*, for 30 min at 4°C, washed twice in ice-cold phosphate-buffered saline, and again centrifuged. The pellet (1–2 ml, 1–5 × 10^8 cells) was diluted 40-fold with a hypotonic buffer (1 mM Tris Cl, pH 7.0) and stirred gently for 2 hr on ice in the presence of 400 units of recombinant *Serratia marcescens* nuclease (Benzonase, grade II, protease-free; Merck). 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 (10 mM Tris Cl, pH 7.4/250 mM sucrose) and homogenized with a Dounce B homogenizer (glass/glass, tight pestle, 30 strokes) in the presence of 800 units of Benzonase. The crude membrane fraction was layered on top of a 38% (wt/vol) sucrose solution in 5 mM Tris Hepes (pH 7.4) and centrifuged in a Beckman SW41 rotor at 280,000 × *g* for 2 hr 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 −80°C until use in transport assays. Protein content was measured by the Lowry method.

Transport of ^{3}H-labeled GSH *S*-conjugates was measured by a rapid filtration technique using nitrocellulose filters (0.45-μm pore size; Schleicher & Schuell) presoaked in TS buffer. Membrane vesicles were rapidly thawed and then incubated at 37°C in the presence of 4 mM ATP, 10 mM $MgCl_2$, 10 mM creatine phosphate, 100 μg of creatine kinase per ml, 250 mM sucrose, 10 mM Tris Cl (pH 7.4), and the labeled substrate in a final volume of 110 μl. Samples (20 μl) were taken at the indicated time points and diluted in 1 ml of ice-cold TS buffer. This solution was applied to the presoaked filters and rinsed with 5 ml of ice-cold TS buffer. In control experiments ATP was replaced by 4 mM 5′-AMP or adenosine 5′-[β,γ-methylene]triphosphate. Filters were dissolved and the radioactivity was measured in a liquid scintillation counter. Incubations with [^{3}H]LTC_4 (1.25 nM) were stopped after 30 sec, and incubations with DNP-[^{3}H]GS (50 μM), after 10 min, by dilution in 1 ml of ice-cold TS buffer.

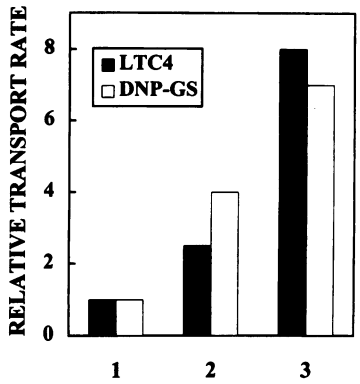

FIG. 1. ATP-dependent uptake of GSH *S*-conjugates ([^{3}H]LTC_4 or DNP-[^{3}H]GS) into membrane vesicles of human SCLC cell lines with different levels of *MRP* expression. Bars: 1, GLC_4; 2, GLC_4/ADR_{pr}; 3, GLC_4/ADR. ATP-dependent transport was calculated by subtracting the transport in the presence of 5′-AMP from those in the presence of ATP. Values are means of quadruplicate determinations. Similar results were obtained with vesicles from several different membrane preparations. (GLC_4, n = 3; GLC_4/ADRpr, n = 2; GLC_4/ADR, n = 4.)

RESULTS

The multidrug-resistant cell line GLC_4/ADR was derived from the human SCLC line GLC_4 (12), and its 150-fold resistance to doxorubicin appears to be attributable to amplification of the *MRP* gene (15) in combination with a decrease in topoisomerase IIα (45). The resistance of the GLC_4/ADR cell line is dependent on the intracellular level of GSH, as pretreatment of the cells with an inhibitor of GSH synthesis substantially reduced resistance (46). This made it of interest to test whether GLC_4/ADR cells contain the GSH *S*-conjugate transporter and can export drug conjugates from the cell.

Transport of GSH *S*-conjugates was determined in membrane vesicles enriched for plasma membranes. Some of these vesicles are inside-out and can be used to study ATP-dependent uptake of GSH *S*-conjugates. Two substrates were used: the high-affinity substrate LTC_4 (35) and the low-affinity substrate DNP-GS (38, 40). Both GSH *S*-con-

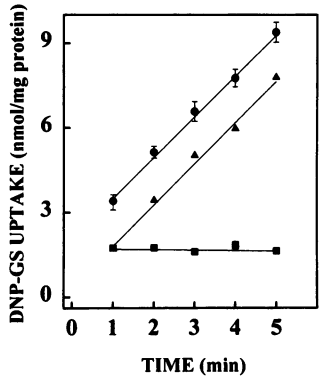

FIG. 2. Time-dependent uptake of DNP-[^{3}H]GS (100 μM) into membrane vesicles prepared from GLC_4/ADR cells in the presence of 4 mM MgATP (●) or of 4 mM MgAMP (■); ATP-dependent transport (▲) = $values_{ATP}$ − $values_{AMP}$. Data are means ± SD of three independent determinations. The transport rate for ATP-dependent DNP-[^{3}H]GS transport in vesicles from GLC_4/ADR cells was 1.46 ± 0.25 nmol·min^{-1}·mg^{-1} of protein, and in vesicles from GLC_4 cells it was 0.2 ± 0.05 nmol·min^{-1}·mg^{-1}.

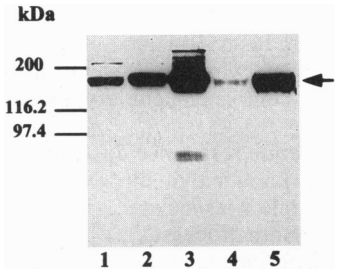

FIG. 3. Immunoblot analysis of MRP in membrane preparations from lung cancer cells. Lanes: 1, GLC_4; 2, GLC_4/ADR_{pr}; 3, GLC_4/ADR; 4, SW1573/S1; 5, the stable *MRP* transfectant S1(MRP). Membrane proteins (30 μg per lane) were size fractionated in an SDS/7.5% polyacrylamide gel and transferred to nitrocellulose by electroblotting. MRP was detected with monoclonal antibody MRPm6 (21) and visualized by enhanced chemiluminescence (Amersham). The positions of protein markers (BDH) of 97.4 kDa (phosphorylase *b*), 116.2 kDa (β-galactosidase), and 200 kDa (myosin heavy chain) are indicated by bars. The migration position of MRP is indicated by an arrow.

jugates were taken up into the inside-out membrane vesicles in a MgATP-dependent manner (Fig. 1). 5'-AMP (Fig. 2) or the nonhydrolyzable ATP analogue adenosine 5'-[β,γ-methylene]triphosphate (data not shown) did not support uptake, indicating that ATP hydrolysis is required for transport. The apparent K_m value for DNP-GS transport in membrane vesicles from GLC_4/ADR cells was 30 μM. The transport rates correlated with the level of MRP (Figs. 1 and 3): low in the nonresistant GLC_4 cells, high in the GLC_4/ADR cells, and intermediate in a partial revertant of GLC_4/ADR, GLC_4/ADR_{pr}, with a 10-fold greater resistance to doxorubicin and MRP levels intermediate between GLC_4/ADR and GLC_4 (Fig. 3).

To verify the association between MRP and the GSH *S*-conjugate transport capacity in a more direct way, we used lung cancer cells stably transfected with a cDNA construct encoding MRP (20). Membrane vesicles from nontransfected SW1573/S1 cells have a low endogenous MRP level (Fig. 3) and a low basal transport activity (Fig. 4). In vesicles from cells stably transfected with *MRP* cDNA [S1(MRP) cells] transport activity was increased 8-fold for LTC_4 and 7-fold for DNP-GS (Fig. 4). No increase in transport was detectable with vesicles prepared from the mock-transfected S1*neo* cells. These cells were transfected with the same *neo* plasmid as S1(MRP), but without the *MRP* cDNA insert, and they have gone through the same Geneticin (G418) selection as the S1(MRP) cells (20).

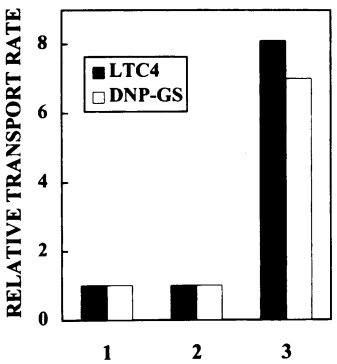

FIG. 4. ATP-dependent uptake of GSH *S*-conjugates ([^{3}H]LTC_4 or DNP-[^{3}H]GS) into membrane vesicles from non-SCLC cells transfected with the *MRP* cDNA and controls. Bars: 1, untransfected SW1573/S1 cells; 2, mock-transfected S1*neo* cells; 3, *MRP*-transfected S1(MRP) cells. Similar results were obtained with three different membrane preparations.

These experiments show that overexpression of *MRP* in lung cancer cells results in the increased activity of a GSH *S*-conjugate carrier. To test whether this carrier resembles the GS-X pump previously characterized in mammalian cells (30–41), we studied the effect of several substrates and inhibitors of the GS-X pump on the carrier activity of our transfectants. GSH disulfide (GSSG), thought to be an endogenous substrate for the GS-X pump (30, 33, 38–41), inhibited ATP-dependent transport of both DNP-GS (data not shown) and LTC_4 (Table 1) in vesicles from *MRP*-transfected cells. GSH *S*-conjugates resulting from the GST-mediated conjugation (47) of lipid peroxidation products such as 4-hydroxynonenal are known to be excellent substrates (40) for the export pump. Indeed, *n*-nonyl-GS was even more effective as an inhibitor than GSSG (Table 1). In contrast, no inhibition was detected with GSH (Table 1), which is not a substrate for the carrier, or with dithiothreitol (0.5 mM), used to prevent GSH oxidation. Furthermore, no inhibition (Table 1) was seen with the uncharged cardiac glycoside ouabain (1 mM), which inhibits the Na^+/K^+-ATPase but not the GS-X pump (31, 35, 40). Doxorubicin had no effect on DNP-GS (data not shown) or LTC_4 transport (Table 1), and vincristine and vinblastine inhibited ATP-dependent LTC_4 transport only at high concentrations (Table 1). A lack of inhibition of the GSH *S*-conjugate carrier by doxorubicin and partial inhibition by high concentrations of *Vinca* alkaloids have been described (35). Finally, transport was inhibited by orthovanadate (IC_{50} = 50 μM for DNP-GS; Table 1), but this is not a very specific property, as this compound is known to inhibit other ATP-dependent transporters, such as the hepatic bile salt carrier (48) and the Pgps (3, 6), as well. Taken together, these results suggest that overexpression of *MRP* increases a GSH *S*-conjugate transporter activity that is very similar, if not identical, to the carrier activity known to be present in mammalian cells.

DISCUSSION

The simplest interpretation of our results is that MRP transports GSH *S*-conjugates and is identical to the GS-X pump (30–41). Although Awasthi *et al.* (41) have suggested that this transporter might be a 38-kDa protein, an unusually low molecular mass for an ATP-dependent plasma membrane

Table 1. Effect of inhibitors on ATP-dependent [^{3}H]LTC_4 transport into plasma membrane vesicles from S1(MRP) cells

Treatment		[^{3}H]LTC_4
Drug	Conc., μM	uptake, %
None	—	100
GSSG	100	75 ± 6*
	500	48 ± 2*
Nonyl-GS	10	14 ± 2*
	100	1 ± 1*
GSH	5000	106 ± 3
Ouabain	1000	100 ± 7
Doxorubicin	100	100 ± 10
Vincristine	10	90 ± 1
	100	52 ± 3*
Vinblastine	10	91 ± 2
	100	36 ± 3*
Vanadate	50	60 ± 2*

Plasma membrane-enriched vesicles from the stable *MRP* transfectant S1(MRP) were incubated with [^{3}H]LTC_4 (1.25 nM) in the presence or absence (control) of the indicated compounds. Relative transport rates (percent of control) were calculated by subtracting the values in the presence of ATP from those in the presence of 5'-AMP (means ± SD from at least two experiments, each performed in triplicate.

*Bonferroni *P* value < 0.001 (ANOVA).

transporter, more recent work by Leier *et al.* (36) has shown that photoaffinity labeling of mastocytoma membranes with LTC_4 specifically labels a 190-kDa plasma membrane protein. This is the molecular mass of MRP (17, 19, 20), and Leier *et al.* (36) have actually speculated that the LTC_4 transporter might be MRP. Our demonstration that MRP is present in all tissues analyzed (15), even erythrocytes (G.J.R.Z., unpublished data), just like the GSH *S*-conjugate transporter, is in agreement with this interpretation. A remaining problem is that MRP confers resistance to drugs, such as doxorubicin and vincristine (19, 20), that are not known to undergo major modifications in cells, or at least no modifications that would turn the drug into a plausible substrate for the GS-X pump (60, 61).

Three explanations can be considered for this discrepancy. (*i*) It is possible that negatively charged complexes can be formed in the cell, but that these have escaped detection—for instance, because of their instability. (*ii*) The GS-X pump might be more versatile than previously thought and might be able to transport both conjugated and unconjugated drugs, as suggested by Awasthi *et al.* (41). The negative competition experiments in Table 1 do not exclude this possibility. There is actually some inhibition of LTC_4 transport by high concentrations of vinblastine and vincristine. Such an inhibition has also been observed for the hepatic GS-X pump (35) and has been attributed to nonspecific effects of amphipathic compounds on the membrane environment of the pump. This may be the case, but the inhibition might also be due to competition for transport by the pump. Another possibility to explain the lack of strong competition would be the existence of two alternative MRP states with separate activities for drugs and drug conjugates. (*iii*) MRP might not be the GS-X pump but instead activate an endogenous GS-X pump.

Although none of these three alternatives is excluded at present, we prefer alternative *i* because it provides the simplest explanation for the strong inhibition of *DL*-buthionine (*S,R*)-sulfoximine (BSO) on MRP-mediated drug resistance. BSO inhibits GSH synthesis and has no other known effects on cells (28). We have previously shown that pretreatment of GLC_4/ADR cells with BSO largely abolishes doxorubicin resistance (46), and Versantvoort *et al.* (49) have recently extended this observation to another human lung cancer line that overexpresses *MRP*, the COR-L23/R line (18). They found that BSO treatment of these cells largely reverses the resistance to daunorubicin, vincristine, and rhodamine and also reverses the diminished drug accumulation of the resistant cells. No effect of BSO was observed on the parental cell line. This strongly indicates that GSH is required to allow expression of resistance mediated by MRP. If MRP is the GS-X pump, this requirement is simply explained. The argument is not conclusive, however. If a decrease in cellular GSH would damage the plasma membrane, this could result in increased drug influx and reversal of resistance.

The link between MRP and the GS-X pump, established here, also links MDR to types of drug resistance not usually associated with MDR, such as resistance to cisplatin, to alkylating agents, and to arsenite. Drug resistance involving GSH conjugates is a complex affair, as pointed out by Ishikawa (31). Resistance involves at least three processes (*i*) the ability to make drug conjugates, (*ii*) the ability to export these conjugates from the cell, and (*iii*) the ability to maintain high levels of GSH notwithstanding continuous loss of drug–GSH conjugates from the cell. Each of these processes must run fast enough to match the continuous influx of drug into the cell. This may be the reason why it is often difficult to get high-level drug resistance by this mechanism, as this would require the simultaneous upregulation of three different enzyme systems: GSH biosynthesis, GST, and GS-X pump. The resistance phenotype obtained can be expected to depend on the selecting drug and on the basal activity of each of these systems in the cell studied. This complexity may also explain why it has been difficult to reproduce this type of resistance by transfection experiments in which only one component of this complex machinery, such as GST, is added in excess (24).

The case of arsenite resistance deserves special comment. It has long been known that the excretion of arsenite by hepatocytes into bile is associated with an increased excretion of GSH (50). Both processes are abolished in the TR^- rat (R. P. J. Oude Elferink, personal communication), suggesting that excretion requires the GS-X pump and that arsenite is excreted as the $As(GS)_3$ complex recently detected by NMR (51, 52). Arsenite resistance can occur in mammalian cells (53) and in protozoa (54, 55). The resistance in *Leishmania* was found to be associated with overexpression of a Pgp encoded by the *pgpA* gene (56). In transfection experiments this gene was able to confer resistance to the oxyanions arsenite and antimony, but only to a low and somewhat variable level (57, 58). All attempts to reproduce the high level of resistance observed in cells selected for resistance have failed. Papadopoulou *et al.* (58) have advanced several plausible explanations for these negative results, but the experiments on MRP provide an additional one. Cole *et al.* (14) noted that MRP most closely resembles PgpA within the ABC transporter superfamily. The resemblance is reinforced by the observation that the GLC_4/ADR line is cross-resistant to arsenite (6-fold; C.M., unpublished data). PgpA of *Leishmania* may therefore be a GS-X pump (or trypanothione-X pump), and oxyanion resistance may involve the export of GSH–oxyanion complexes rather than free oxyanions. The high resistance obtained by selection of *Leishmania* with arsenite could be due to simultaneous selection for increased GSH biosynthesis and increased export of GSH–arsenite conjugates. If transfection provides only high levels of the export pump, only low resistance may be the result. Although GSH–arsenite complexes can form nonenzymically (52), there is a strong correlation between arsenite resistance and raised GSTπ levels in Chinese hamster ovary cells (59). It is therefore possible that complex formation is accelerated by GST, adding to the complexity of arsenite resistance.

The link between MRP and the GS-X pump documented in this paper sheds further light on the reported association between drug resistance and raised cellular levels of GSH and GSTs. Thus far the emphasis has been on detoxification of drugs and of the peroxidation products that some drugs generate. Efficient export of drug conjugates or cytotoxic peroxidation products from the cell may be essential to generate resistance to the continued presence of drug. Export of detoxification products could therefore be the bottleneck in many forms of resistance. Although the picture sketched here has many speculative elements, most of these are testable.

We thank Tineke Leijnema and Hans Koning (University Hospital Groningen) and Marcel de Haas (Netherlands Cancer Institute) for technical assistance, Dr. J. H. Beijnen (Netherlands Cancer Institute) and Dr. R. P. J. Oude Elferink (Academisch Medisch Centrum, University of Amsterdam) for helpful advice, and Dr. M. M. Gottesman (National Institutes of Health, Bethesda, MD) and Dr. M. Ouellette (Université Laval, Canada) for comments on the manuscript. This work was supported by a collaborative project of the University of Amsterdam and the Netherlands Cancer Institute (to G.J.R.Z.) and grants from the Dutch Cancer Society (NKI 91-18 to P.B. and GUKC 90-18 to N.H.M. and E.G.E.V.) and the Biomed and Health Research Program (PL 931436 to P.J.).

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