Coordinated Action of Glutathione S-Transferases (GSTs) and Multidrug Resistance Protein 1 (MRP1) in Antineoplastic Drug Detoxification

MECHANISM OF GST A1-1- AND MRP1-ASSOCIATED RESISTANCE TO CHLORAMBUCIL IN MCF7 BREAST CARCINOMA CELLS*

(Received for publication, April 15, 1998, and in revised form, May 28, 1998)

Charles S. Morrow‡, Pamela K. Smitherman, Sri K. Diah, Erasmus Schneider§, and Alan J. Townsend

From the Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, North Carolina 27157 and §Wadsworth Center, Albany, New York 12201

To examine the role of multidrug resistance protein 1 (MRP1) and glutathione S-transferases (GSTs) in cellular resistance to antineoplastic drugs, derivatives of MCF7 breast carcinoma cells were developed that express MRP1 in combination with one of three human cytosolic isozymes of GST. Expression of MRP1 alone confers resistance to several drugs representing the multidrug resistance phenotype, drugs including doxorubicin, vincristine, etoposide, and mitoxantrone. However, co-expression with MRP1 of any of the human GST isozymes A1-1, M1-1, or P1-1 failed to augment MRP1-associated resistance to these drugs. In contrast, combined expression of MRP1 and GST A1-1 conferred a 4-fold resistance to the anticancer drug chlorambucil. Expression of MRP1 alone failed to confer resistance to chlorambucil, showing that the observed protection from chlorambucil cytotoxicity was absolutely dependent upon GST A1-1 protein. Moreover, using inhibitors of GST (dicumarol) or MRPI (sulfipyrazone), it was shown that in MCF7 cells resistance to chlorambucil requires both intact MRP1-dependent efflux pump activity and, for full protection, GST A1-1 catalytic activity. These results are the first demonstration that GST A1-1 and MRP1 can act in synergy to protect cells from the cytotoxicity of a nitrogen mustard, chlorambucil.

The glutathione S-transferases (GSTs) catalyze the conjugation with glutathione of a number of electrophilic xenobiotics, including several carcinogens, mutagens, and antitumor drugs (1–6). Usually, but not invariably, these electrophiles are made less reactive by conjugation with glutathione, and the conjugates are thought to be less toxic to the cell. Consequently, GSTs are believed to play an important role in the defense of cells against these xenobiotics.

Several antineoplastic drugs, particularly the reactive electrophilic alkylating agents, can form conjugates with glutathione both spontaneously and in GST-catalyzed reactions (7–14). Despite these catalytic activities, the role of GSTs in the protection of cells from the cytotoxicities of these cancer drugs remains equivocal due to the inconsistent results obtained by different laboratories (5, 15–25). Although some investigators have found associations between cellular resistance to some anticancer drugs and expression of particular isozymes of GST, other investigators have found no such associations in other cell lines. In MCF7 breast carcinoma cells, increased expression of Alpha, Mu, or Pi class GSTs failed to confer any consistent, significant resistance to a variety of anticancer drugs, including drugs known to be substrates of GSTs (19, 20, 24). We recently proposed that conjugation of some of these drugs and toxins with glutathione may represent only partial detoxification. In this view, export of the glutathione conjugate is required to fully potentiate the GST-mediated protection. The identification of MRP1 as an important glutathione-conjugate efflux pump (26–30) raises the possibility that MRP1 and GST may act in synergy to confer cellular resistance to some of these compounds (31). This hypothesis was recently validated for the model carcinogen, 4-nitroquinoline-1-oxide (31). These studies showed that GST P1-1-associated protection from 4-nitroquinoline-1-oxide cytotoxicity was dependent upon concomitant expression of MRP1. Additionally, GST P1-1-associated protection from 4-nitroquinoline-1-oxide-mediated DNA adduct formation was greatly enhanced by co-expression of MRP1.

Previous studies failed to demonstrate an association between increased MRP1 expression and resistance to alkylating agents in MCF7/VP cells (32). In another study examining paired cell lines that differed in the levels of MRP1 expressed, increased MRP1 was associated with chlorambucil resistance in one but not the other two paired cell lines (33). These inconsistent relationships between increased MRP1 and alkylating agent sensitivity indicate that other factors, such as GST or glutathione levels, may be important in determining whether or not MRP1 will mediate protection from the cytotoxicities of some alkylating cancer drugs. Additionally, properties of some of the bifunctional alkylating agents and their metabolites make them particularly interesting candidates for GST/MRP1-mediated detoxification. Even after formation of monoglutathionyl derivatives, some of these bifunctional alkylating agents retain significant reactivity at the unmodified alkylating group and may therefore remain cytotoxic. Moreover, glutathione conjugates of some of these compounds, the nitrogen mustards melphalan and chlorambucil, are known to be transported by MRP1-containing membrane vesicles in vitro (27, 33).
We have developed cellular models to examine the role of GST/MRP1 synergy in the emergence of anticancer drug resistance. Although cell-free, in vitro analyses of toxin conjugation and transport are informative, cellular models of MRP1 and GST function are essential to determine the cytoprotective consequences of coordinated MRP1 and GST expression and to determine the precise mechanisms of cellular detoxifications. Accordingly, from MCF7 cells that express extremely low levels of MRP1 and GST, we have developed derivative sublines that express MRP1 alone or in combination with representative of three major classes of human cytosolic GSTs, GST A1-1, M1-1, and P1-1. Results show that GST A1-1 operates in synergy with MRP1 to confer resistance to the antineoplastic nitrogen mustard chlorambucil. The studies indicate the mechanism of synergy involves GST catalytic activity as well as MRP1-mediated efflux of glutathione conjugates.

**EXPERIMENTAL PROCEDURES**

**Drugs and Chemicals—**Mitoxantrone, 1,3-bis(2-chloroethyl)-1-nitrosourea, tiopeta, and hepsulfan were provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program of the National Cancer Institute (Bethesda, MD). Gentamicin was from Life Technologies, Inc., and hygromycin was from Calbiochem. All other drugs were from Sigma. Stock solutions stored at −30 °C were prepared fresh: chlorambucil (100 mM in ethanol), doxorubicin (5 mM in H2O), vincristine (1 mM in H2O), mitoxantrone (5 mM in dimethyl sulfoxide), dicumarol (50 mM in 0.1 N NaOH), and thiotepa (200 mM in dimethyl sulfoxide).

The following were prepared fresh: chlorambucil (100 mM in ethanol), melphanal (16.4 mM in acidified ethanol (~0.4 × HCl)), 1,3-bis/2-chloroethyl-1-nitrosourea (250 mM in 71% ethanol), and tiopeta (100 mM in H2O).

**Cell Lines and Tissue Culture—**Cells were grown at 37 °C, 5% CO2 in DMEM supplemented with 10% fetal calf serum. All cell lines were derived from cloned parental MCF7 breast carcinoma cell lines, MCF7/WT (GST−/−MRP1−/−), and the MDR derivative MCF7/VP (GST−/−MRP1+/+). Cells expressing human isozymes of GST, GST A1-1, GST M1-1, or GST P1-1 were established by stable transfection of MCF7/WT and MCF7/VP cells with pcDNA3 (Invitrogen, Carlsbad, CA) or pCEP4 lacking GST cDNA inserts (MCF7/WT-neo, MCF7/VP-neo), pcDNA3 containing cDNA inserts (35–37) (MCF7/VP a; pCEP4 vector (34) containing cDNA inserts (35–37) (MCF7/WT a); or the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) containing the cDNA insert encoding the human GST A1 (MCF7/VP a; pCEP4 plasmid expression vector (CLONTECH). The protein was expressed following transformation into E. coli DH5a. The cultures were grown at 37 °C to an OD at 600nm of 0.8 and then chilled, pelleted, and resuspended in 0.1 volume of 10 mM KPO4 (pH 8) plus 5 mM EDTA. The suspension was warmed to 20 °C, incubated in lysosome (0.2 mg/ml) for 10 min, chilled on ice, sonicated, and then centrifuged at 10,000 × g for 20 min. The supernatant was adjusted to 0.2 mM NaCl and loaded onto a glutathione affinity column (Sigma G-4510) equilibrated with 50 mM Tris buffer (pH 7.4) plus 0.2 mM NaCl. The column was washed with 10 volumes of equilibration buffer, and then GSTA1-1 was eluted with buffer containing 50 mM Tris (pH 9.6), 0.2 M NaCl, and 5 mM glutathione. The eluate was neutralized with dilute HCl, dialyzed against 10 mM KPO4, (pH 7.4) plus 50% glycerol, and stored at −20 °C.

**Biochemical and in Vitro GST Enzyme Analyses—**Glutathione levels were measured by the enzymatic recycling method (39). GST activities were determined using 1-chloro-2,4-dinitrophenyl (CDNB) as substrate (40). Enzyme kinetic and in vitro inhibition analyses were accomplished using affinity-purified, recombiant human GST A1-1. For these assays, CDNB served as the varied substrate, and glutathione concentration was fixed at 2 mM. Activity was monitored spectrophotometrically at 340 nm and peak as described by Habig et al. (40).

Northern blots of total cellular RNA were done as described (41) using probes derived from the entire human GST A1, GST M1, or GST P1 cDNAs (36, 37, 42) or the 5' 2629 base pairs of the MRPI cDNA (43). The cDNA probes were labeled with [α-32P]dCTP by random priming (44). Western blot analyses of GST isoforms were accomplished as described previously using affinity-purified rabbit polyclonal antibodies directed against human GST A1, GST M1, or GST P1 subunits (19). Western blotting of membrane protein preparations for MRP1 was done with the QCRL1 antibody kindly provided by Dr. S. P. Cole (45).

**In Vivo (Intact Cells) Analyses of the Effects of Inhibitors on MRP1-mediated DNP-SG Efflux and GST Activity—**For the analysis of DNP-SG efflux, MCF7/VP cells were plated to a density of 0.6 × 106 cells/well in 6-well plates. 24 h later, cells were incubated in DMEM plus 1% fetal calf serum or in HBSS equilibrated to 25 °C in 5% CO2. At indicated times, cells were washed and acidified to 10% perchloric acid. Cells were lysed in 10% perchloric acid.

**In Vivo (Intact Cells) Analyses of the Effects of Inhibitors on MRP1-mediated DNP-SG Efflux and GST Activity—**For the analysis of DNP-SG efflux, MCF7/VP cells were plated to a density of 0.6 × 106 cells/well in 6-well plates. 24 h later, cells were incubated in DMEM plus 1% fetal calf serum or in HBSS equilibrated to 25 °C in 5% CO2. At indicated times, cells were washed and acidified to 10% perchloric acid. Cells were lysed in 10% perchloric acid. Medium and lyse samples were prepared for reverse phase high performance liquid chromatography analysis as described (31). Details of DNP-SG chromatography will be described elsewhere. Briefly, acid-soluble samples were eluted isocratically from a C18 reverse phase column in 20% methanol plus 0.1% trifluoroacetic acid. Chromatograms were monitored spectrophotometrically at 340 nm, and DNP-SG levels were determined from the areas of peaks eluting at the position of authentic DNP-SG by comparison with DNP-SG standards. Concentrations of DNP-SG reference standards were determined using ε340 = 10.2 cm3·mol−1·dm−3.

The effect of sulfinpyrazone or dicumarol on GST activity in vitro was determined as follows. MCF7/VPa cells (0.5 × 105/well) were seeded in 6-well plates. 24 h later, cells were incubated in DMEM plus 1% fetal calf serum ± 2 mM sulfinpyrazone or in HBSS ± 0.1 mM dicumarol at 37 °C in 5% CO2. At indicated times, cells were washed and incubated in HBSS equilibrated to 25 °C containing inhibitor (2 mM sulfinpyrazone or 0.1 mM dicumarol) or vehicle plus 10 μM CDNB. Relative in vivo GST activities were determined as the rate of total DNP-SG formation (DNP-SG in medium plus cell lysates) for 2 and 5 min at 25 °C. DNP-SG levels were measured chromatographically as described above. The levels of DNP-SG formation increased linearly with time throughout the 5 min incubation periods.

**RESULTS**

**Characterization of Cell Lines—**MCF7 breast carcinoma cells were chosen to study the effects of expression of MRP1 alone or in combination with isozymes of GST. These cell lines constitute a good model system for such studies because parental MCF7/WT cells express extremely low levels of cytosolic GSTs and MRP1. Additionally, a MDR derivative of MCF7/WT, MCF7/VP, is available that has as its primary genetic and phenotypic change the overexpression of high levels of MRP1 (32). Like MCF7/WT, MCF7/VP cells have very low endogenous cytosolic GST levels. Neither MCF7/WT nor MCF7/VP contain detectable MDR1 (P-glycoprotein) or the MRP2 (cMOAT or cMPR) isoform (32).3

Parental MCF7/VP cells were stably transfected with expression vectors containing cDNAs encoding the human GST A1-1 (MCF7/VPa), GST M1-1 (MCF7/VPa), or GST P1-1 (MCF7/VPa). Additionally, control cell lines were generated by stable

---

3 C. S. Morrow, P. K. Smitherman, S. K. Diah, E. Schneider, and A. J. Townsend, manuscript in preparation. 4 C. S. Morrow, P. K. Smitherman, S. K. Diah, E. Schneider, and A. J. Townsend, unpublished data.
transfection of parental MCF7/WT and MCF7/VP cells with empty expression vectors encoding antibiotic resistance to hygromycin (hyg) or geneticin (neo) but not GST. Expression of GST isozyme-specific protein and mRNA was detected only in cell lines transfected with GST expression vectors (Fig. 1). In these cells (MCF7/VPα, MCF7/VPμ, and MCF7/VPπ), relatively high levels of GST activity were confirmed by enzyme assays (Table I). GST activities remained uniformly low in the parental and control cell lines (Table I). High levels of MRP1 expression (membrane protein and mRNA) were seen only in parental MCF7/VP cells and their transfected derivatives. Co-expression of GST isoforms had no effect on the levels of MRP1 (Fig. 2). Finally, glutathione levels were comparable in all of the cell lines tested (Table I).

Transfection of control vectors devoid of GST cDNA inserts into parental MCF7/WT and MCF7/VP cells had no effect on GST levels (MCF7/WT-neo and -hyg; MCF7/VP-neo and -hyg), glutathione levels (MCF7/WT-neo and -hyg; MCF7/VP-neo and -hyg), or MRP1 expression (Table I). Moreover, cytotoxicity profiles of parental cells MCF7/WT and MCF7/VP were similar to the corresponding cells transfected with control vectors (MCF7/WT-neo and -hyg; MCF7/VP-neo and -hyg). Thus, data reported in the text below will not differentiate between the parental cells and their corresponding derivatives transfected with control vectors.

**Effect of GSTs on the Sensitivities to Drug Cytotoxicities of MRP1-expressing MCF7 Cells**—The effect of MRP1 expression, alone or in combination with three different isozymes of cytosolic GST, on cellular sensitivities to various cytotoxic anticancer drugs was tested. Many of these data are summarized in Table II. A comparison of the relative resistance of MCF7/WT versus MCF7/VP cells to four drugs of the MDR phenotype, VP-16, doxorubicin, vincristine, and mitoxantrone, confirmed previous findings (32) that increased expression of MRP1 alone confers resistance to these drugs. Although VP-16, doxorubicin, and vincristine are not known to form conjugates with glutathione, we wondered whether these drugs or their metabolite derivatives might be unrecognized, toxic substrates of any of the three cytosolic GST isozymes tested and, if so, whether the GST isoforms would augment MRP1-mediated resistance to these drugs. Although mitoxantrone can form glutathione conjugates, it is not known whether any of the cytosolic GSTs tested can catalyze these reactions. However, as shown in Table II, expression of relatively high levels of GST A1-1 (MCF7/VPα cells), GST M1-1 (MCF7/VPμ cells), or GST P1-1 (MCF7/VPπ cells) had no significant effect on the level of MRP1-associated resistance to these MDR-related drugs.

In contrast to the MDR phenotype-associated drugs, a variety of alkylating anticancer drugs are known to form glutathione conjugates both spontaneously and in GST-catalyzed reactions (7–14). However, MRP1 has not consistently been associated with resistance to these drugs. Nevertheless, a number of glutathione conjugates, including monogluthathionyl derivatives of chlorambucil and melphalan, are known to be substrates of MRP1-dependent transport in isolated membrane vesicles (27, 33). Therefore, we investigated whether MRP1 could support resistance to some of these alkylating agents if co-expressed with the GST isozyme appropriate to accelerate conjugate formation. There are reports that implicate GST catalysis in detoxification reactions involving all of the alkylating agents listed in Table II (13, 15–18, 21–23, 25, 46). However, neither MRP1 nor the isoforms of GST tested had any significant effect on relative resistance to melphalan, 1,3-bis(2-chloroethyl)-1-nitrosourea, thiotepa, or hepsulfan. In contrast, co-expression of GST A1-1 and MRP1 conferred significant resistance to chlorambucil, a nitrogen mustard closely related to melphalan (Fig. 3). Indeed, cumulative results from 12 independent experiments showed that GST A1-1 expression is associated with ~4-fold (3.74 ± 0.19 S.E.) resistance to chlorambucil in MCF7/VPα cells compared with MCF7/VP cells (p < 0.0001). Resistance to chlorambucil was GST A1-1 isozyme-specific because co-expression of MRP1 with GST M1-1 or GST P1-1 had no effect on cellular sensitivity to chlorambucil (not shown).
Dicumarol was chosen as a candidate inhibitor because it has been reported to inhibit a rodent Alpha class GST and was found to be potent for this GST A1-1-catalyzed reaction (4). Inhibition of the conversion of CDNB to DNP-SG by purified, recombinant GST A1-1 was accomplished using affinity-purified GST A1-1 and CDNB as the variable substrate (see under “Experimental Procedures”). Under these conditions, kinetic parameters estimated from the data shown in the Hane’s plots are: $V = 64.3 \mu\text{mol/min/mg enzyme}$ and $K_{m} = 0.36 \text{mM}$ for CDNB; and $K_{m} = 4.4 \mu\text{M}$ and $K_{i} = 3.6 \mu\text{M}$ for dicumarol. B. Inhibition of GST A1-1 in vivo was done as described (see under “Experimental Procedures”). Cells were incubated with 0.1 mM dicumarol (open circles, + dicumarol) or vehicle (closed circles, − dicumarol) at 37 °C, and at the indicated times, the rate of CDNB conversion to DNP-SG was measured in intact cells. Shown are mean values ± the range of duplicate determinations, expressed as the fraction of 0 time controls.

Dicumarol had no effect on the kinetics of MRP1-dependent DNP-SG efflux (data not shown). Based upon these preliminary results, the effect of GST A1-1 inhibition by dicumarol on MCF7/VPα cell sensitivity to chlorambucil was examined.

Treatment of MCF7/VPα cells with 0.1 mM dicumarol sufficed to inhibit GST activity by ≥ 80% resulted in nearly complete reversal of GST A1-1-associated resistance (Fig. 5A). Moreover, dicumarol-mediated sensitization was selective for GST A1-1-expressing MCF7/VPα cells because dicumarol had no significant effect on the sensitivities of MCF7/WT and MCF7/VP cells to chlorambucil cytotoxicity (Fig. 5, A and B).

**Expression of GST A1-1 Protein and Catalytic Activity Are Required for MCF7/VPα Resistance to Chlorambucil**—Inspection of cytotoxicity profiles of MCF7/WT versus MCF7/VP reveals that MRPI alone does not confer resistance to chlorambucil (Fig. 3). Indeed, as shown here and reported previously (32), expression of MRPI alone is associated with modest sensitization to chlorambucil cytotoxicity. This indicates that the resistance to chlorambucil in MRPI-positive cells is absolutely dependent upon GST A1-1 protein expression. To confirm this relationship and to eliminate the possibility that MCF7/VP cells express a resistance to chlorambucil is absolutely dependent upon GST A1-1, we tested whether inhibition of GST A1-1 by dicumarol would reverse resistance in MCF7/VPα cells.

Dicumarol was chosen as a candidate inhibitor because it has been reported to inhibit a rodent Alpha class GST and was expected to inhibit the human ortholog, GST A1-1 (47). Additionally, dicumarol is lipophilic and therefore should freely traverse the cell membrane of intact cells. To determine whether the inhibitory properties and potency of dicumarol would likely be sufficient to inhibit cellular GST A1-1 at sub-cytotoxic levels, we first examined the kinetics of dicumarol inhibition of the conversion of CDNB to DNP-SG by purified, recombinant GST A1-1 in vitro. These results (Fig. 4A) show that dicumarol inhibition is of the mixed type and is quite potent for this GST A1-1-catalyzed reaction ($K_{i} = 3.6 \mu\text{M}$, $K_{m} = 4.4 \mu\text{M}$) (48). The effect of exogenously added dicumarol on GST A1-1 activity in vivo is examined in Fig. 4B. These data show that 0.1 mM dicumarol inhibited GST activity in MCF7/VPα cells by at least 80%, that inhibition was maximal within 15 min, and that inhibition remained at this level throughout the 1-h incubation. Treatment of intact cells with 0.1 mM dicumarol had no effect on the kinetics of MRPI-dependent DNP-SG efflux (data not shown). Based upon these preliminary results, the effect of GST A1-1 inhibition by dicumarol on MCF7/VPα cell sensitivity to chlorambucil was examined.

**Effect of MRP and GST expression on cellular sensitivities to the cytotoxicities of antineoplastic drugs**

*Relative resistance = IC_{50} of cell line ÷ IC_{50} of MCF7/VP cell line. Values are the means of at least duplicate experiments. For values derived from ≥ 3 determinations, shown are ± 1 S.D. from the mean. ND, not determined.*

**Table II**

| Drug                        | MCF7/WT | MCF7/VP | MCF7/VPα | MCF7/VPβ | MCF7/WT | MCF7/VPα |
|-----------------------------|---------|---------|----------|----------|---------|----------|
| MDR-associated              |         |         |          |          |         |          |
| VP-16                       | 0.03 ± 0.002 | 1.0     | 1.1      | 0.95     | 0.95    | 0.95     |
| Doxorubicin                 | 0.09 ± 0.002 | 1.0     | 1.2      | 1.1      | 0.7     |
| Vinorelbine                 | 0.39     | 1.0     | 1.1 ± 0.2 | 1.0 ± 0.1 | 1.1 ± 0.2 |
| Mitoxantrone                | 0.10 ± 0.13 | 1.0     | 1.4 ± 0.4 | 1.2 ± 0.2 | 1.0 ± 0.2 |
| Alkylating agents           |         |         |          |          |         |          |
| Melphalan                   | 1.0 ± 0.2 | 1.0     | 1.1 ± 0.1 | 1.1      | 1.2     |
| 1,3-bis(2-chloroethyl)-1-nitrosourea | 1.3     | 1.0     | 1.3      | 1.3      | 0.75    |
| Thiotepa                    | 0.83     | 1.0     | 1.3      | ND       | ND      |
| Hepsulfon                   | 1.1      | 1.0     | 1.0      | 1.4      | 0.95    |

**Fig. 3. GST A1-1 confers resistance to chlorambucil in MRPI-expressing MCF7 cells.** Shown are the profiles for chlorambucil cytotoxicity (1-h drug exposure) in parental MCF7/WT (WT) and MCF7/VP (VP) cells and in MCF7/VP cells transfected with GST A1-1 (MCF7/VPα, VPα). Error bars represent ± 1 S.D. from the mean proportion of cells surviving ($n = 8$) at the indicated chlorambucil concentrations.

**Fig. 4. Inhibition of GST A1-1 by dicumarol, in vitro and in vivo.** A. kinetic analysis of dicumarol inhibition of GST A1-1 in vitro was accomplished using affinity-purified GST A1-1 and CDNB as the variable substrate (see under “Experimental Procedures”). Under these conditions, kinetic parameters estimated from the data shown in the Hane’s plots are: $V = 64.3 \mu\text{mol/min/mg enzyme}$ and $K_{m} = 0.36 \text{mM}$ for CDNB; and $K_{m} = 4.4 \mu\text{M}$ and $K_{i} = 3.6 \mu\text{M}$ for dicumarol. B. Inhibition of GST A1-1 in vivo was done as described (see under “Experimental Procedures”). Cells were incubated with 0.1 mM dicumarol (open circles, + dicumarol) or vehicle (closed circles, − dicumarol) at 37 °C, and at the indicated times, the rate of CDNB conversion to DNP-SG was measured in intact cells. Shown are mean values ± the range of duplicate determinations, expressed as the fraction of 0 time controls.
Required for Chlorambucil Resistance in MCF7/VP cells—To evaluate the requirement for MRP1 in the observed chlorambucil resistance in MCF7/VP cells, we used sulfinpyrazone to inhibit MRP1-mediated glutathione conjugate efflux activity. It is perhaps even more remarkable that sulfinpyrazone completely reverses MCF7/VPα resistance to chlorambucil but has little effect on MCF7/WT or MCF7/VP cells (Fig. 7, A and B). Thus, sulfinpyrazone sensitization to chlorambucil toxicity is selective for GST A1-1-expressing cells.

MRP1-dependent Glutathione Conjugate Efflux Activity Is Absolutely Required to Potentiate GST A1-1-Mediated Resistance to Chlorambucil—We conclude that MRP1-dependent glutathione conjugate efflux activity is absolutely required to potentiate GST A1-1-mediated resistance to chlorambucil in MCF7 cells under the conditions used.

**DISCUSSION**

Our results establish that MRP1 and GST A1-1 act in synergy to confer resistance to chlorambucil in MCF7 cells. The co-dependence of resistance upon both GST A1-1 and MRP1 is remarkable. First, that GST A1-1 activity alone does not confer protection indicates that the glutathione conjugate(s), or other metabolites, of chlorambucil, the formation of which is favored in the presence of GST A1-1, may be toxic to the cells. Because the conjugate(s) and other derivatives of chlorambucil formed are more water-soluble than the parent compound and, therefore, less permeable to the plasma membrane, they may accumulate to high intracellular levels in the absence of MRP1 or another suitable efflux mechanism. This may explain why GST A1-1, at the levels achieved, is insufficient to confer protection from chlorambucil cytotoxicity in the absence of MRP1-dependent efflux activity. It is perhaps even more remarkable that MRP1 alone fails to confer protection from chlorambucil cytotoxicity. This finding is particularly significant because glutathione conjugation of chlorambucil can occur non-enzymatically (10, 49) and because glutathione conjugates of chlorambucil, especially the monogluthathionyl derivative, are

---

4 S. Diaih, unpublished data.
chlorambucil cytotoxicity is expressed as fold sensitization (IC₅₀ from the mean fraction surviving. B. control) or presence (scribed under “Experimental Procedures.”

chlorambucil in MCF7/VP efflux with sulfinpyrazone completely reverses resistance to presence of 2 mM sulfinpyrazone).

with chlorambucil for 1 h in the absence (-, closed symbols, vehicle control) or presence (+, open symbols) of 2 mM sulfinpyrazone as described under “Experimental Procedures.” Error bars signify ± 1 S.D. from the mean fraction surviving. B, effect of sulfinpyrazone on chlorambucil cytotoxicity is expressed as fold sensitization (IC₅₀ indicated cell line in the absence of sulfinpyrazone + IC₅₀ cell line in the presence of 2 mM sulfinpyrazone). Bars represent mean values (± 1 S.E.) of four independent experiments.

reportedly substrates of MRP1-dependent transport in isolated membrane vesicles (33). Thus, it is not immediately obvious why MRP1 alone does not afford some protection from chlorambucil toxicity. These issues have important implications for understanding the dynamics of drug detoxification and the relative importance of the components of drug detoxification studied—components that include phase II drug conjugation (GST/glutathione) and phase III drug/conjugate efflux (MRP1).

GST A1-1 is known to catalyze the formation of the monoglutathionyl, but not the diglutathionyl, derivative of chlorambucil from glutathione and chlorambucil (49). However, the catalytic rate enhancement is relatively modest (10, 11, 49). Indeed, Meyer et al. (49) suggest that, rather than increasing the overall extent of conjugation with glutathione, the major effect of the human GST alpha class isozymes, A1-1, A1-2, and A2-2 on chlorambucil metabolism may be to increase the proportion of monoglutathionyl chlorambucil relative to diglutathionyl chlorambucil and other derivatives (including hydroxy- and phosphate-substituted metabolites) (49). This altered profile is believed to be the combined consequence of 1) GST Alpha class catalysis of monoglutathionyl chlorambucil formation, and 2) the ability of these GSTs to sequester monoglutathionyl chlorambucil at the enzyme active site with high affinity (49).

Because the monoglutathionyl chlorambucil retains one of the reactive chloroethyl groups, this metabolite of chlorambucil is only partially detoxified. Although GST A1-1 may sequester the monoglutathionyl derivative and thereby mitigate some toxicity, with continued exposure to chlorambucil, this GST:monoglutathionyl chlorambucil interaction will become saturated and will inhibit further catalysis (49, 50). Thus, in the absence of MRP1-dependent efflux of the monoglutathionyl derivative, the detoxification capacity of GST A1-1 may be quickly exceeded. It is possible that the other derivatives may also have some toxicities at high intracellular levels.

Our transfection data show that GST A1-1 protein is required for MRPI-associated resistance to chlorambucil in MCF7 cells. Moreover, results from in vivo inhibition of GST A1-1 with dicumarol indicate that GST catalytic activity is also an important requirement for maximum resistance. We have recently confirmed that purified GST A1-1 does bind radiola beled monoglutathionyl chlorambucil with considerable avidity. Hence, both catalytic activity and chlorambucil conjugate binding could be important mechanisms of GST A1-1-associated cytoprotection. In our view, GST A1-1 may serve to catalyze the substitution of glutathione to one of the chloroethyl groups of chlorambucil. These monoglutathionyl derivatives, formed both enzymatically and non-enzymatically, can then be sequestered as relatively benign complexes with GST A1-1 until they can be delivered to MRP1 for export. In the absence of MRP1, as monoglutathionyl chlorambucil accumulates intracellularly, the catalytic activity GST A1-1 is compromised by product inhibition and the binding capacity of the enzyme for monoglutathionyl chlorambucil is exceeded. Consequently, the levels of chlorambucil remain high, and the levels of reactive, free monoglutathionyl chlorambucil and its other derivatives accumulate intracellularly resulting in increased cytotoxicity. In the absence of GST A1-1, not only is the monoglutathionyl derivative free to react with cellular macromolecules, but the distribution of chlorambucil metabolites is shifted away from the monoglutathionyl forms to other derivatives that are significantly poorer substrates for MRP1-mediated efflux. The potential importance of such GST A1-1-dependent changes in the profile of chlorambucil glutathione conjugates and derivatives is underscored by the recent findings of Barnoun et al. (33). These investigators show that the monoglutathionyl chlorambucil is by far the best chlorambucil derivative for MRPI-dependent transport in isolated membrane vesicles in vitro.

The three isozymes of GST tested confer no protection to MRP1-expressing MCF7/VP cells against the cytotoxicities of the four other alkylating agents examined. The reason for this is unknown. The GSTs examined may have little impact in vivo on the metabolism of thiota, hepsulfan, or 1,3-bis(2-chloroethyl)-1-nitrosourea. Additionally, it is not known whether MRP1 can support the efflux of glutathione conjugates of thiota or hepsulfan. Some GST isozymes are reported to catalyze the denitrosation of 1,3-bis(2-chloroethyl)-1-nitrosourea, but these studies have not identified stable glutathione conjugate intermediates (15, 22). Thus, there may be no stable glutathi one conjugate of 1,3-bis(2-chloroethyl)-1-nitrosourea to serve as a potential MRP1 substrate. Particularly interesting is the failure of GST A1-1 and MRP1 to confer resistance to melphalan, a nitrogen mustard closely related to chlorambucil. The explanation for the difference in melphalan and chlorambucil resistance is unknown. Structural differences in these drugs may result in GST A1-1 having distinctly different effects on the metabolic profiles of melphalan and chlorambucil or their glutathione conjugates in vivo. Alternatively, glutathione conjugates of melphalan may be less efficiently exported by MRP1. Regardless of the explanation, our results show that MRP1/GST resistance synergy is both specific for a particular GST isozyme:drug pair and highly drug-selective, even among structurally related drugs.

The magnitude of MRP1-mediated resistance to four drugs of the MDR phenotype was not significantly augmented by coexpression of GST A1-1, GST M1-1, or GST P1-1. This result
was not surprising for the drugs doxorubicin, VP-16, and vincristine, which are not known to form stable conjugates with glutathione. However, it was important to examine these drugs because it was possible that unrecognized metabolites or derivatives of these compounds might form glutathione conjugates that could influence their export by MRP1. In contrast, mitoxantrone reacts with glutathione to form conjugates (51). However, mitoxantrone is not known to be a substrate of cytosolic GSTs, although it is reported to be a substrate of microsomal GST (52). The failure of the cytosolic GSTs tested to augment MRP1-mediated resistance to mitoxantrone is consistent with these data.

In summary, we demonstrate that GST A1-1 can act in synergy with MRP1 to confer resistance to the alkylating agent chlorambucil. The concept of coordinated action of phase II GST-dependent and phase III MRP1-dependent (and other membrane transport proteins) processes will very likely prove to be of general importance for detoxification of a variety of xenobiotic compounds, electrophiles that include genotoxic carcinogens (31) and anticancer drugs. The mechanism of GST A1-1/MRP1 resistance to chlorambucil depends upon both GST A1-1 catalytic activity and MRP1-dependent efflux activity. Moreover, because MRP1 is required to potentiate GST A1-1-associated resistance to chlorambucil, this suggests the possibility that some of the glutathione conjugates or other metabolites may themselves be important cellular toxins. Additionally, the removal of these conjugates by MRP1 may be required to relieve product inhibition and thereby maintain continued GST catalysis of drug conjugation. Finally, GST/MRP1 resistance synergy is highly specific for the particular xenobiotic: GST isozyme pair. There are multiple known and putative membrane-associated transport proteins related to MRP1 (53). It is possible that the complete detoxification of xenobiotic-glutathione conjugates will prove to be similarly dependent upon the specific membrane-associated transport protein expressed.

REFERENCES

1. Black, S. M., and Wolf, C. R. (1991) Pharmaco. Ther. 51, 139–154
2. Coles, B., and Ketterer, B. (1990) Crit. Rev. Biochem. Mol. Biol. 25, 47–70
3. Hayes, J. D., and Pullford, D. J. (1995) Crit. Rev. Biochem. Mol. Biol. 30, 445–600
4. Mannervik, B., and Danielson, U. H. (1988) Crit. Rev. Biochem. 23, 283–337
5. Mannervik, B., and Cowan, K. H. (1990) Cancer Cells 35, 1577–1581
6. Tew, K. D. (1994) Cancer Res. 54, 2410–2415
7. Aikou, T., Ebitani, T., Tsuchiya, K., and Kadoya, M. (1997) Cancer Res. 57, 13033–13037
8. Black, S. M., Beggs, J. D., Hayes, J. D., Bartoszek, A., Muramatsu, M., Sakai, M., and Wolf, C. R. (1990) Biochem. J. 268, 309–315
9. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 75, 919–925
10. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
11. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
12. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
13. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
14. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
15. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
16. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
17. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
18. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
19. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
20. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
21. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
22. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
23. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
24. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
25. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
26. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
27. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
28. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
29. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
30. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
31. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
32. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
33. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
34. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
35. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
36. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
37. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
38. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
39. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
40. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
41. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
42. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
43. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
44. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
45. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
46. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
47. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
48. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
49. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
50. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
51. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
52. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102
53. Bocan, J., Frisk, K., and Ketterer, B. (1997) Br. J. Cancer 76, 1098–1102