Biochemical Pharmacology, Vol. 56, pp. 1013–1022, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00240-8

ELSEVIER

Combined Expression of Multidrug Resistance Protein (MRP) and Glutathione *S*-Transferase P1-1 (GSTP1-1) in MCF7 Cells and High Level Resistance to the Cytotoxicities of Ethacrynic Acid but Not Oxazaphosphorines or Cisplatin

Charles S. Morrow, Pamela K. Smitherman and Alan J. Townsend*

Department of Biochemistry, Wake Forest University School of Medicine, Winston-Salem, NC 27157-1016, U.S.A.

ABSTRACT. We tested the hypothesis that combined increased expression of human glutathione *S*-transferase P1-1 (GSTP1-1), an enzyme that catalyzes the conjugation with glutathione of several toxic electrophiles, and the glutathione–conjugate efflux pump, multidrug resistance protein (MRP), confers high level resistance to the cytotoxicities of anticancer and other drugs. To accomplish this, we developed MCF7 breast carcinoma cell derivatives that express high levels of GSTP1-1 and MRP, alone and in combination. Parental MCF7 cells, which express no GSTP1-1 and negligible MRP, served as control cells. We found that either MRP or GSTP1-1 alone conferred significant resistance to ethacrynic acid cytotoxicity. Moreover, combined expression of GSTP1-1 and MRP conferred a high level of resistance to ethacrynic acid that was greater than resistance conferred by either protein alone. Increased MRP was also associated with modest resistance to the oxazaphosphorine compounds mafosfamide, 4-hydroxycyclophosphamide, and 4-hydroperoxycyclophosphamide. However, coordinated expression of GSTP1-1 with MRP failed to augment this modest resistance. Similarly, GSTP1-1 had no effect on the sensitivities to cisplatin of MCF7 cells regardless of MRP expression. These results establish that coordinated expression of MRP and GSTP1-1 can confer high level resistance to the cytotoxicities of some drugs, including ethacrynic acid, but that such resistance is variable and does not apply to all toxic drugs that can potentially form glutathione conjugates in either spontaneous or GSTP1-1-catalyzed reactions. BIOCHEM PHARMACOL **56**;8:1013–1022, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. drug resistance; detoxification; efflux; conjugation; cellular models

The GSTs† (EC 2.5.1.18) are a multigene family of isozymes that catalyze the conjugation with glutathione of many toxic, electrophilic compounds [1–3]. Generally, but not always, the conjugates formed are less toxic to the cell. Consequently, GSTs are believed to play important roles in the protection of cells from these toxic substrates that include antineoplastic drugs, carcinogens, and mutagens [4–6].

Increased levels of GSTs are often associated with the emergence of resistance to some antineoplastic agents in drug-selected cell lines [4–6]. To obtain more direct evidence in support of the role of GST isozymes in drug resistance, GST expression vectors were stably transfected into MCF7 cells—cells that normally express very low levels of GST. In these studies, increases in cytosolic GSTs generally failed to confer significant resistance to antineoplastic drugs, including drugs known to be substrates of the GST isozymes transfected [7–9]. These results led to the suggestion that other factors necessary to potentiate GST-mediated resistance may be limiting in MCF7 cells [6]. These co-factors of resistance might include glutathione levels or GS-X export. Indeed, conjugates of glutathione and their metabolites, if not efficiently transported out of the cells, may themselves be cytotoxic—either directly or by inhibition of other glutathione-dependent reactions [10–14].

Recently, MRP, an energy-dependent, membrane-associated drug efflux pump responsible for one form of P-glycoprotein-independent MDR, has been shown to support the efflux of several conjugates of glutathione [15–19]. The finding that MRP is an important GS-X transporter led us to propose that coordinated expression of MRP and GST may confer a level of resistance to some cytotoxic compounds that is greater than that achievable by the expres-

* Corresponding author: Dr. Charles S. Morrow, Department of Biochemistry, Wake Forest University School of Medicine, Medical Center Blvd., Winston-Salem, NC 27157-1016. Tel. (336) 716-9478; FAX (336) 716-7671; E-mail: cmorrow@wfubmc.edu

†*Abbreviations:* EA-SG, glutathione conjugate of ethacrynic acid; GST, glutathione *S*-transferase; GS-X, glutathione conjugate; 4-OOH-CP, 4-hydroperoxycyclophosphamide; IC_{50}, drug concentration at which cell proliferation is inhibited 50% of control; maf, mafosfamide; MDR, multidrug resistance or resistant; MRP, multidrug resistance protein; and 4-OH-CP, 4-hydroxycyclophosphamide.

Received 4 May 1998; accepted 18 June 1998.

sion of either protein alone. In this view, drug detoxification would involve the sequential formation of drug–glutathione conjugates in GST-catalyzed reactions followed by conjugate efflux by MRP-mediated transport. Accordingly, the failure of increased GST isozymes to confer resistance in transfected MCF7 cells to the drugs tested previously may have been due to the extremely low level of MRP expressed in these cells [20]. Indeed, even though the human pi class GST, GSTP1-1, efficiently catalyzes the conjugation of 4-nitroquinoline 1-oxide with glutathione, we recently showed that high level protection from the cytotoxicity of 4-nitroquinoline 1-oxide by GSTP1-1 in MCF7 cells is attained only when MRP is expressed concomitantly with GSTP1-1 [21, 22].

The experiments reported herein were designed to test whether co-expression of MRP and GSTP1-1 offers a level of protection from certain cytotoxic drugs not achieved by the expression of either protein alone. This was accomplished by stable transfection with *GSTP1* expression vectors into MCF7/WT cells, which express negligible MRP and no GSTP1-1, and into MCF7/VP cells, MDR variants that express high level MRP but no GSTP1-1 [20]. We examined the cytotoxicities of several drugs towards parental (MCF7/WT and MCF7/VP) and GSTP1-1-transfected derivative MCF7 cells. The drugs were chosen on the basis of their ability to form glutathione conjugates either spontaneously or in GST-catalyzed reactions. They included ethacrynic acid, three oxazaphosphorines (maf, 4-OH-CP, and 4-OOH-CP), and cisplatin. The MCF7 clone pairs were matched for similar levels of GSTP1-1 expression. Ethacrynic acid and the oxazaphosphorines were chosen because either the parent compound (ethacrynic acid) or some of the metabolites (oxazaphosphorines) are known to be substrates of GSTP1-1 [23–26]. Cisplatin was chosen because it can form glutathione conjugates that are transported by membrane efflux pumps related to MRP [27–30]. While GSTP1-1 has not been shown to be directly involved in metabolic transformation of cisplatin, results from some laboratories have implicated increased GSTP1-1 in the development of cisplatin resistance [31, 32].

MATERIALS AND METHOD

Tissue Culture

MCF7 breast carcinoma cells, MCF7/WT and their MDR derivative, MCF7/VP [20], were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and penicillin/streptomycin (Life Technologies) at 37°, 5% CO_2.

Expression Vectors and Stable Transfections

The plasmid vector ΔpREP7 was derived from pREP7 (Invitrogen). It was modified to remove sequences encoding the Epstein Barr Virus origin of replication and nuclear antigen and thus is unable to replicate as an episome. The ΔpREP7 was made by excision of the *Xba*I/*Avr*II fragment, blunt end formation with Klenow fragment, and re-circularization with T4 DNA ligase. *GSTP1* expression vectors were made by inserting the complete GSTP1 cDNA (encoding the *GSTP1*A* allele [33, 34]) into pcDNA3 (Invitrogen) or ΔpREP7 to create the plasmids pcDNA3π and ΔpR7π, respectively.

The *GSTP1* expression vectors (pcDNA3π and ΔpR7π) and empty control vector (pcDNA3) were stably transfected into the cell lines MCF7/WT and MCF7/VP. This was accomplished by the calcium phosphate method [35] followed by selection in 1 mg/mL of geneticin (Life Technologies) or 0.4 mg/mL of hygromycin (Calbiochem). Clones expressing increased levels of GSTP1-1 activity were identified from geneticin (pcDNA3-based vector transfections) or hygromycin (ΔpREP7-based vector transfections) resistant colonies by GST assay (see below). Stably transfected clones were grown in selecting drug until ~48 hr prior to the cytotoxicity experiments (see below). GST enzyme assays of the various cloned cell lines were performed periodically to assure stable levels of GSTP1-1 expression.

Biochemical Assays

The cytosolic GST activities towards 1-chloro-2,4-dinitrobenzene (CDNB) substrate were determined for cell lysates derived from control and transfected cell lines [36]. Total glutathione levels were determined by the enzymatic recycling method [37]. For glutathione assays, cells were washed and suspended in ice-cold phosphate-buffered saline plus 1 mM EDTA. An aliquot was sonicated, and the supernatant was assayed for protein concentration [38]. The remaining

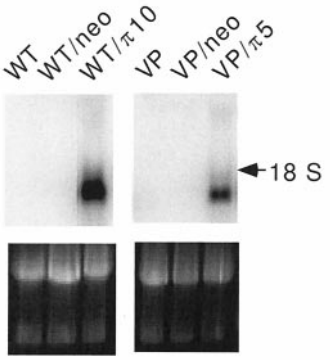

FIG. 1. Expression of GSTP1 mRNA in parental and transfected MCF7 cells. Northern blot analysis of GSTP1 expression was done with total cellular RNA (10 μg/lane), as described in Materials and Methods. Shown are blots hybridized with radiolabeled GSTP1 cDNA inserts (upper panels) and the ethidium bromide stained gel prior to northern transfer (lower panels). RNA samples were derived from parental MCF7/WT (WT) and MCF7/VP (VP) cells and their corresponding derivatives transfected with empty expression vectors, MCF7/WTneo (WT/neo) and MCF7/VPneo (VP/neo), or transfected with *GSTP1* expression vectors, MCF7/WTπ10 (WT/π10) and MCF7/VPπ5 (VP/π5).

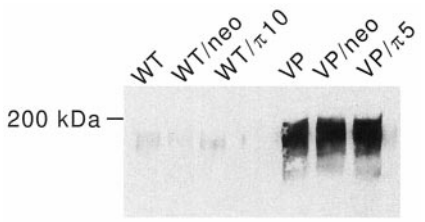

FIG. 2. Expression of MRP membrane protein in parental and transfected MCF7 cells. Protein (50 μg/lane) samples were obtained from membrane preparations of parental MCF7/WT (WT) and MCF7/VP (VP) cells and their corresponding derivatives transfected with empty expression vectors, MCF7/WTneo (WT/neo) and MCF7/VPneo (VP/neo), or transfected with *GSTP1* expression vectors, MCF7/WTπ10 (WT/π10) and MCF7/VPπ5 (VP/π5). Electrophoresis and Western blot analysis were accomplished as described in Materials and Methods using the MRP-specific primary antibody QCRL-1 [41].

cells were lysed in sulfosalicylic acid (2% final concentration), and the acid-soluble material was used for glutathione assay.

Northern and Western Blot Analyses

Expression of GSTP1 mRNA was determined by northern blot analysis of total cellular RNA [35] using an [α-^{32}P]dCTP labeled GSTP1 cDNA probe [39, 40]. Western blot analysis of membrane-associated MRP was accomplished as described [41], using the QCRL-1 monoclonal antibody supplied by S.P.C. Cole.

Drug Cytotoxicity Assays

Ethacrynic acid and cisplatin were from the Sigma Chemical Co. 4-OH-CP was from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics, National Cancer Institute. Mafosfamide and 4-OOH-CP were provided by Dr. J. Pohl, Asta Medica, FRG. Drugs were stored at −80° as the following stock solutions: ethacrynic acid (100 mM in 95% ethanol), maf (20 mM in H_2O), cisplatin (6.67 mM in dimethyl sulfoxide), 4-OH-CP (100 mM in dimethyl sulfoxide) and 4-OOH-CP (10 mM in 95% ethanol).

Cells were plated at a density of 300/well in 96-well microtiter plates. Twenty-four hours later, medium was changed to medium containing drug or vehicle. All drug exposures, except those with ethacrynic acid, were for 1 hr in serum-free medium. Following drug treatment, medium was replaced with medium containing 10% fetal bovine serum. For ethacrynic acid, drug or vehicle exposures were continuous in medium containing 10% fetal bovine serum. Six days following initial exposures to drug, cells were fixed and stained according to the sulforhodamine B cytotoxicity assay described [42].

RESULTS

Transfection and Properties of Model Cell Lines

Previous studies showed that MCF7/WT cells express no GSTP1-1 and negligible membrane-associated MRP [20, 22, 40]. An MDR derivative of MCF7 cells, MCF7/VP, expresses a very high level of membrane-associated MRP but, like MCF7/WT, this cell line does not express GSTP1-1 [20]. These parental cell lines (MCF7/WT and MCF7/VP) were transfected with *GSTP1* expression or empty control vectors as described in Materials and Methods. Northern blot analysis confirmed that GSTP1 mRNA is undetectable in parental and control vector transfected MRP minus cells (MCF7/WT and MCF7/WTneo) and MRP positive cells (MCF7/VP and MCF7/VPneo), whereas GSTP1 mRNA is highly expressed in the GSTP1-transfected clones (MCF7/WTπ10 and MCF7/VPπ5) (Fig. 1). Western blot analysis of membrane proteins derived from parental and transfected cells revealed that GSTP1 did not influence the level of MRP expression—a level that remained negligible in MCF7/WT- and high in MCF7/VP-derived cells (Fig. 2).

To assess the influence of GSTP1-1 on drug sensitivities, it was necessary to establish MCF7/WT and MCF7/VP clone pairs that are matched for comparable levels of both GST activity and glutathione. Accordingly, total GST activities and glutathione levels were determined in the parental and transfected clones (Table 1). The table shows that GST activity was very low in parental (MCF7/WT and MCF7/VP) and empty vector-transfected (MCF7/WTneo and MCF7/VPneo) clones. Increased and high level GST activities (~400 nmol/min/mg of protein, Table 1), completely attributable to the transfection-derived GSTP1-1 isozyme, were nearly identical in the GSTP1 vector transfected clones, MCF7/WTπ10 and MCF7/VPπ5. The glutathione levels were similar in all of the cell lines tested.

TABLE 1. GST activities and glutathione levels of model cell lines

Cell line	Description	MRP	GSTP1-1	GST activity*	Glutathione†
MCF7/WT	Parental MCF7	−	−	<10	92.3 ± 15.6
MCF7/WTneo	Empty vector transfected MCF7/WT	−	−	<10	81.0 ± 10.3
MCF7/WTπ10	*GSTP1* vector transfected MCF7/WT	−	+	405 ± 61	88.9 ± 12.9
MCF7/VP	Parental MDR derivative of MCF7	+	−	<10	90.8 ± 9.1
MCF7/VPneo	Empty vector transfected MCF7/VP	+	−	<10	81.7 ± 11.8
MCF7/VPπ5	*GSTP1* vector transfected MCF7/VP	+	+	408 ± 46	77.7 ± 15.2

*Expressed in nmol/min/mg protein (mean ± 1 SD, N ≥ 3).
†Expressed in nmol/mg protein (mean ± 1 SD, N ≥ 5).

Combined Expression of GSTP1-1 Plus MRP and High Level Resistance to Ethacrynic Acid

Figure 3 shows the results of ethacrynic acid cytotoxicity towards control (MCF7/WT, MCF7/WTneo, MCF7/VP, and MCF7/VPneo) and GSTP1 transfected clones (MCF7/WTπ10 and MCF7/VPπ5). The upper panel (Fig. 3A) shows a representative cytotoxicity profile, and the middle panel (Fig. 3B) shows the relative resistances (IC_{50} values normalized to IC_{50} of MCF7/WT) determined from five independent experiments. Stable transfection of the empty vectors had no significant effect on the sensitivity of cells to ethacrynic acid (compare MCF7/WT with MCF7/WTneo and MCF7/VP with MCF7/VPneo). Thus, these relative resistance data from MRP minus and MRP positive control cells (GSTP1 minus) were pooled, separately, in the bottom panel (Fig. 3C). These data show that expression of MRP alone (MCF7/VP and MCF7/VPneo or VP pool) resulted in a 1.7- to 2.0-fold increase in resistance to ethacrynic acid cytotoxicity when compared with MRP minus, GSTP1 minus cell lines (MCF7/WT and MCF7/WTneo or WT pool). These differences between MRP non-expressing and MRP expressing cells that lack GSTP1-1 were highly significant ($P < 0.0001$, *t*-test). Expression of GSTP1-1 alone (MCF7/WTπ10) conferred a 5.5-fold resistance to ethacrynic acid when compared with control cells (MCF7/WT and MCF7/WTneo or WT pool). Moreover, the combined expression of MRP and GSTP1-1 (MCF7/VPπ5) conferred a high level of resistance (~9-fold) to ethacrynic acid that was greater than that observed in cells expressing either MRP or GSTP1-1 alone. Three pairs of MCF7/WT and MCF7/VP clones matched for similar levels of GSTP1-1 expression were analyzed (results not shown). Without exception, high level MRP expression in MCF7/VP derivatives conferred ~2-fold resistance to ethacrynic acid over GSTP1-1-matched MCF7/WT derivatives. When MCF7/VP (MRP positive) and MCF7/WT (MRP minus) derivatives were considered separately, resistance to ethacrynic acid was positively correlated with the level of GSTP1-1 expressed ($R > 0.96$).

Effect of GSTP1-1 and MRP Expression on Cellular Sensitivities to the Cytotoxicities of Oxazaphosphorines and Cisplatin

Several metabolites of the oxazaphosphorine form conjugates with glutathione in both non-enzymatic and GST-catalyzed reactions [23–25, 43]. As some of these metabolites are substrates of GSTP1-1 [23, 24], we determined whether expression of GSTP1-1 and MRP could influence the sensitivities of MCF7 cells to the cytotoxicities of three oxazaphosphorines, maf, 4-OH-CP, and 4-OOH-CP. The upper panels of Fig. 4 (Fig. 4A) show representative cytotoxicity profiles, and the middle panels (Fig. 4B) show relative resistance (IC_{50} values normalized to the IC_{50} of MCF7/WT) determined from three independent experiments. Increased expression of GSTP1-1 had no significant

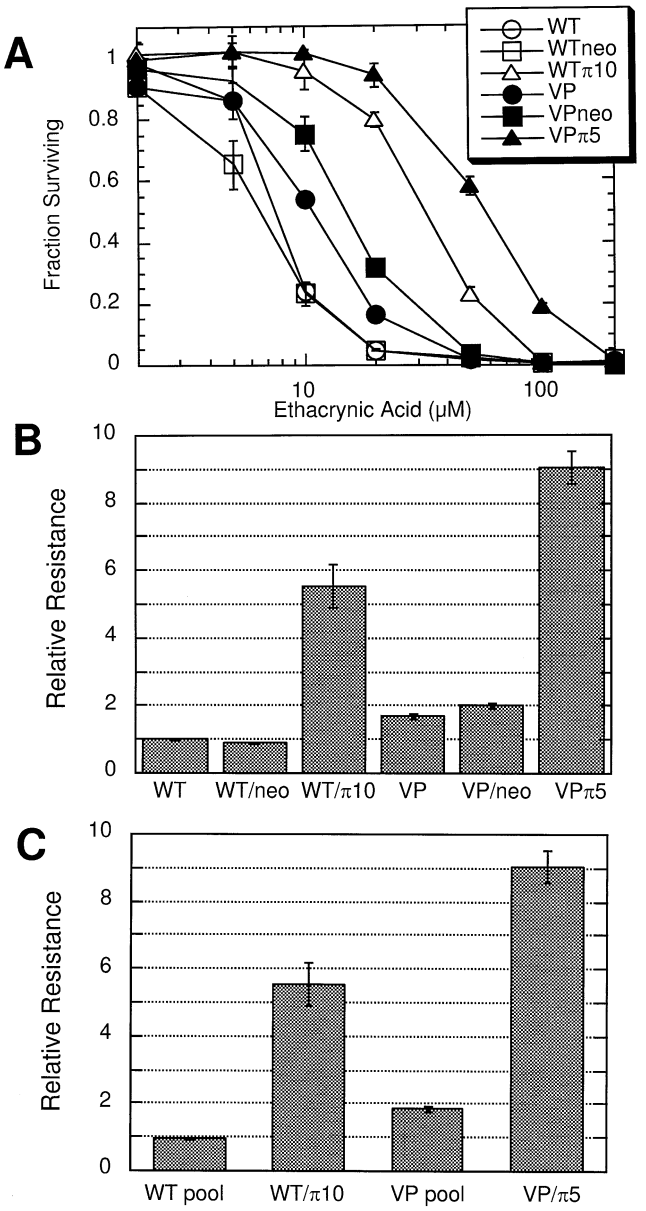

FIG. 3. Effect of GSTP1-1 and MRP on ethacrynic acid cytotoxicity. The cytotoxicity of continuous exposure to ethacrynic acid was examined in: GSTP1-1 minus/MRP minus MCF7 cell—MCF7/WT (WT) and MCF7/WTneo (WT/neo); GSTP1-1 positive/MRP minus MCF7 cells—MCF7/WTπ10 (WT/π10); GSTP1-1 minus/MRP positive MCF7 cells—MCF7/VP (VP) and MCF7/VPneo (VPneo); and GSTP1-1 positive/MRP positive MCF7 cells—MCF7/VPπ5 (VP/π5). Panel A shows a representative cytotoxicity profile. Error bars represent ± one standard deviation from the mean of 8 replicate wells. Panel B shows the relative resistance (IC_{50} cell line ÷ IC_{50} MCF7/WT cells) of the various cell lines. Bars represent the mean values ± one standard error of five independent experiments. Panel C is derived from panel B. The data from GSTP1-1 minus clones were pooled: the WT pool includes cytotoxicity data from MCF7/WT and MCF7/WTneo clones, while the VP pool includes data from MCF7/VP and MCF7/VPneo clones.

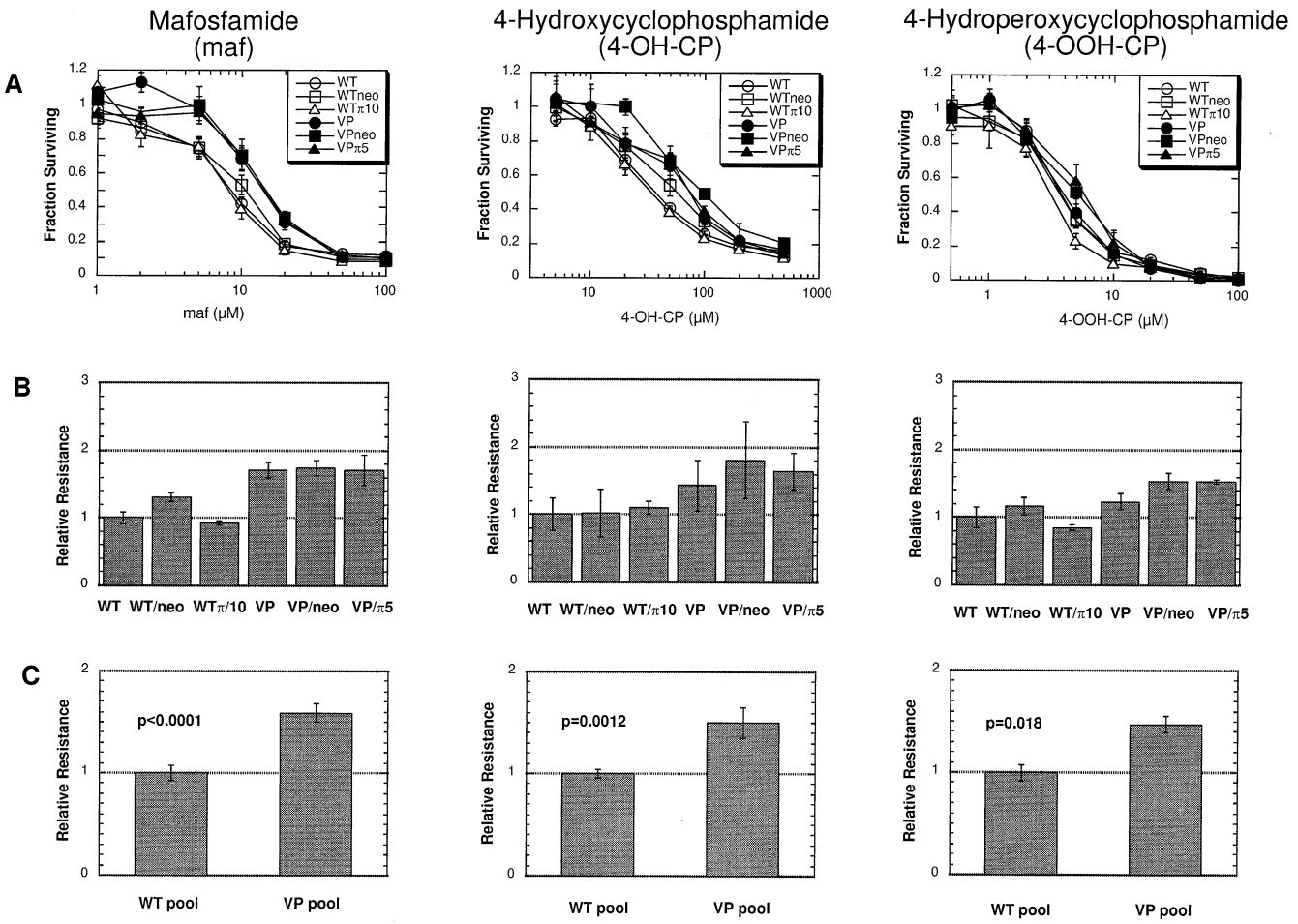

FIG. 4. Effect of GSTP1-1 and MRP on oxazaphosphorine cytotoxicities. Shown are the cytotoxicities of the three oxazaphosphorines, maf (left panels), 4-OH-CP (middle panels), and 4-OOH-CP (right panels). Cells were exposed to drug or vehicle for 1 hr, as described in Materials and Methods. Cell lines and derivations of the three panels (A, B, and C) are as described in Fig. 3 except that in panel C the WT pool included MCF7/WT (WT), MCF7/WTneo (WT/neo) and MCF7/WTπ10 (WT/π10), while the VP pool included MCF7/VP (VP), MCF7/VPneo (VP/neo), and MCF7/VPπ5 (VP/π5) cells. Mean values represented in panels are: (A) ± one standard deviation, N = 8; (B) ± one standard error, N = 3; and (C) ± one standard error, N = 9. The *P* values (C) refer to the relative resistances of the WT pool versus the VP pool (*t*-test).

effect on the cytotoxicities of any of these compounds in either the absence (compare MCF7/WTπ10 with MCF7/WT and MCF7/WTneo) or presence of concomitant MRP expression (compare MCF7/VPπ5 with MCF7/VP and MCF7/VPneo). However, increased expression of MRP (±GSTP1-1) tended to confer a slight decrease in sensitivities. This modest, MRP-associated resistance is apparent in the lower panel (Fig. 4C), where data pooled from all MRP minus, MCF7/WT cell lines (WT pool) are compared with data pooled from all MRP positive MCF7/VP cell lines (VP pool). These data show that ~1.5-fold resistance to all three oxazaphosphorines is associated with expression of MRP. These differences in MCF7/WT versus MCF7/VP sensitivities were statistically significant (maf, $P < 0.0001$; 4-OH-CP, $P = 0.0012$; and 4-OOH-CP, $P = 0.018$; *t*-test).

In some reports, development of resistance to cisplatin has been associated with increased GSTP1-1 [31, 32]. However, expression of GSTP1-1, even at the relatively high levels achieved, failed to confer resistance to MCF7/WTπ10 and MCF7/VPπ5 cells relative to their respective MRP minus (MCF7/WT and MCF7/WTneo) and MRP positive (MCF7/VP and MCF7/VPneo) controls (Fig. 5, A and B). However, expression of MRP (VP pool, Fig. 5C) appeared to confer a slight (1.3-fold), but statistically significant ($P = 0.0063$, *t*-test), resistance to cisplatin when compared with MRP minus cells (WT pool, Fig. 5C).

DISCUSSION

The studies presented in this report tested the postulate that combined expression of GSTP1-1 and MRP can confer *cellular* resistance to some cytotoxic compounds, including anticancer agents, that is greater than the cellular resistance conferred by either protein alone. This hypothesis is based upon observations that GSTs can catalyze the conjugation with glutathione of some of these toxins and that

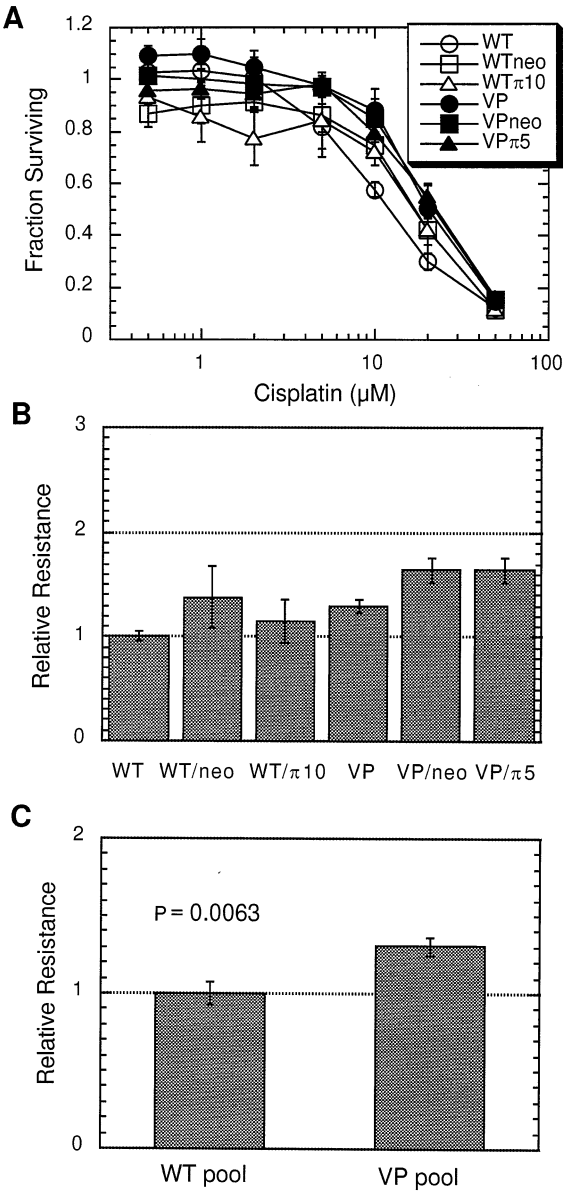

FIG. 5. Effect of GSTP1-1 and MRP on cisplatin cytotoxicity. The cytotoxicity of cisplatin towards the various cell lines was determined in 1-hr drug exposures, as described in Materials and Methods. Cell lines and derivations of the three panels (A, B, and C) are as described in Fig. 4. Mean values represented in panels are: (A) ± one standard deviation, N = 8; (B) ± one standard error, N = 3; and (C) ± one standard error, N = 9.

MRP-containing membrane vesicles can support GS-X transport [3, 15–19].

The studies herein provide insights that could not be drawn from prior *in vitro*, cell-free studies alone. Indeed, as has been shown by previous transfection studies, the demonstration that a cytotoxic compound is a potential substrate of a GST isozyme *in vitro* does not necessarily mean that the GST isozyme will confer resistance to the cytotoxin in intact cells [7–9]. Furthermore, the finding that a particular glutathione-toxin conjugate is an *in vitro* substrate of MRP-associated transport using membrane vesicles is not proof that MRP can confer cellular resistance to this toxin. For example, intracellular conditions may influence substrate availability or modulate enzyme kinetic parameters. In intact cells, the efficiency of glutathione–xenobiotic conjugate formation may be insufficient to influence cytotoxicity significantly. Alternatively, for some compounds, GST-dependent rate enhancement of conjugate formation may be relatively low. Here, non-enzymatic glutathione conjugation may predominate in cells, rendering GST levels irrelevant to detoxification. Finally, the activity of the MRP transporter may differ considerably between isolated membrane preparations and intact cells for a variety of reasons, including altered co-factor levels, alternative pathways of glutathione-conjugate processing and metabolism, or other factors that may be difficult to anticipate in cell-free studies alone. Indeed, we have shown that the apparent K_m of MRP towards *S*-(2,4-dinitrophenyl)-glutathione in intact MCF7/VP cells was considerably different from that determined using isolated membrane vesicles (Diah S and Morrow C, unpublished results). Moreover, if the toxin–glutathione conjugate is both stable and non-toxic, MRP-mediated efflux may offer no additional protection from cytotoxicity than that conferred by increased conjugation alone (e.g. by increased GST levels). Hence, the use of intact cells with defined alterations in the levels of MRP and GSTP1-1 provides both novel and complementary information to *in vitro* biochemical studies.

Chronic exposure of cells to cytotoxic drugs can result in the selection of multiple phenotypic and genetic changes. Thus, the use of MCF7/VP cells, which were selected for resistance to VP-16, was potentially problematic. However, a thorough characterization of MCF7/VP cells demonstrated that they are similar to, if not indistinguishable from, MCF7/WT cells with respect to doubling times, morphology, very low GST levels, glutathione levels, and undetectable P-glycoprotein (Table 1 and [20]). Indeed, besides high level MRP expression in MCF7/VP cells, the only reported molecular difference between MCF7/WT and MCF7/VP cells is a modest decrease in MCF7/VP topoisomerase II sensitivity to VP-16-induced cleavable complex formation [20]. This phenotypic change in topoisomerase II sensitivity is probably unrelated to ethacrynic acid, cisplatin, or oxazaphosphorine resistance. Thus, high level MRP is very likely the only molecular change in parental MCF7/VP cells germane to altered sensitivities to the drugs tested in the present report.

Ethacrynic acid was chosen as a model toxin for our studies for several reasons. Ethacrynic acid is a good substitute for some isozymes of GST including GSTP1-1, and increased GST has been associated with resistance to ethacrynic acid cytotoxicity [7–9, 26, 44]. Some cell lines selected for resistance to ethacrynic acid express increased levels of MRP [45], and MRP-containing membrane vesicles have been shown to transport glutathione conjugates of ethacrynic acid *in vitro* [46]. Finally, the cellular fate and cytotoxicity of ethacrynic acid are of practical interest

because this drug has been used clinically as a diuretic and, in cancer chemotherapy trials, as an inhibitor of GST [47].

Our results establish that MRP, alone or in combination with GSTP1-1, can confer resistance to ethacrynic acid. These results are the first documentation that simultaneous expression of GSTP1-1 and MRP can operate to confer high level cellular resistance to ethacrynic acid (Fig. 3). Increased expression of GSTP1-1 alone, while protective, is insufficient to cause this same high level resistance. This observation suggests that the glutathione conjugate of ethacrynic acid formed in GSTP1-1-catalyzed reactions may itself be toxic to the cell. The mechanism of the putative toxicity is unknown but may involve some direct toxicity of EA-SG. Alternatively, EA-SG may serve as an intracellular reservoir of ethacrynic acid that can later be released as free, toxic ethacrynic acid. Indeed, the formation of EA-SG from ethacrynic acid has been shown to be a reversible reaction [48]. Finally, accumulation of intracellular EA-SG may limit, via EA-SG product inhibition, further detoxification by GSTP1-1-mediated catalysis of EA conjugation [49, 50]. In this view, MRP-dependent efflux of EA-SG may augment GST-associated detoxification by: (1) eliminating EA-SG, which itself may be toxic, (2) eliminating EA-SG as a reservoir of free EA by the reverse conjugation reaction, or (3) eliminating EA-SG product inhibition of GST.

The oxazaphosphorine compounds include the clinically important anticancer drugs cyclophosphamide, mafosfamide, and ifosfamide. They are metabolized to derivatives that can form conjugates with glutathione [24, 25, 43]. Several of these metabolites, including acrolein [23], 4-OH-CP [24], and ifosfamide mustard [25], are known to be substrates of GSTP1-1. Heretofore, it was unclear whether GSTP1-1 alone or in combination with MRP could confer cellular resistance to activated oxazaphosphorines. There is considerable evidence that some of these glutathione conjugates, including the glutathione conjugates of acrolein and its metabolites, are toxic to the cell [51, 52]. For the glutathione conjugate of acrolein, toxicity may be a direct consequence of conjugate-mediated oxygen radical formation [51] or may involve the re-release of acrolein from its glutathione conjugate via the reverse reaction [52]. Similarly, it has been suggested that the glutathione conjugate of 4-OH-CP, 4-glutathionylcyclophosphamide, can undergo *trans*-thiolation reactions leading to the re-release of the penultimate toxins, the tautomers 4-OH-CP/aldophosphamide [24]. Therefore, the possibility that these potentially toxic glutathione conjugates of oxazaphosphorine metabolites may be substrates of MRP-mediated export is a particularly important issue.

The present studies show that, despite the high level of GSTP1-1 achieved in transfected cells, GSTP1-1 expression has no effect on sensitivities to the cytotoxicities of the oxazaphosphorines 4-OH-CP, 4-OOH-CP, and maf in MCF7 cells (Fig. 4). The failure of GSTP1-1 to augment resistance indicates that either: 1) the formation of glutathione conjugates is only a minor mechanism of oxazaphosphorine detoxification; or 2) at the level of intracellular metabolites formed, non-enzymatic conjugation with glutathione is more important than GSTP1-1-catalyzed detoxification. However, the finding that MRP alone confers some resistance raises the possibility that toxic metabolites of oxazaphosphorines, which may include glutathione conjugates, are substrates for MRP-associated export.

Cisplatin is an interesting model compound to test for GSTP1-1/MRP-associated resistance. On the one hand, increased MRP is not generally associated with cisplatin resistance [53, 54], and GSTP1-1 has not been shown to catalyze chemical transformations of cisplatin. However, some laboratories have reported an association between increased GSTP1-1 and cisplatin resistance [31, 32]. Additionally, cisplatin can form complexes with glutathione [27] that are potential substrates of MRP or related transporter proteins [15, 29, 30]. Indeed, Ishikawa *et al.* [29] reported findings that suggested that increased MRP expression can be associated with cisplatin resistance. Moreover, they found that glutathione conjugates of cisplatin can inhibit MRP-mediated transport of labeled leukotriene C_4—results indicating that platinum–glutathione complexes may be substrates of MRP. Our data clearly show that relatively high level GSTP1-1 expression had no effect on the cisplatin sensitivity of MCF7 cells regardless of their MRP status (Fig. 5, A and B). In contrast, MRP confers a very modest but statistically significant resistance to cisplatin in MCF7/VP cells (Fig. 5C). These data are consistent with the findings of Ishikawa *et al.* [29] and suggest at least a limited role for MRP in some forms of cisplatin resistance.

In summary, our results are the first validation that, in intact transgenic cell lines, combined expression of GSTP1-1 and MRP can confer high level resistance to ethacrynic acid that is greater than that achieved by expression of either protein alone. However, this effect on cellular resistance by combined GSTP1-1 and MRP expression is not observed against all drugs that can potentially form glutathione conjugates, including drugs that are substrates of GSTP1-1. Finally, our results suggest that previously unrecognized substrates of MRP may include oxazaphosphorine metabolites or their glutathione conjugates.

This work was supported by National Institutes of Health Grants CA 70338 and CA 55214.

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