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Cellular Response to a Glutathione *S*-Transferase P1-1 Activated Prodrug

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ABSTRACT

TER286 [γ-glutamyl-α-amino-β(2-ethyl-*N,N,N′,N′*-tetrakis(2-chloroethyl)phosphorodiamidate)-sulfonyl-propionyl-(*R*)-(−) phenylglycine] is a novel nitrogen mustard prodrug that is preferentially activated by glutathione *S*-transferase P1-1 (GSTP1-1). A human promyelocytic leukemia /TER286-resistant cell line was selected by chronic, long-term exposure to the prodrug. Although resistance was not readily achieved, eventually a 5-fold resistant clone was isolated. Cross-resistance to melphalan occurred, but not to doxorubicin (Adriamycin), taxol, and γ-glutamyl-*S*-(benzyl)cysteinyl-*R*(−)-phenyl glycine diethyl ester, a GSTP1-1 inhibitor. The protein and transcript levels and enzymatic activity of GSTP1-1 were reduced significantly in the selected resistant line. GSTα levels were unchanged, and GSTμ was undetectable. Although glutathione levels were elevated in human promyelocytic leukemia/TER286 cells, no changes in the expression of thiol-related genes including γ-glutamylcysteine synthetase, γ-glutamyl transpeptidase, or multidrug resistance protein were found. A 7-fold increase in catalase expression in the resistant cell line indicated an adaptive response to oxidative and electrophilic stress, and this was also reflected in the lower prevalence of drug-induced DNA single-strand breaks in the resistant cells. Mouse embryo fibroblast GSTP1-1$^{-/-}$ cells exhibited 2-fold resistance to TER286 compared with GSTP1-1$^{+/+}$ cells. NIH3T3 cells transfected with combinations of γ-GCS and multidrug resistance protein exhibited enhanced resistance to TER286, although the degree of resistance was impaired by cotransfection of GSTP1-1. These results are consistent with responses in the TER286-resistant cells indicative of GSTP1-1-mediated mechanism of activation. In consequence, these data support the rationale that tumors expressing high levels of GSTP1-1 will be more sensitive to the cytotoxic effects of the drug.

The latent prodrug TER286 [γ-glutamyl-α-amino-β(2-ethyl-*N,N,N′,N′*-tetrakis(2-chloroethyl)phosphorodiamidate)-sulfonyl-propionyl-(*R*)-(−) phenylglycine] is the lead clinical candidate from a group of rationally designed glutathione (GSH) analogs that exploit high glutathione *S*-transferase P1-1 (GSTP1-1 or GSTπ) levels in solid tumors and drug-resistant cell populations (Kauvar, 1996). Drug design was based on extensive literature showing that overexpression of GST in human tumors is associated with malignancy, poor prognosis, and the development of drug resistance (Tew, 1994; Hayes and Pulford, 1995). Thus, selective targeting of susceptible tumor phenotypes is a strategy that should result in the "release" of more active drug in malignant cells compared with normal tissue, thereby achieving an improved therapeutic index.

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In TER286, the sulfhydryl of a GSH conjugate has been oxidized to a sulfone. The tyrosine-7 in GSTP1-1 promotes a β-elimination reaction that cleaves the compound (Fig. 1). The cleavage products are a GSH analog and a phosphorodiamidate, which in turn spontaneously form aziridinium species, the actual alkylating moieties. The cytotoxic moiety has tetrafunctional alkylating properties, similar in concept to bifunctional nitrogen mustards that react with cellular nucleophiles with a short half-life (Lyttle et al., 1994; Satyam et al., 1996).

TER286 exhibits activity against a variety of tumors and tumor cell lines. In vitro studies have shown that elevated GSTP1-1 in transfectant cell lines correlates with increased sensitivity to TER286 cells (Morgan et al., 1998). Similarly, drug-resistant cell lines that overexpress GSTP1-1 are more sensitive to TER286. In an ex vivo clonogenic assay against human solid tumors, TER286 showed activity against 15 of 21 lung tumors and 11 of 20 breast tumors tested. Additionally, effective antitumor activity was found in vivo using

ABBREVIATIONS: TER286, [γ-glutamyl-α-amino-β(2-ethyl-*N,N,N′,N′*-tetrakis(2-chloroethyl)phosphorodiamidate)-sulfonyl-propionyl-(*R*)-(−) phenylglycine]; GSH, glutathione; GSTP1-1 or GSTπ, glutathione *S*-transferase P1-1; HL60, human promyelocytic leukemia; WT, wild type; MEF, mouse embryo fibroblast; γ-GCS, γ-glutamylcysteine synthetase; γ-GT, γ-glutamyl transpeptidase; MRP, multidrug resistance protein; TER199, γ-glutamyl-*S*-(benzyl)cysteinyl-*R*(−)-phenyl glycine diethyl ester; CDNB, 1-chloro-2,4-dinitrobenzene; BSO, buthionine sulfoximine; RT-PCR, reverse transcriptase-polymerase chain reaction.

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xenografted human tumors in nude mice, with only mild bone marrow toxicity (Morgan et al., 1998).

Acquired drug-resistant cell lines (selected by incremental chronic drug response) are useful models for the study of phenotypic adaptations that confer survival advantage, because these will often reflect the drug's mechanism of action. Although resistance to TER286 proved unusually difficult to achieve, eventually a 5-fold resistant clone was isolated. Unlike the vast majority of anticancer drug-resistant cell lines, human promyelocytic leukemia (HL60)/TER286 exhibited a significant decrease in the expression and activity of GSTP1-1. Other changes reflect the alkylating properties of the drug, and overall the data support the rationale that tumors expressing high levels of GSTP1-1 will be more sensitive to cytotoxic effects of the drug.

Materials and Methods

GST-Activated Latent Drug. The synthesis and biochemical characterization of TER286 are described in Satyam et al. (1996). For cell culture, the drug was dissolved in dimethyl sulfoxide (final concentration <1%).

TER286-Resistant Cell Lines. HL60/TER286 are cell lines cloned from the parental HL60/wild type (WT) cell line. Selection of TER286-resistant cell lines was initiated at 4 μM, with escalation in 1-μM increments to a final concentration of 10 μM. A resistant clone was established after 9 months of drug selection. HL60/TER286 cells are maintained in 10 μM TER286 and express 5-fold resistance to the drug. These cell lines are grown as a suspension in RPMI 1640 medium supplemented with 300 μg/ml L-glutamine, 5 μg/ml penicillin/streptomycin, and 20% fetal bovine serum and split twice a week.

Mouse Embryonic Fibroblast (MEF) Cell Lines. A GSTP1-$1^{-/-}$ mouse model was developed previously (Henderson et al., 1998). The murine GSTP1-1 gene cluster was deleted, completely abolishing the coding sequences of P1 and leaving five exons for the coding region of P2. To identify phenotypes linked with GSTP1-1 inactivation, we have established MEF cell lines from GSTP1-$1^{+/+}$ and GSTP1-$1^{-/-}$ mice. Timed pregnant mice were euthanized by cervical dislocation, and the uterus was aseptically removed for dissection of the embryos. Embryos were harvested at 14 days. Tissue was finely chopped and rinsed, and the pieces were seeded onto the culture surface in a medium containing serum. Cultures were kept at 37°C for 18 to 24 h. Once the tissue pieces began to adhere, the medium was replaced until a substantial outgrowth of cells was observed, at which point the cells were passaged. Primary MEF cell lines were then aliquoted and preserved in liquid nitrogen.

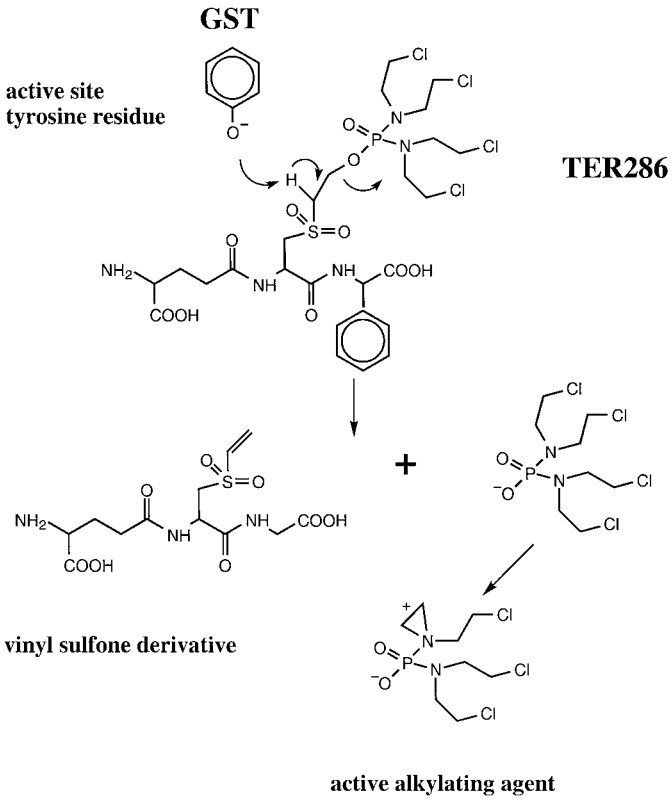

Fig. 1. TER286 is a prodrug that requires GSTP1-1 for activation.

Once a culture was initiated, cells were maintained in minimum Eagle's medium with 10% fetal bovine serum, 1[time] nonessential amino acids, 5 μg/ml penicillin/streptomycin, and 292 μg/ml glutamine. The passage numbers for the lines indicated the number of culture passages.

Transfected Cell Lines. NIH3T3 cells were transfected with pcDNA3.1 plasmid vector (Invitrogen, Carlsbad, CA) containing either human GSTP1-1 and/or human γ-glutamyl cysteine synthetase (γ-GCS) heavy and light chain (regulatory and catalytic) subunits. Human GSTP1-1 was amplified using polymerase chain reaction (PCR) from mRNA prepared from human colon HT29 cells. A Kozak sequence was encoded in the sense primer for amplification (Kozak, 1978). After PCR amplification, the fragment was ligated into a PCR vector using the TA cloning kit (Invitrogen, Carlsbad, CA). Clones were sequenced to ensure the integrity of the insert. GSTP1-1 was then directionally cloned into pcDNA3.1 hygromycin into the *Hin*dIII and *Xba*1 sites. γ-GCS heavy- and light-chain subunits were provided by Dr. Timothy Mulcahy (University of Wisconsin, Madison) and cloned into the *Not*1 site of pcDNA3.1. NIH3T3 and 3T3/MRP cells (3T3/MRP overexpress MRP; Breuninger et al., 1995) were transfected using the lipofectamine lipid transfection method. The various DNA plasmid constructs were added alone (GSTP1-1) or in combination (γ-GCS heavy and light, or GSTP1-1, γ-GCS heavy, and γ-GCS light). 3T3/pc (mock transfectant) were made by transfecting NIH3T3 and 3T3/MRP cells with pcDNA3.1 that did not contain any gene inserts. After a 20-h exposure to DNA/lipofectamine, cells were allowed to grow in drug-free medium for 24 h before colony selection in medium containing 175 μg/ml hygromycin (Sigma Chemical Co., St. Louis, MO). Several colonies were chosen and characterized for each transfected cell line made. Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 5 μg/ml penicillin/streptomycin, and 300 μg/ml glutamine in 5% CO_2.

Western Blot Analysis. Cytosolic fractions were prepared from HL60/WT cells and HL60/TER286. Protein concentration was determined according to the method of Bradford (Bio-Rad Laboratories, Hercules, CA). Western blots were performed as described previously using 8 or 12.5% SDS/polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membranes (Ausbel et al., 1994). Chemiluminescence was used to determine immunoreactivity (ECL; Amersham Pharmacia Biotech, Arlington Heights, IL). Equivalence of loading was confirmed by immunoreactivity to actin using a monoclonal antibody (Amersham Pharmacia Biotech, Piscataway, NJ).

Whole cell extracts were used to determine the expression of GSTP1-1, γ-GCS heavy and light, MRP, and catalase in the appropriate cell lines. Rabbit polyclonal antibodies for γ-GCS light and heavy subunits were made in our laboratory from peptide GLLSQGSPLSWEETK for GCS heavy and from peptide LLTHNDPKELLSEAS for GCS light. These antibodies were diluted 1:1000 in 5% milk. MRP antisera were provided by Dr. Gary Kruh (Fox Chase Cancer Center, Philadelphia, PA) and diluted 1:10 in 5% milk. The polyclonal GSTP1-1 antibody was obtained from Biotrin Technologies (Dublin, Ireland) and diluted 1:3000 in 5% milk. The polyclonal catalase antibody (Rockland, Gilbertsville, PA) was diluted 1:5000 in 5% milk. Blots were probed with a monoclonal antibody against α-actin (Amersham Pharmacia Biotech) to verify equivalent loading.

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Blotting grade affinity purified goat anti-mouse IgG-horseradish peroxidase conjugate (Bio-Rad Laboratories) was diluted 1:5,000 or 1:10,000, and anti-rabbit IgG-horseradish peroxidase conjugate (Amersham Pharmacia Biotech) was diluted 1:3000 or 1:5000.

GSH Assay. GSH levels were measured using a colorimetric assay (Bioxytech GSH400, Portland, OR) according to the manufacturer's instructions.

GST Enzyme Activity Assay. Activity of HL60/WT and HL60/TER286 cytosolic fractions toward the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with GSH was measured spectrophotometrically according to the method of Habig et al. (1974).

Reverse Transcriptase (RT)-PCR. The Qiagen RNeasy Total RNA kit (Qiagen Inc., Chatsworth, CA) was used to isolate total RNA from logarithmically growing cells. First-strand cDNA synthesis was performed in a 20-μl reaction volume containing: 1 μg of total RNA, 2.5 mM random hexamers, 0.01 M dithiothreitol, 0.2 M dATP, dCTP, dGTP, and dTTP, 50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, and 200 U of Superscript II RT (Life Technologies, Germantown, MD). The PCR was performed in a 100-μl reaction volume containing: 20 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM sense and antisense primers, 5 U of *Taq* DNA polymerase, and 50 ng of cDNA. The PCR program consisted of an initial 30-s denaturation period at 95°C. Each cycle (total of 23–30 cycles) consisted of 30 s at 95°C to denature the genomic DNA, 30 s at the appropriate annealing temperature, and 30 s of extension time at 72°C. The following annealing temperatures were used: GSTP1-1 mouse = 50°C, GSTP1-1 human = 62°C, GSTα = 55°C, γ-GCS heavy and γ-GCS light = 59°C, γ-glutamyl transpeptidase = 58°C, MRP = 58°C, and catalase = 60°C. Products were analyzed on a 2% MetaPhor (FMC BioProducts, Rockland, ME) agarose gel and stained with ethidium bromide.

Drug Sensitivity Assays. Drug sensitivity was determined using the cell titer 96 aqueous nonradioactive cell proliferation assay (Promega Corp., Madison, WI) [MTS(3-[4,5-dimethylthiazol-2-yl]-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2-H-tetrazolium] according to the manufacturer's instructions. A sample (100 μl) of cell suspension (1×10^4 cells) was dispensed into 96-well plates containing various concentrations of doxorubicin (Adriamycin), cisplatinum, melphalan, taxol, TER286, and the GSTP1-1 inhibitor TER199 [γ-glutamyl-*S*-(benzyl)cysteinyl-*R*(−)-phenyl glycine diethyl ester] in standard solvents. After 72 h of incubation at 37°C in a humidified 5% CO_2 atmosphere, 20 μl of freshly prepared MTS reagent was added to each well. Using a 7520 microplate reader (Cambridge Technologies Inc., Watertown, MA), production of formazan was assayed by reading absorbance at 490 nm.

DNA Damage Assay. The alkaline comet assay was performed as described by Olive et al. (1993). Briefly, HL60/WT and HL60/TER286 cells grown to 75% confluency were treated with 10 μM TER286. At 0, 1, and 24 h after treatment 2×10^4 cells were recovered by low-speed centrifugation, rinsed with PBS, and resuspended in 225 μl of 0.75% low melting temperature agarose. Cells were spread on microscope slides precoated with 1% agarose, covered with a 24 × 60-mm coverslip, and left on ice for 5 min or until they became semisolid. The coverslip was removed and the slide submerged in lysis buffer (30 mM NaOH, 1 M NaCl, 0.1% Sarkosyl) for 1 h. Slides were washed twice for 20 min with a solution of 30 mM NaOH and 2 mM EDTA, followed by electrophoresis at 23 V for 10 min and staining with 2.5 μg of propidum iodide. For each time point, >50 cells were visualized using a charge coupled device camera. DNA damage was quantified as an increase in tail moment as determined by NIH Image software.

Results

Selection of resistant cell lines was initiated at 4 μM TER286, with escalation in 1-μM increments to a final concentration of 10 μM. Although this standard protocol is usually efficient in developing resistant clones, for TER286, cells that initially survived and passaged did not successfully establish long-term resistant cultures. After 9 months, one resistant clone was established. The sensitivity of this line to various drugs at specific selection stages is shown in Table 1. As shown in Fig. 2, at 10 μM, the IC_{50} value for HL60/TER286 was 42.8 ± 3.3 μM compared with 9.4 ± 4.3 μM for the wild type. These cells grew normally when maintained in 10-μM drug; that is, the rates of proliferation and morphology were not affected by TER286.

To assess cross-resistance, cell survival was determined 72 h after exposure to the cytotoxic agents, and the resultant IC_{50} values are shown in Table 1. Cross-resistance to the bifunctional nitrogen mustard melphalan (HL60/TER286 IC_{50} = 2.3 μM; HL60/WT IC_{50} = 9.4 μM) was found. However, no cross-resistance was demonstrated to Doxorubicin, taxol, cisplatin, or TER199, a GSTP1-1 inhibitor. (Table 1).

Buthionine sulfoximine (BSO) is a potent specific inhibitor of γ-GCS, the rate-limiting enzyme in de novo biosynthesis of GSH. Furthermore, depletion of intracellular GSH by BSO significantly enhances the cytotoxicity of many alkylating agents including melphalan (Hayes and Pulford, 1995). To investigate the effect of GSH depletion in HL60/WT and HL60/TER286 cells on resistance to melphalan and TER286, cells were treated with 50 μM BSO before drug exposure. BSO-induced GSH depletion increased melphalan cytotoxicity (IC_{50}) in both HL60/WT and HL60/TER286 by 53 ± 6% and 55 ± 20%, respectively. Similarly, TER286 cytotoxicity

TABLE 1

Cross-reactivity profile for HL60/WT and HL60/TER286

Cell survival to other classes of chemotherapeutics was assessed 72 h after exposure to the agents. Response was assessed at intermediate steps in the selection process (4–10 μM). IC_{50} values are shown (μM) except where noted.

Drug	WT	TER286	Resistance Ratios
10 μM			
TER286	9.4 ± 4.3	42.8	5
TER199	22.8 ± 4.3	27.0 ± 2.2	1
Melphalan	2.5 ± 0.2	6.4 ± 0.8	3
7.0 μM			
TER286	7.5 ± 2.8	36.4 ± 5.3	5
TER199	13.7 ± 1.2	14.7 ± 2.8	1
Melphalan	1.3 ± 0.6	6.1 ± 2.5	5
Taxol[a]	1.3 ± 0.3	1.6 ± 0.2	1
Doxorubicin[a]	7.5 ± 31.0	61.3 ± 21.7	1
6.5μM			
TER286		27.4 ± 8.6	4
TER199		13.0 ± 1.0	1
Melphalan		6.2 ± 2.4	5
Taxol[a]		1.5 ± 0.3	1
Doxorubicin[a]		56.0 ± 14.0	1
5.0 μM			
TER286	7.0	14.5	2
TER199	22.5	27.5	1
Melphalan	1.3	3.1	2
Taxol[a]	3.0	2.5	1
Doxorubicin[a]	88.3	60.3	1
Cisplatin	5.0	6.5	1
4.0 μM			
TER286		10.3	1
TER199		27.5	1
Melphalan		2.6	2
Taxol[a]		2.2	1
Doxorubicin[a]		48.0	1
Cisplatin		4.5	1

[a] Indicates nanomolar.

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was enhanced by 40 ± 18% and 59 ± 36% in HL60/WT- and HL60/TER286-resistant cells, respectively (Fig. 3).

GSTP1-1 is the major isoform in HL60/WT and HL60/TER286 cell lines. The resistant cells showed a 43% decrease in GSTP1-1 protein and 73% reduction in the level of transcript expression compared with WT cells (Figs. 4 and 5). These results are consistent with the general enhancement of the cytotoxicity of nitrogen mustards by depletion of GSH. Thus, similarities between TER286 and melphalan reflect the reduced capacity of cells to detoxify the electrophilic aziridinium species through GSH conjugation. Enzyme activity assays of the conjugation of CDNB with GSH showed that total cytosolic GST activity was significantly lower in HL60/TER286 cells (26.95 ± 1.49 μmol min^{-1} mg^{-1}) relative to HL60/WT (47.01 ± 1.60 μmol min^{-1} mg^{-1}; $P < .00001$). The quantitative relationship between drug cytotoxicity and decreased GSTπ levels was approximately equivalent. Although the IC_{50} values suggest a 4- to 5-fold difference between HL60/WT and HL60/TER286, the extent of resistance varied over the concentration range shown in Fig. 2. The apparent 2-fold change in GSTπ levels could account for much of the resistance, although other concomitant adaptive changes may also participate. Expression of GSTα mRNA (Fig. 4) was not significantly different between HL60/WT and HL60/TER286 cells, whereas GSTμ was not detectable in either cell line.

GSH levels in HL60/TER286 cells were increased by 47% compared with HL60/WT cells (8.0 ± 0.75 nmol versus 5.4 ± 0.4 nmol/1 × 10^6 cells; $P < .001$). Because intracellular GSH can be synthesized de novo by γ-GCS or salvaged by γ-GT, the expression of both enzymes was examined. The protein and transcript levels of γ-GCS were not significantly changed. (Figs. 4 and 5). Similarly, transcript levels of γ-GT were not different in HL60/WT versus HL60/TER286 cells (Fig. 5). These results suggest that increased expression of de novo and/or salvage enzymes for GSH synthesis does not provide a straightforward causal link to increased GSH levels. Moreover, although increased MRP levels can influence GSH by increasing efflux, MRP levels, determined at the transcript and protein levels, were unaltered in HL60/TER286 (Figs. 4 and 5).

Because TER286 releases electrophilic metabolites, the possibility for indirect production of reactive oxygen species exists. Therefore, the expression of a number of reactive oxygen species protective gene products was measured (Fig. 4). Although there was no change in selenium-dependent GSH peroxidase and superoxide dismutase (data not shown), catalase protein and transcript were increased 7- and 2-fold, respectively, in HL60/TER286 cells (Figs. 4 and 5).

A modified DNA "comet" assay was used to quantitate DNA breaks in individual cells following TER286. The con-

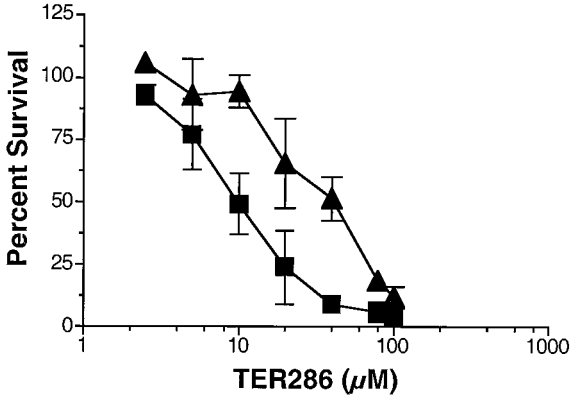

Fig. 2. HL60/TER286 (▲) cells show 5-fold resistance to the prodrug compared with the HL60/WT (■) cell line. IC_{50} values, HL60/TER286 = 42.8 ± 3.3 μM; HL60/WT = 9.4 ± 4.3 μM. Cell survival was assessed 72 h after exposure to TER286 using the cell titer 96 aqueous nonradioactive cell proliferation assay (Promega Corp.).

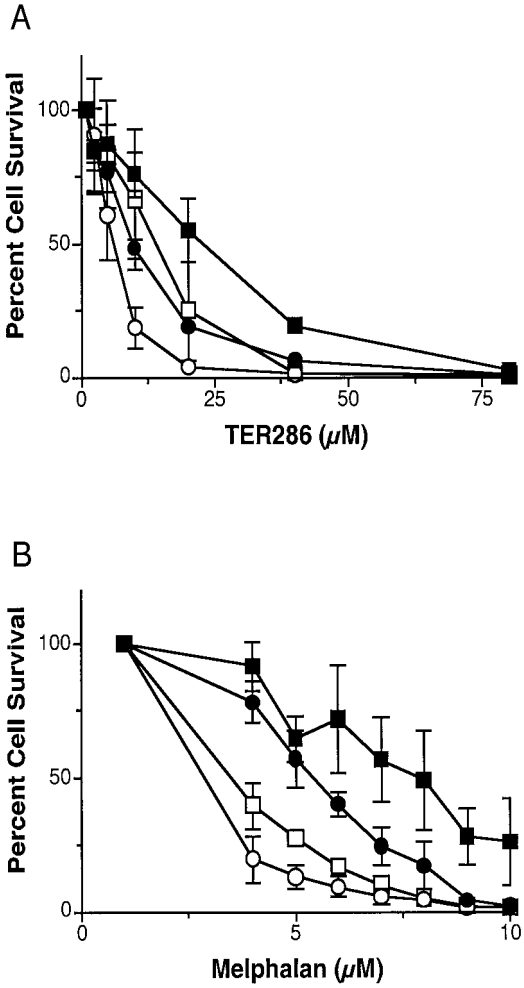

Fig. 3. Depletion of intracellular GSH by BSO enhances the cytotoxicity of melphalan and TER286 in HL60/WT and HL60/TER286-resistant cells. A and B show the response to TER286 and melphalan, respectively, in HL60/WT (●) and HL60/TER286-resistant (■) cells. Twenty-four hours before exposure to either drug, HL60/WT cells (○) and HL60/TER286-resistant cells (□) cells were treated with 50 μM BSO. Cell survival was assessed 48 h after drug exposure.

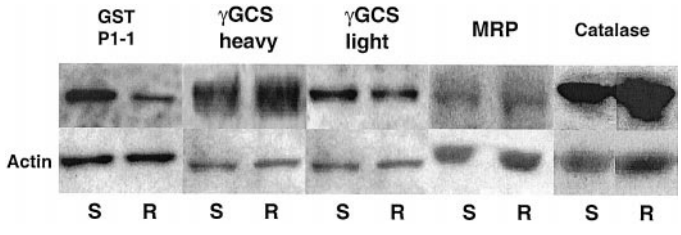

Fig. 4. Changes in protein expression associated with resistance to TER286. Protein expression was determined by Western blot analysis, and densitometry was performed with NIH image analysis using actin as control. S, HL60/WT; R, HL60/TER286.

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centration of TER286 used was 10 μM, the same concentration at which the HL60/TER286 cells are maintained. Basal DNA damage was not significantly different in HL60/WT versus HL60/TER286 cells (Fig. 6A and B). One hour after exposure to TER286, tail moments were smaller in HL60/TER286 than in HL60/WT cells (Fig. 6, C and D). There was also a trend toward smaller tail moments in HL60/TER286 cells 24 h after exposure to TER286 (Fig. 6, E and F). Figure 6G shows the change in tail moment (DNA damage) as a function of time after TER286 exposure. These results show a decrease in DNA lesions in HL60/TER286 cells 1 h after TER286 exposure. The rate of DNA repair after 24 h does not appear to differ between HL60/WT and HL60/TER286-resistant cells.

We prepared MEF cell lines from 14-day-old embryos and measured their sensitivity to TER286 and other anticancer drugs (Table 2). The absence of GSTP1-1 expression in the GSTP1-1$^{-/-}$ cells was confirmed by Western blot and RT-PCR (Fig. 7). These cells exhibited a 2-fold resistance to TER286 compared with GSTP1-1$^{+/+}$ cells (Table 2). No cross-

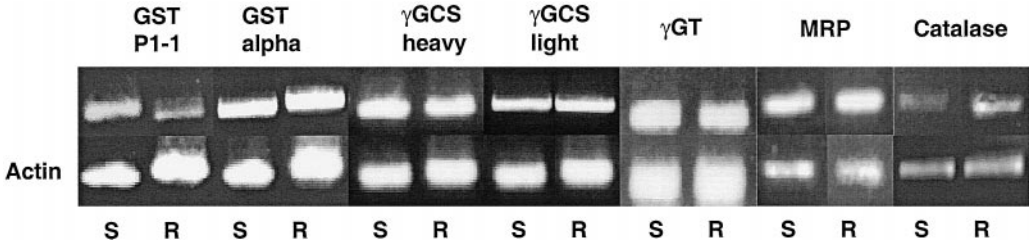

Fig. 5. Changes in transcript expression associated with resistance to TER286. mRNA expression was measured by RT-PCR, and densitometry was performed using NIH image analysis using actin as control. S, HL60/WT; R, HL60/TER286.

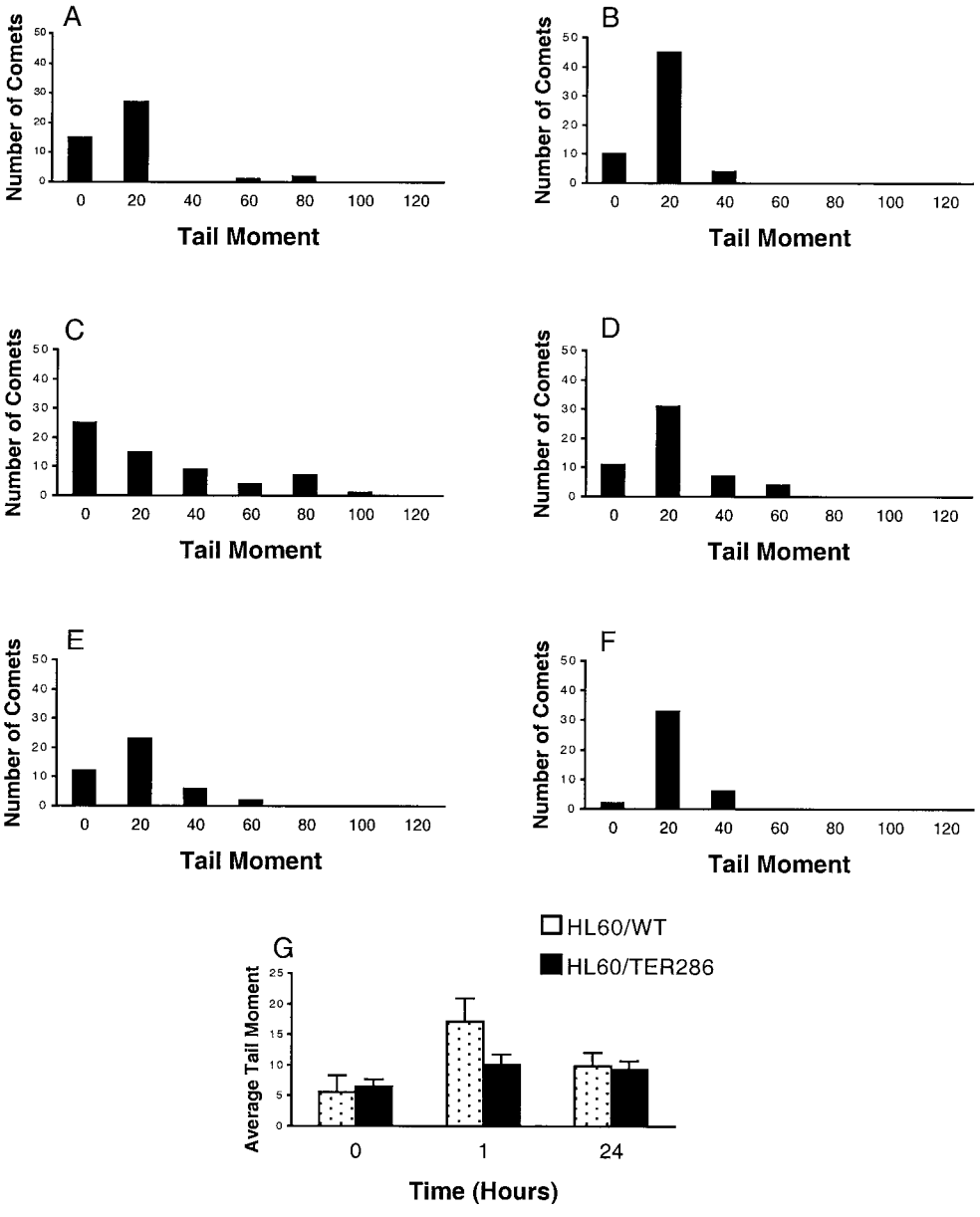

Fig. 6. A, TER286 induced single-strand DNA breaks in HL60/WT and HL60/TER286 cells. The alkaline comet assay technique was used to measure the change in tail moment of at least 50 cells treated with 10 μM TER286. A and B show histograms for HL60/WT and HL60/TER286, respectively, without the drug. C and D show tail moments 1 h after treatment with 10 μM TER286. E and F show tail moments 24 h after TER286. G represents the average ± S.E.M. for tail moment as a function of time in HL60/WT versus HL60/TER286 cells.

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resistance was observed to taxol, mitoxantrone, cisplatin, melphalan, Doxorubicin, or TER199.

Because TER286 was designed to release aziridinium species, we developed multiple transfectant cell lines to assess the importance of coordinate regulation of GSH pathways in effecting drug response. NIH3T3 cells were transfected with γ-GCS heavy and light subunits and MRP1 alone or in various combinations. The influence of GSTP1-1 was then assessed by cotransfection. Although precise quantitation of the effective overexpression of each protein was not attempted, immunoblot assays showed MRP increased 4-fold, γ-GCS 2-fold, and GSTP1-1 5-fold over mock transfectants (O'Brien et al., 2000). Our earlier data have demonstrated a 2-fold increase in sensitivity in NIH3T3 cells transfected with GSTP1-1 alone (Morgan et al., 1998). Table 3 shows the drug response of the various cotransfectants to TER286. Transfection of either γ-GCS or MRP alone enhanced resistance to TER286 by ~2-fold. The two together resulted in a 6-fold elevation in the IC_{50} values. When GSTP1-1 was cotransfected with γ-GCS and MRP, cytotoxicity was decreased with an enhanced resistance of only 4-fold.

Discussion

Of the GST gene family, the GSTP1-1 isozyme is the most frequently expressed at high levels in a number of human cancers. The prevalence of this protein, particularly in lung, breast, colon, and ovarian tumors (Schisselbauer et al., 1992; O'Brien and Tew, 1996), has provided a strategy for drug design that could lead to an enhanced therapeutic index. Although previous in vitro studies with purified GSTs have shown the extent of specificity for activation of TER286 by GSTP1-1 (Morgan et al., 1998), our present data provide a more detailed analysis of the importance of this isozyme in contributing to the cytotoxicity of TER286.

We have used three distinct model cell systems to study the pharmacology of TER286. First, we developed an acquired drug-resistant cell line. The establishment of TER286-resistant clones was not readily achieved. Although cells frequently survived the initial selecting drug treatment and partially repopulated the culture, recovery of a fully viable population was routinely difficult to obtain. In light of the relative ease with which HL60 cells usually can be made resistant to anticancer drugs, this result was somewhat surprising. This may indicate that resistance to TER286 is governed by multiple factors or that survival response pathways are not readily invoked after chronic drug exposure. One of the characteristics of GSTP1-1 as an enzyme has been the general low catalytic efficiency with broad substrate "specificity". Our most recent data (Adler et al., 1999) implicate GSTP1-1 directly in the regulation of c-Jun NH_2-terminal kinase (JNK)-mediated stress response. This is a ligand binding, noncatalytic function for the protein and may provide a partial explanation for the high GSTP1-1 levels in many tumors, in which kinase cascade pathways involving JNK may be imbalanced. Although for TER286, the β-elimination reaction catalyzed by GSTP1-1 does not inactivate the protein, it may serve to compartmentalize it away from the JNK ligand binding function. This may influence the stoichiometry that controls kinase-mediated proliferative/apoptotic pathways and may be a contributory factor in the difficulty experienced in establishing a TER286-resistant cell line.

The emergence of resistance to TER286 resulted in decreased expression and enzymatic activity of GSTP1-1, supporting a mechanism of action based on the rational design of the drug. Additionally, the results showing increased resistance in the GSTP1-$1^{-/-}$ MEF cell lines and increased sensitivity in the NIH3T3 overexpressing GSTP1-1 confirm that levels of GSTP1-1 have a direct impact on cytotoxicity. These data suggest that the toxic effect of the drug occurs via the alkylating moieties, the aziridinium species after release from the parent compound.

HL60/TER286 cells showed cross-resistance to the alkylating agent melphalan. The resistant phenotype was accompanied by increased GSH levels, a mechanism commonly associated with resistance to alkylating agents (Tew, 1994; Hayes and Pulford, 1995). These enhanced GSH levels did not seem to be a consequence of induced overexpression of the primary enzymes responsible for de novo (γ-GCS) or salvage (γ-GT) synthesis of the tripeptide. Similarly, MRP expression was unaltered in resistant cells. We have shown a coordinated increased expression of γ-GCS, GSTP1-1, and MRP in cells selected for resistance to ethacrynic acid (Ciaccio et al., 1996; Tew et al., 1998), a drug with Michael addition properties. There is evidence that Michael acceptors may induce expression of γ-GCS via antioxidant response elements (Mulcahy et al., 1997; Wild et al., 1998). On the other hand, TER286 produces aziridinium moieties characteristic of other nitro-

TABLE 2

Cross-reactivity profile for GSTP1-$1^{+/+}$ and GSTP1-$1^{-/-}$ MEF cells

Cell survival to other classes of chemotherapeutics was assessed 72 h after exposure to the agents. IC_{50} values are shown (μM).

Drug	GSTP1-$1^{+/+}$	GSTP1-$1^{-/-}$	Resistance Ratio
TER286	50.0 ± 10.0	82.5 ± 2.5	1.7
TER199	54.0 ± 12.9	54.6 ± 12.1	1.0
Taxol	0.1 ± 0.0	0.1 ± 0.0	1.0
Mitoxantrone	0.7 ± 0.3	0.7 ± 0.2	1.0
Cisplatin	26.3 ± 14.0	25.3 ± 16.0	1.0
Melphalan	49.0 ± 9.0	52.6 ± 11.0	1.1
Doxorubicin	0.4 ± 0.1	0.4 ± 0.1	1.0

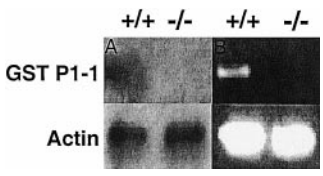

Fig. 7. GSTP1-1 expression in GSTP1-$1^{+/+}$ and GSTP1-$1^{-/-}$ MEF cells. GSTP1-1 protein (A) and transcript (B) were measured by immunoblot and RT-PCR, respectively, as described previously. MEF cell lines were developed from a previously established GSTP1-$1^{-/-}$mouse model (Henderson et al., 1998).

TABLE 3

Effect of TER286 on NIH3T3 cells transfected with GSTP1-1(π), γ-GCS, MRP, or appropriate combinations compared with sham-transfected parental cells (3T3/pc)

Cell Lines	IC_{50}	Resistance Ratio
	μM	
3T3/pc	10.7 ± 0.7	
3T3/π	6.3 ± 2.4	0.6
3T3/GCS	20.0 ± 0.6	1.9
3T3/MRP	23.7 ± 2.0	2.2
3T3/GCS/MRP	63.0 ± 0.0	5.9
3T3/π/GCS/MRP	40.0 ± 6.0	3.7

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gen mustards. This difference may account for the absence of evidence for inducible expression of the cadre of GSH-related detoxification gene products in HL60/TER286 cells. The overall importance of GSH in contributing to resistance was demonstrated by the fact that BSO, in depleting GSH through inhibition of γ-GCS, reversed resistance to melphalan. In contrast, BSO-induced GSH depletion partially sensitized HL60/TER286 cells to the prodrug. These results suggest that acquired resistance to TER286 is only partially mediated by increased GSH, but may ultimately be a consequence of multiple adaptations.

It is not currently known how the production of vinyl sulfone impacts on the efficacy of TER286. Its electrophilic characteristics would predict reactivity with cellular nucleophiles, and because GS-conjugates are primary substrates for MRP (Keppler et al., 1997; O'Brien et al., 1999), a GS-vinyl sulfone could prove to be an effective substrate for MRP. In addition, enhanced expression of catalase in HL60/TER286 suggests that chronic drug exposure either directly or indirectly produces reactive oxygen species intracellularly. The vinyl sulfone could be a contributory factor in chain reactions leading to lipid peroxidation and/or the generation of hydrogen peroxide (Comporti, 1989) and, similarly, these could lead to the production of GSH conjugates. Thus, although the vinyl sulfone component of the drug is unlikely to be important to the therapeutic alkylating activity, it may prove to have some pharmacological significance.

Reactive oxygen species could also contribute to the DNA damage, but direct alkylation by the aziridinium intermediates is a more probable contributing factor. The alkaline comet assay data suggest a slightly higher level of damage in the WT cells after 1 h of drug exposure; however, the repair rates appear to be similar for both cell lines. These results suggest that there is no significant adaptive response in DNA repair mechanisms. This finding is consistent with the absence of any adaptive change in expression of DNA-dependent protein kinase (data not shown), an enzyme associated with DNA strand breakage that is overexpressed in HL60 cells resistant to Doxorubicin (Shen et al., 1999). These results also suggest that the alkylating activity of TER286 is more likely causally linked to DNA strand breakage.

To further investigate the mechanism of action of TER286, a second cell model system using MEF cell lines established from GSTP1-1$^{-/-}$ mice has been developed (Henderson et al., 1998). An approximate 2-fold level of resistance to TER286 was found in the GSTP1-1$^{-/-}$ cells. There was no apparent cross-resistance or sensitivity to any of the common anticancer drugs including other nitrogen mustards. Such data are consistent with the reduced capacity of the null phenotype to activate the drug. Because the GSTP1$^{-/-}$ MEF cells have other GSTs, drug activation does occur at a much less efficient rate. Thus, unlike cyclophosphamide, in which cytochrome P4502B6 is required to activate the prodrug (Chang et al., 1993), in vitro cytotoxicity of TER286 can be achieved even in the absence of the primary activating enzyme.

Our earlier report (Morgan et al., 1998) showed that transfection of GSTP1-1 enhanced the sensitivity of recipient cells to TER286. This is essentially consistent with the data for the null MEF cells. However, the alkylating aziridinium species and the vinyl sulfone (Fig. 1) both are likely to form thioether conjugates with GSH, and these are subject to possible transport by MRP. Thus, we developed a third model to investigate the influence of cotransfection of GSTπ with γ-GCS regulatory and catalytic subunits (rate-limiting enzyme in de novo GSH biosynthesis) and MRP. The 3T3/GCS cells exhibited higher intracellular GSH levels (2-fold) and the 3T3/MRP greater MRP levels (5-fold) (O'Brien et al., 2000) compared with parental lines. These values were consistent for the multiple transfectant lines. Similarly, GSTP1-1 protein levels were higher (5-fold) in 3T3/π and 3T3/π/GCS/MRP lines, corresponding to a 2-fold higher enzyme activity as measured with the CDNB assay, a general substrate for all GST isozymes. Transfection of either γ-GCS or MRP increased resistance to TER286 in NIH3T3 cells by approximately 2-fold. When combined, a greater than additive 6-fold value was obtained. Thus, these data imply that the alkylating species and the vinyl sulfone may be subject to GSH conjugation and that these conjugates may be effluxed by the membrane pump MRP. Coordinate forced overexpression enhanced the efficiency of this protective function. Transfection of GSTP1-1 only (3T3/π; Table 3) confirmed our earlier data showing an enhancement in cytotoxicity (Morgan et al., 1998). When GSTP1-1 was cotransfected with γ-GCS and MRP, a decrease of the resistance ratio from 6.3 to 4.0 was observed. This result may reflect the shift in the balance of drug activation versus detoxification, in which the additional GSTP1-1 modifies the cellular kinetics of the drug. It is also of interest that previous studies with electrophilic drugs have suggested that the rate of conjugation to GSH may be enhanced by GST catalysis (Bolton et al., 1991; Ciaccio et al., 1991). Obviously, this could provide a paradox for a drug such as TER286, in which activation and deactivation reactions could be catalyzed by the same enzyme. However, for drugs such as chlorambucil, human GSTα isozymes have a significantly more biologically favorable K_{cat} value than GSTπ (Ciaccio et al., 1990), implying that GSTπ isozyme may not provide the most efficient detoxification route available to the cell.

In summary, we have shown that cellular responses to chronic TER286 exposure are indicative of GSTP1-1 mediated mechanism of activation. Additional changes reflect the alkylating properties of the drug. Together, these results support the design rationale that tumors expressing high levels of GSTP1-1 will show enhanced sensitivity to the drug.

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