Cancer Chemother Pharmacol (1996) 37: 363–370 © Springer-Verlag 1996

ORIGINAL ARTICLE

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Isozyme-specific glutathione S-transferase inhibitors potentiate drug sensitivity in cultured human tumor cell lines

Received: 18 October 1994, Accepted: 14 May 1995

Abstract Novel glutathione (GSH) analogs, previously shown to inhibit glutathione S-transferase (GST) activity at about 1 μ*M* in vitro, were tested for their ability to potentiate the killing of cultured tumor cells by chemotherapeutic drugs. When tested at doses up to 200 μ*M*, the analogs were neither toxic nor capable of potentiating drug toxicity unless the diethyl ester (DEE) form was used for treatment of the cells. HPLC analysis revealed rapid internalization of the DEE and intracellular conversion to a monoethyl ester form that accumulated in the cell, followed by a more gradual loss of the second ester to generate the active parent form. For the four GSH analogs tested, the ability of the DEE forms to potentiate chlorambucil (CMB) toxicity in HT-29 human colon adenocarcinoma cells strongly correlated with the in vitro ability of the parent form to inhibit recombinant human P1-1. This isozyme is the dominant form of GST present in HT-29 cells. Of the four analog DEEs tested, γ-glutamyl-S-(benzyl)cysteinyl-R(−)-phenyl glycine (TER 117) DEE was the most effective in potentiating CMB toxicity in several cell lines: HT-29, HT4-1 (HT-29 subclone), SKOV-3 ovarian carcinoma, and SK VLB (vinblastine-resistant variant of SKOV-3) cells. γ-Glutamyl-S-(octyl)cysteinyl-glycine (TER 143) DEE potentiated mitomycin C (MTC) toxicity in HT4-1 and SK VLB cells while TER 117 DEE did not. TER 117 DEE enhanced melphalan effects on xenografts of HT4-1 in mice to a similar extent as that achieved with the previously described nonspecific GST inhibitor, ethacrynic acid. Taken together, our results indicate that cell-permeable analogs of GSH can potentiate cytotoxicity of common chemotherapeutic drugs and this effect has a strong positive correlation with the ability of the analogs to inhibit specific GST isozymes.

Key words GST inhibitor · Potentiate · Drug sensitivity

Abbreviations *ADR* adriamycin *CMB* chlorambucil *CDNB* 1-chloro-2,4-dinitrobenzene *DEE* diethyl ester *DMF* dose modification factor *EA* ethacrynic acid *EtOH* ethanol *GGT* γ-glutamyl transpeptidase *GR* glutathione reductase *GSH* glutathione *GSSG* oxidized glutathione *GST* glutathione S-transferase IC_{50} concentration required to produce 50% inhibition *MTC* mitomycin C *PBS* phosphate buffered saline *L-PAM* melphalan *FBS* fetal borne serum *DMSO* dimethyl sulfoxide.

Introduction

Glutathione-S-transferases (GSTs; E.C. no. 2.5.1.18) detoxify a variety of xenobiotic compounds by enzymatic conjugation of the compound with glutathione (GSH). GSH conjugates have been demonstrated for several important chemotherapeutic drugs, especially alkylating agents such as chlorambacil (CMB) [8, 9], melphalan (L-PAM) [11], and hepsulfam [3]. Human GST P1-1 is capable of catalyzing GSH conjugation to either CMB [9] or hepsulfam [3], although human alpha class GST isozymes are more effective at the reaction for CMB [9]. Human liver microsomal GST also generates GSH conjugates of L-PAM [11]. Overexpression of one or more of the GST isozymes in cells exposed to the toxic agent should therefore confer resistance to these drugs. Correlation of drug resistance with the overexpression of GSTs has been demonstrated in a variety of cells, including several human

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tumor cell lines [5, 16, 30, 34, 45, 47]. Similarly, GST overexpression, including that of GST P1-1, has also been observed in many clinical tumor specimens as compared to their normal tissue counterparts [19, 29, 37]. Recent evidence shows that elevated levels of total GST [38] and overexpression of GST P1-1 [22, 42] as well as a novel GST isozyme [24] occurs in association with the development of drug resistance in tumors of patients undergoing cancer chemotherapy. Further, elevated levels of GST P1-1 have been correlated with poor prognosis in node-negative breast cancer patients [15].

Several investigators have reported increased drug sensitivity of tumor cells in culture by treatment with inhibitors of GST such as ethacrynic acid (EA), [18, 39, 41], piriprost [41], indomethacin [17], and gossypol [14]. EA, which has been shown to potentiate drug effects two to three fold in cell culture [41], also results in significant retardation of tumor growth in mice [10]. In a phase I clinical trial with EA, systemic GST activity was reduced by about 60% in some patients without severe collateral toxicities [31]. Anecdotal clinical evidence for striking reversal of drug resistance with EA in a patient with leukemia has recently been reported [35]. None of the GST inhibitors described to date shows strong isozyme specificity for GST inhibition. EA, the best-characterized compound, decreases intracellular GSH levels [41], affecting normal cells as well as tumor cells [10, 31]. Further, all of these established potentiators have independent pharmacological activities [4, 21, 49] that make them less suitable for research and clinical use than a pure GST inhibitor. Because different GST isozymes have different substrate specificities and overexpression of GSTs in tumors appears to be limited to a subset of isozymes [19], enzyme inhibitors that are selective for the overexpressed GST may provide a means of modulating drug resistance in the tumor cells while sparing normal cells. We have previously demonstrated that GSH analogs, specifically modified by functionalization of the cysteinyl sulfur and/or amino acid replacement of glycine, will selectively inhibit GST P1-1, A1-1, M1a-1a, and M2-2 [13]. Below we describe the use of several of these analogs as potentiators of drug sensitivity in human tumor cell lines in culture.

Materials and methods

Cell lines

HT-29 (human colon adenocarcinoma) cells were kindly provided by Dr. Roberto Ceriani (Cancer Research Fund of Contra Costa County, Walnut Creek, Calif.). EA-sensitive (HT4-1) and-resistant ($HT\text{-}EA^R$) subclones of HT-29 were derived by Kuzmich et al. and their characterization and culture conditions have been described in detail elsewhere [23]. The SKOV-3 (human ovarian carcinoma) cell line and its vinblastine-resistant variant, SK VLB were kindly provided by Victor Ling (Ontario Cancer Institute, Toronto, Ontario, Canada) and have been further described by Bradley, et al. [6]. All cell lines were used in the log-phase of growth unless otherwise specified.

Chemicals

Adriamycin (ADR), CMB, L-PAM, mitomycin C (MTC), and EA were all obtained from Sigma Chemical Co. (St. Louis , Mo). CMB and EA were dissolved in 100% ethanol (EtOH), ADR and MTC were dissolved in water, and L-PAM was dissolved in 0.1 *N* HCl in 100% EtOH. For injections, L-PAM and EA were prepared as previously described [10]. The synthesis [26, 27] and GST inhibitory activity [13] of the GSH analogs have been described in detail elsewhere. Nomenclature for the GSH analogs, their GST inhibitory activity, and toxicity in HT-29 cells are detailed in Table 1. All analogs were dissolved in ethanol, dimethyl sulfoxide (DMSO), or water just prior to use in cell culture. The same amount of solvent added to culture medium served as the vehicle control. TER 117 DEE was dissolved in water for use in the tumor xenograft model.

GSH-dependent enzymes

Recombinant GST M1a-1a was obtained from Dr. Bengt Mannervik (Uppsala University, Uppsala, Sweden); recombinant M2-2 was obtained from Dr. Gordon Rule (University of Virginia); recombinant A1-1 and P1-1 were purified from *E. coli*, using the procedure of Kolm et al.[1]. Bovine kidney γ-glutamyl transpeptidase (GGT) and its substrates (glutamyl *p*-nitroaniline and glycylglycine) were obtained from Sigma. Baker's yeast glutathione reductase (GR) was also obtained from Sigma. All other chemicals were reagent grade from various suppliers.

Table 1. Definition, K_i values, and toxicity[a] of GSH analogs used (*ND* not determined)

GSH analog (unesterified)	Abbreviation	GST: (K_i in μ*M*)[b] P1-1	A1-1	M1a-1a	M2-2	Toxicity (IC_{50} in μ*M*) Unesterified	DEE[c]
γ-Glutamyl-S(benzyl)cysteinyl-R(−)-phenyl glycine	TER 117	0.4	20	25	31	> 200	22
γ-Glutamyl-S(hexyl)cysteinyl-R(−)-phenyl glycine	TER 135	0.85	5.8	41	97	> 200	24
γ-Glutamyl-S(naphthyl)cysteinyl-glycine	TER 211	1.2	4.2	0.01	1.5	> 200	47
γ-Glutamyl-S(octyl)cysteinyl-glycine	TER 143	1.9	0.27	1.2	ND	> 200	40
Ethacrynic acid		4.0	2.0	3.0	ND		

[a]As determined in HT-29 cells after a 4-h exposure of $2x10^5$ cells/ml in serum-free medium and assayed using the modified clonogenic method.
[b]K_i values of inhibition of GSH conjugation to CDNB

GSH-dependent enzyme activity

All GSH analogs were tested in the unesterified form. Assay volumes were adjusted to accommodate the use of 96-well plates. Analysis of selective inhibition of 1-chloro-2,4-dinitrobenzene (CDNB) conjugation to GSH by GST isozymes is described in detail by Flatgaard, et al. [13] and K_i values for the analogs are given in Table 1. IC_{50} values for GGT were determined by monitoring the transfer of glutamyl from glutamyl *p*-nitroaniline (final assay concentration, 0.5 m*M*) to glycylglycine (final assay concentration, 10 m*M*) in the presence of various concentrations of GSH analogs. Assays were performed in 190 m*M* Tris-HCl, pH 8.0. Absorbance at 405 nm was monitored at 30°C for 5 min, using a Thermomax plate reader (Molecular Devices, Menlo Park, Calif.). GR IC_{50} values were determined by monitoring the disappearance of NADPH in the presence of oxidized glutathione (GSSG) in the presence of various concentrations of GSH analogs. Assay concentrations of GSSG and NADPH were 0.5 m*M* and 0.2 m*M*, respectively, in 1 m*M* EDTA and 100 m*M* potassium phosphate, pH 7.4. Decreasing absorbance at 340 nm was monitored at 30°C for 5 min, using the Thermomax plate reader.

GST profiles of cell lines

Cells were harvested and washed twice in serum-free medium. Cells were washed once in calcium magnesium-free PBS. The pellets were then snap-frozen and stored at -80°C prior to homogenization. Cells were homogenized according to the method of Castro et al. [7]. The homogenization buffer consisted of 10 m*M* sodium phosphate, pH 7.0, 0.16 *M* potassium chloride, 100 μ*M* Pefabloc SC (Centrachem, Stamford, Ct.), 1 μg/ml leupeptin (Sigma), 2 m*M* EDTA and 2 m*M* dithiothreitol [28]. GSTs were isolated from cytosolic fractions by affinity chromatography using an affinity HPLC column. Reversed-phase analysis of the GSTs on a J. T. Baker 7105–00 Wide Pore Octyl HPLC column (25 cm × 4.6 mm., VWR Scientific, Brisbane, Calif.), determination of protein concentrations, and determination of CDNB-conjugating activity were performed as previously described [7].

Clonogenic assay

Cells were seeded at 300 cells/well in 2 ml medium in six well plates in the presence of EA, GSH analog, or vehicle. EA and GSH analogs were used at concentrations that resulted in $\geq 85\%$ survival when compared to vehicle-treated cells. After incubation for 1–2 h, varying doses of CMB, ADR, or MTC were added. At least three replicate wells were plated for each test condition. Plates were incubated for 2 weeks, and colonies were then fixed in 95% EtOH and stained with crystal violet for colony counting. IC_{50} values were determined for the drug in the presence or absence of EA or GSH analog. Dose modification factors were calculated by dividing the IC_{50} value of drug without inhibitor treatment by the IC_{50} value of the drug with inhibitor treatment.

Modified clonogenic assay.

Cells were suspended at 2×10^5 cells/ml in serum-free medium in the presence of vehicle, EA or GSH analog. EA and GSH analogs were used at concentrations that resulted in $\geq 90\%$ survival when compared to vehicle-treated cells. Cells were incubated for 2 h, then varying doses of CMB were added. At the end of a second 2-h incubation, cells were diluted to $7.5–10 \times 10^3$/ml in medium containing 10% fetal calf serum, and plated in quadruplicate at 200 μl/well in Falcon Microtest III 96-well plates. Plates were incubated for 6 days and assayed by a modified methylene blue method [32]. Briefly, cells were fixed with 1.25% glutaraldehyde (prepared from 25% glutaraldehyde; Sigma) in PBS then stained with 0.05% methylene blue in distilled water. Plates were washed several times in distilled water to remove unretained dye, and retained dye was resolubilized in 0.03 *N* HCl. Dye concentration was measured at 650 nm in a V_{max} plate reader (Molecular Devices). IC_{50} values were determined for the drug in the presence or absence of EA or GSH analog. Dose modification factors were calculated as described for the clonogenic assay.

Tumor xenografts in mice

Male *scid* mice were subcutaneously implanted with HT4-1 tumors from donor mice. When tumors reached approximately 100 mm^3, mice were randomized into six treatment groups and treated on days 1–7 as follows: 5 mg/kg L-PAM, 10 mg/kg EA, 60 mg/kg TER 117 DEE, 5 mg/kg L-PAM and 10 mg/kg EA, 5 mg/kg L-PAM and 60 mg/kg TER 117 DEE, or vehicle. Mice were monitored for weight changes and tumor volumes were determined by measurement with calipers. Tumor growth was monitored until the average tumor size reached 1500 mm^3 for all groups except L-PAM with EA., this group failed to reach 1500 mm^3 tumor volume even after 72 days when the study was discontinued. The statistical significance of the differences between the treatment and control groups in the tumor xenograft model was determined using ANOVA.

Metabolism of TER 117 DEE in HT-29 cells

TER 117 DEE, at 50 μ*M*, was added to 10^6 HT-29 cells/ml in culture medium containing 1% FBS or tubes containing medium with FBS only. At various times, triplicate tubes containing cells were centrifuged and the supernatants were carefully removed. Cell pellets, supernatants, and samples of 50 μ*M* TER 117 DEE in medium only were extracted in acetonitrile. Analysis was performed by reverse-phase HPLC. Appropriately synthesized compounds were coinjected to verify the indentity of the DEE, the monoethyl esters, and free TER 117, and data are expressed as the percentage of total peak area.

Similar analysis of TER 143 DEE was not possible because of poor detection by HPLC, requiring concentrations in the incubation mixture that were toxic to the cells.

Results

GST profiles of cell lines

As seen in Table 2, all cell lines expressed P1-1 as the predominant GST isozyme. HT-29 cells contained trace amounts of both A1 and A2 as well as M1 and M2. HT4-1 had low levels of M2 but other isozymes except P1-1 were at trace or undetectable levels. SKOV-3 cells expressed moderate levels of A2-2. Trace levels of M1-1 were also seen in this cell line, while SK VLB contained only P1-1 and trace amounts of M2-2. Since within a GST isozyme class (Alpha, pi, mu) heterodimers can occur, the dimeric form of the alpha and mu GSTs in HT-29 or HT4-1 cells could not be assigned. M3-3 has been detected in about 50% of the cell lines (Morgan et al., unpublished observation) and in tissues [20] we have examined but was not found in any of these cell

Table 2. Reversephase HPLC analysis of cytosolic GSTs expressed in tumor cell lines exhibiting drug resistance to alkylating agents. Values are expressed as mOD/min per mg cytosolic protein and are the means ± SEM of three determinations (*ND* not detectable)

Cell Line	GST protein levels (μg/mg cytosolic protein) P1	A1	A2	M1	M2	M3	Total CDNB activity
HT-29	4.89	< 0.1	ND	< 0.1	< 0.1	–	5504 ± 172
HT4-1[a]	3.96	–	–	< 0.1	0.39	–	5204 ± 261
SKOV-3	1.32	–	0.31	< 0.1	–	–	3191 ± 54
SK VLB[b]	1.95	–	–	–	< 0.1	–	4215 ± 136[c]

[a]HT-29 subclone retaining wild-type sensitivity to ethacrynic acid.
[b]Vinblastine-resistant subclone of SKOV-3.
[c]$P < 0.01$ vs SKOV-3

Table 3. Role of esterification in the capacity of GSH analogs to potentiate CMB toxicity in HT-29 cells in culture. Values are the means ± SD of two or three experiments (DMF) dose modification factor.

GSH analog	Unesterified Dose tested (μ*M*)	DMF	Diethyl ester Dose tested[a] (μ*M*)	DMF
TER 117	100	1.08 ± 0.01	12.5	1.65 ± 0.04
TER 135	100	1.1 ± 0.02	12.5	1.27 ± 0.02
TER 211	200	1.02 ± 0.02	12.5	1.21 ± 0.01
TER 143	not tested		5	0.86 ± 0.02

[a]The test dose was determined from the toxicity curve and analogs were used at the dose at which ≥ 90% survival occurred in the presence of the analog alone.

lines. HT-29 and HT4-1 cells contained similar amounts of GST activity, while SK VLB cells had 32% more total GST activity than SKOV-3 cells. This difference in GST activity is similar to the change in P1-1 levels which are increased 48% in the SK VLB cells.

Inhibition of GSH-dependent enzymes by GSH analogs

Table 1 summarizes an analysis of in vitro inhibition of GST isozymes by the four GSH analogs used in our cell culture studies. All four compounds were very effective at inhibiting CDNB conjugation by GST P1-1 with K_i values of 0.4–1.9 μ*M*, with TER 117 being the most effective inhibitor of P1-1. TER 143 had the lowest K_i for A1-1 while TER 211 was the best inhibitor of M1a-1a and M2-2 with a 120-fold selectivity for M1a-1a over other GSTs tested. By comparison, EA was nonselective with respect to GST isozyme and, in our hands, the K_i values were 2–5 μ*M* for three of the four human GSTs tested. All of the GSH analogs had at least ten fold lower K_i values for specific isozymes than EA and 3–120-fold selectivity for that isozyme vs any other isozyme tested. None of the compounds, including EA, was inhibitory of either GR or GGT (data not shown). These data not only indicate the selectivity of the GSH analogs for specific GST isozymes but also suggest that there is some degree of specificity with respect to other GSH-related enzymes.

Toxicity and potentiation effects

The modified clonogenic assay was used for initial screening of the GSH analogs since this format allows for the rapid screening of a number of compounds. Under the conditions used, none of the unesterified compounds resulted in any decrease in survival of HT-29 cells at any dose up to 200 μ*M* (Table 1). By comparison, the DEEs of all four compounds generated IC_{50} values of 20–40 μ*M*, indicating that the presence of esters is important for the observed toxic effects.

When the four DEEs were evaluated for their ability to potentiate CMB killing of HT-29 cells, only TER 117 DEE produced greater than a 50% enhancement of toxicity (Table 3). Two other DEEs, TER 135 DEE and TER 211 DEE, produced more modest effects. The fourth DEE, TER 143 DEE, failed to potentiate CMB toxicity. The unesterified forms of all four compounds showed negligible potentiation of the toxic effects of CMB (Table 3). We also tested the phenyl glycyl monoethyl ester of TER 117 for toxicity and potentiation of CMB; this compound was not toxic at doses up to 200 μ*M* and did not potentiate CMB in HT-29 cells (data not shown). Likewise, a mixture of glycyl and glutamyl monoethyl esters of TER 143 was nontoxic and failed to potentiate CMB toxicity in HT-29 cells. For the analogs containing phenyl glycine, the R (−) configuration was essential for both toxicity and potentiation. The DEE of the *R* (−) diastereomer of TER 135 had an IC_{50} value of 24 μ*M* and enhanced CMB toxicity by about 30% while the DEE of the *S* (+) diastereomer had an IC_{50} value of 170 μ*M* and failed to potentiate (data not shown).

As indicated in Fig. 1, the relative capacity of the GSH analog DEEs to potentiate CMB toxicity in HT-29 cells correlated positively with the potency of the parent compounds as inhibitors of GST P1-1 in vitro. Rank correlation analysis yielded a significance of 0.04.

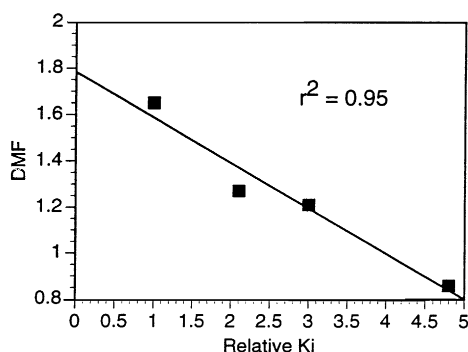

Fig. 1 Correlation of dose modification factor (DMF) produced by GSH analog DEEs with the relative K_i (P1-1) of the unesterified GSH analog ($r^2 = 0.95$ for a linear regression analysis)

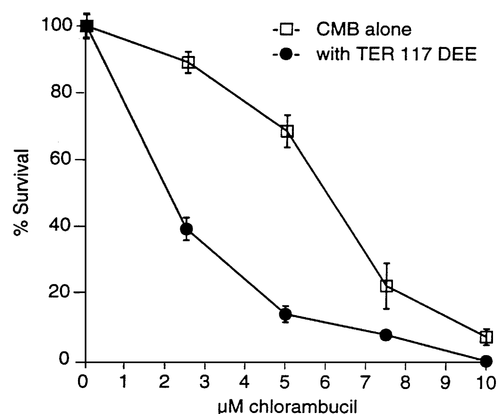

Fig. 2 Efficacy of TER 117 DEE in HT4-1 cells. Potentiation of CMB toxicity: □ CMB alone, ● CMB in the presence of 25 μ*M* TER 117 DEE. Values are the means ± SD of five determinations

Table 4. Ability of selected GSH analogs to potentiate drug toxicity as demonstrated in a clonogenic assay. Values are from individual experiments except for HT4-1 with CMB and TER 177 DEE; value is mean ± SEM of three experiments. (*DMF* dose modification factor, *ND* not determined)

Cell line	GSH analog (test dose)	CMD	DMF[a] ADR	MTC
HT4-1	TER 117 DEE (25 μ*M*)	2.39 ± 0.5	1.20	1.03
	TER 143 DEE (5 μ*M*)	1.74	1.13	1.56
SKOV-3	TER 117 DEE (25 μ*M*)	1.24	1.14	1.03
	TER 143 DEE (2.5 μ*M*)	1.03	1.24 (5 μ*M*)[a]	ND
SK VLB	TER 117 DEE (25 μ*M*)	ND	2.5	0.82 (5 μ*M*)[a]
	TER 143 DEE (5 μ*M*)	ND	1.06	1.63

[a]Test dose was different from that indicated on the left

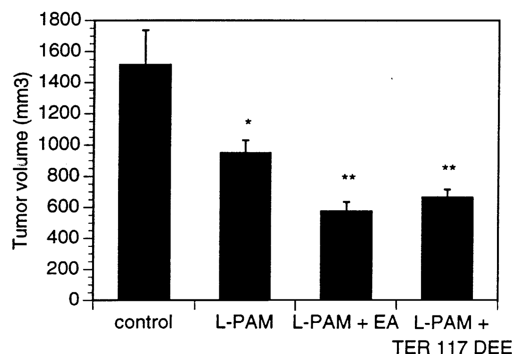

Fig. 3. Effect of EA or TER 117 DEE on L-PAM modulation of HT4-1 tumor size in *scid* mice. Values are the means ± SEM ($n = 15$)*. $P < 0.05$ for L-PAM vs control; **P < 0.005 for L-PAM + inhibitor vs L-PAM and ≤ 0.001 for L-PAM + inhibitor vs control

Clonogenic assays.

TER 117 DEE and TER 143 DEE were also tested in a standard clonogenic assay, using three cell lines with three chemotherapeutic drugs. Data for these two compounds are shown in Table 4. Doses of TER 117 DEE that could be used were generally five to tenfold higher than could be achieved with TER 143 DEE without significant toxicity. In HT4-1 cells, TER 117 DEE produced a greater than twofold enhancement of CMB toxicity in HT4-1 cells (Fig. 2) while TER 143 DEE treatment resulted in a lesser, but still significant, effect. In separate studies, TER 117 DEE was also effective in potentiating L-PAM in HT4-1 cells, with a mean dose modification factor of 1.75. Likewise, TER 117 DEE was the better potentiator of CMB in SKOV-3 cells. Potentiation of ADR by the compounds was modest and equivalent in HT4-1 and SKOV-3 cells. In one experiment, TER 117 DEE had a marked enhancing effect on ADR toxicity in SK VLB cells. However, this was an inconsistent finding. For MTC treatment of HT4-1 and SK VLB cells, TER 143 DEE, but not TER 117 DEE, was an effective potentiator.

HT4-1 tumor growth

As seen in Fig. 3, HT4-1 tumor growth was moderately retarded (27%) by L-PAM treatment. EA and TER 117 DEE increased the effect of L-PAM on tumor growth to 62% and 56%, respectively. The time for tumors to reach 1000 mm^3 was 36, 50, 61 and 58 days for control, L-PAM alone, L-PAM with EA, and L-PAM with TER 117 DEE, respectively and the time to reach 1000 mm^3 (shown in Fig. 4) was increased 39%, 69% and 61% over control for L-PAM alone, L-PAM with EA, and L-PAM with TER 117 DEE, respectively. Neither EA nor TER 117 DEE alone caused any tumor growth inhibition.

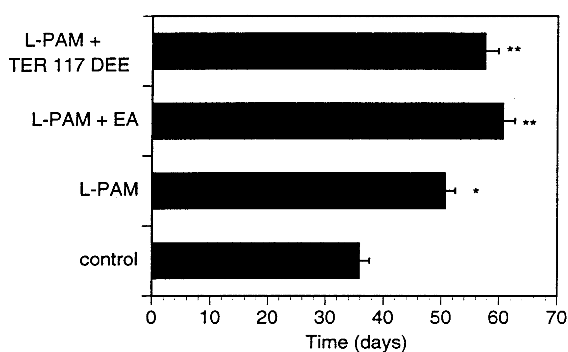

Fig. 4 Effect of EA or TER 117 DEE on L-PAM-induced growth delay of HT4-1 tumors in *scid* mice. Values are the means ± SEM ($n = 21$). *$P < 0.001$ for L-PAM vs control; **$P < 0.005$ for L-PAM + inhibitor vs L-PAM and < 0.001 for L-PAM + inhibitor vs control

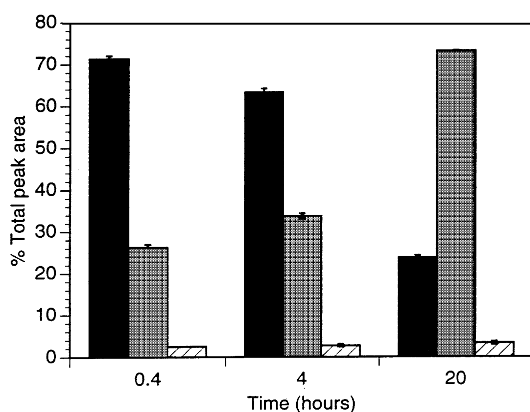

Fig. 5 Metabolism of TER 117 DEE in HT-29 cells. Cells were cultured in the presence of TER 117 DEE for the indicated times. Supernatants and cell pellets were harvested and analyzed by HPLC. Values are means ± SD of three determinations. ■ TER 117 DEE; □ Monoethyl ester; ▩ TER 117

Metabolism of TER 117 DEE in HT-29 cells

As seen in Fig. 5, cell pellets showed that 30% of TER 117 DEE was converted to the phenyl glycyl monoethyl ester by 20 min and this rose to 70% by 18 h. Free TER 117 was detected by 20 min and levels doubled by 18 h. Both increases occurred with a corresponding decrease in DEE. No glutamyl monoethyl ester form was detected at any time point. Over the same time course, TER 117 DEE comprised 90% of the total peak area in both the supernatant and culture medium alone (data not shown).

Discussion

A homologous series of GSH analogs has previously been shown to differ in in vitro inhibitory capacity for several different GST isozymes. These analogs have submicromolar K_i values for specific GSTs and 3–120-fold selectivity within the GST isozyme family. Further, none of these compounds inhibits two other major GSH-metabolizing enzymes. Accordingly, these reagents permit a stringent test of the role of GSTs in drug response, previously indicated by studies with compounds with less selective properties [14, 17, 18, 39, 41]. Since the GSH analogs contain two free carboxyl groups, the resulting charge was expected to inhibit cell uptake of the compounds. To overcome this potential problem, the analogs were tested as the DEE forms for their ability to potentiate drug toxicity. HPLC analysis indicated that the ester groups are removed intracellularly. By comparing the DEEs in HT-29 cells, which contain P1-1 as the predominant GST isozyme, we found that the degree of sensitization to CMB correlated well with the relative ability of the unesterified compound to inhibit P1-1 in vitro. These results support inhibition of GST P1-1 as the mechanism of enhancement of drug toxicity in this cell line. The correlation also indicates the potential for selective inhibition of specific GST isozymes elevated in tumors as an effective method for modulating drug resistance in tumor cells. Corroborative evidence comes from recent work by Nitsu et al. (personal communication). Using antisense mRNA constructs, they showed that a 50% reduction in P1-1 levels and, therefore, P1-1 enzyme activity, sensitized tumor cells to such drugs as ADR.

In addition, we demonstrated the ability of GSH analogs with differential GST inhibitory activity to selectively potentiate L-PAM (TER 117 DEE) or MTC (TER 143 DEE). In two of the three cell lines tested, MTC toxicity was potentiated by TER 143 DEE but not TER 117 DEE. TER 143 DEE has moderate selectivity for alpha class GSTs. GST activity is significantly increased (44-fold) in a human breast tumor cell line resistant to MTC [12] and in a mouse cell line [48], and the latter can be sensitized to MTC with EA [48]. To date, the GST isozyme involved in MTC resistance has not been determined. MTC resistance in colon tumor cell lines has been associated with increased GST levels [40], specifically pi and alpha isozymes [33]. Although alpha class GSTs were not detected in our cell lines, our potentiation data suggest a role for alpha class GSTs in MTC resistance. These results also reinforce the concept of selective GST inhibition as a means of potentiating drug response.

Further support for the efficacy of GSH analogs as potentiators of drug toxicity came from in vivo experiments with HT4-1 tumor xenografts. In this model, a P1-1-selective GST inhibitor was as effective as a nonspecific GST inhibitor in enhancing L-PAM efficacy. That GSH analogs capable of selectively inhibiting GST P1-1 are effective potentiators of CMB, L-PAM, and perhaps of ADR, argues that they may also be effective in tumors overexpressing GST P1-1 and demonstrating resistance to other chemotherapeutic agents such as cisplatin [30, 36] and BCNU [1].

We have obtained evidence that the potentiating effect we have observed is due to the generation of the unesterified form of the analogs intracellularly. Continuous exposure of tumor cells to GSH analog DEEs, as was the case in the clonogenic assay, resulted in more profound enhancement of drug effects than was seen with limited exposure in the modified clonogenic assay, suggesting increased accumulation of the analog intracellularly with the former treatment. HPLC determinations of intracellular levels of the analog at 4 h and 18 h indicate that this is the case. For TER 117 DEE, the phenyl glycyl monoethyl ester becomes the predominant intracellular form. The phenyl glycyl monoethyl ester has been synthesized and had no toxic effect at doses up to 200 μM and failed to potentiate CMB toxicity in HT-29 cells at this dose. Complete de-esterification presumably is an essential prerequisite for the intracellular effects, since we have shown the ethyl esters of our GSH analogs to be extremely poor enzyme inhibitors (unpublished observations). The postulated role of ethyl esters in promoting cellular uptake of the analogs is consistent with the findings by Lo and Thornalley [25], where only the DEE form of glyoxalase inhibitors had an effect on cell survival in culture. Both our results and those of Lo and Thornalley are in contrast to what has been observed with esters of GSH itself [2, 43, 46]. In work by Meister's group, even the monoethyl ester of GSH was able to enter the cells. Although GSH monoethyl esters are active, the diethyl ester form may be more active [44]. Utilization of amino acid transport channels has been suggested as an explanation for the effectiveness of GSH monoesters [25]. However, this mechanism is probably not involved in the uptake of the more complex GSH analogs described here.

All of the analog DEEs used in this study produced significant toxicity in cultured tumor cells, although the mechanism by which the analogs produce their toxic effect has not been directly determined. The amount of toxicity was not specifically associated with replacement of glycine with phenyl glycine since TER 135 DEE was twice as toxic as TER 117 DEE although both contain phenyl glycine. The addition of alkyl groups to the sulfur of cysteine resulted in greater toxicity than did the addition of ring structures.

The data reported here provide a foundation for preclinical testing of GST isozyme-specific inhibitors as chemosensitizers. Results of in vivo studies with TER 117 DEE indicate that, like EA, it can enhance the effect of L-PAM on growth of HT4-1 tumor xenografts in mice. We have recently developed analogs that are more inhibitory for GST P1-1 than TER 117, and are currently testing these compounds in culture. Finally, we are also continuing our work on inhibitors of other GST isozymes with the intention of elucidating the role of the various isozymes in drug resistance as well as developing an array of selective inhibitors that can be matched against individual tumor profiles [20].

Acknowledgements Our thanks to our colleagues at Terrapin: Ms. Julie Montali and Dr. Don Schmidt for providing the HPLC analysis of the cell lines, Mrs. Betsy Hughes for providing the HPLC determination of intracellular products of TER 117 DEE, and to Ms. Karin Bauer , Dr. Jeffrey Flatgaard and Dr. Deb Higgins for the enzyme inhibition analysis. We also wish to express our appreciation to Ms. Jeanette E. Stuart for assisting with the cell culture work conducted at Fox Chase Cancer Center.

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