Biochemical Pharmacology, Vol. 42, No. 7, pp. 1504–1507, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

Enzymatic conjugation of chlorambucil with glutathione by human glutathione *S*-transferases and inhibition by ethacrynic acid

(*Received* 11 *April* 1991; *accepted* 6 *June* 1991)

Chlorambucil (CMB*) is a clinically important alkylating agent employed in the treatment of a number of neoplastic diseases including chronic lymphocytic leukemia. The effectiveness of this and other anticancer agents can be limited by the development of tumor cell resistance. Several mechanisms have been postulated for the alkylator-resistance phenotype, including impaired cellular uptake of drug [1], altered levels of glutathione (GSH) [2], and elevations of glutathione *S*-transferase (GST) [3–5].

GSTs are a structurally and functionally heterogeneous family of enzymes that conjugate GSH to diverse electrophiles [6, 7]. It has been suggested that the increased expression of particular classes or individual subunits of GSTs confers resistance by detoxifying drug substrates [3–5, 8–10]. Early studies revealed overexpression of GST class α in association with alkylator resistance [4, 8, 11], whereas Adriamycin resistance was accompanied by increased expression of GST class π [10, 12]. By contrast, all three GST classes (α, μ and π) were elevated in preneoplastic liver foci induced by *in vivo* treatment with alkylating agents [13]. Recently, where Nakagawa *et al.* [14] and Moscow *et al.* [15] did not find protection against CMB treatment with π cDNA transfection into cultured cells, both Black *et al.* [16] and Puchalski and Fahl [17] noted that transfection of either α or π cDNAs into yeast or mammalian cells, respectively, could confer low levels of resistance to CMB. Further complications for interpretation are provided by studies on transfection of human GSTα cDNA into MCF-7 human breast cancer cells that failed to give resistance against these drugs [18].

Overall, these findings suggest that at least in some cells involvement of α class GSTs in alkylator resistance is relevant. The common finding of overexpression of GSTπ in human tumors [6, 7] lends itself to the still unanswered question as to whether this GST plays a direct role in metabolizing alkylating agents. To sort out differences in functional capacity (detoxification ability) that also determine resistance or sensitivity, we explored which of these two GST classes most efficiently catalyzes the conjugation of CMB to GSH. Kinetic analyses suggest that purified human liver GSTα catalyzes this reaction more efficiently (V_{max}/K_m) than does purified human ovarian GSTπ. Ethacrynic acid (EA), a substrate and inhibitor of GST function that is currently under clinical investigation for use in combination with alkylating agents,† potently inhibited the reaction catalyzed by the GSTα enzyme.

Methods and Results

Affinity and chromatofocusing HPLC purification of human liver GSTα and human ovarian GSTπ. Tissue samples were obtained at the time of surgical resection and were stored at −80° prior to use. Liver tissue from an adult female human was minced and homogenized in 2–3 vol. of 10 mM Tris–HCl, pH 7.8 (Buffer A) using a polytron homogenizer. Ovarian tumor tissue pooled from twelve humans was treated similarly. Liver and ovarian cytosol was prepared from homogenate by differential centrifugation, and GST enzymes were isolated by HPLC using a glutathione affinity column as described previously [19, 20]. Pooled fractions from respective tissues were concentrated and dialyzed against Buffer A and separated into the three GST classes (α, μ and π) by HPLC chromatofocusing. Aliquots (2.5 to 5 mg) were adjusted to pH 9.3 with 25 mM triethylamine (liver) or to pH 7.0 with 25 mM bis-Tris buffer (liver and ovarian) and applied to a mono P HR 5/20 chromatofocusing column (Pharmacia Fine Chemicals, Uppsala, Sweden). pH gradients were run from 9.3–7.0 and 7.0–4.0 using appropriate combinations of Pharmalyte with Polybuffer 96 or Polybuffer 74 (Pharmacia) [20]. The 1-mL fractions were monitored for protein at 280 nm and for GST activity using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. Pooled fractions were analyzed for electrophoretic and GST class homogeneity by isoelectric focusing (IEF), sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and immunoblotting procedures [20].

Four peaks from liver and four peaks from ovarian purified GST samples were resolved by chromatofocusing. Proteins in pools I–III from liver were recognized only by antibodies raised against GSTα protein and, as a class, were electrophoretically homogeneous (immunoblotting and silver staining, data not shown; and IEF, Fig. 1). Also, electrophoretic resolution (SDS–PAGE) and immunodetection of pool IV from ovarian tissue revealed GST class homogeneity and recognition only by antibodies raised against GSTπ. For subsequent experiments, only pools I–III from liver and pool IV from ovarian tissue were employed. Since yields for GSTμ from the liver were very low, this GST class was not used for further study. It is known that different GST forms may arise from dimeric combinations of structurally distinct subunits [21]. GSTπ subunits are also subject to glycosylation which may affect pI values [22]. Approximately consistent with pI values reported by other investigators for GSTs from normal liver tissue [21, 23] and for GSTs from ovarian tumor tissue,‡ pI values (Fig. 1) determined for liver samples were: I, 9.1; II, 8.8; and III containing two subunits, 8.3 and 7.9. Both proteins from pool III were immunoreactive with anti-Ya (α) antibodies, as determined by two-dimensional IEF and gel electrophoresis (data not shown). pI values determined for the mixture of ovarian π subunits or forms were 4.68 (most enriched), 4.75 and 4.81. Using CDNB as substrate, specific activities of these GST preparations were (μmol·min^{-1}·mg^{-1}): liver, pool I, 18.1; pool II, 17.3; pool III, 19.6; ovarian, pool IV, 67.0.

* Abbreviations: CMB, chlorambucil (4[bis(2-chlorethyl)amino]benzene butanoic acid); GS-CMB, monochloromonoglutathionyl CMB; EA, ethacrynic acid ([2,3-dichloro-4-(2-methylene-butyryl)phenoxy]acetic acid); GSH, reduced glutathione (γ-glutamyl-cysteinyl-glycine); GST, glutathione *S*-transferase (EC 2.5.1.18); CDNB, 1-chloro-2,4-dinitrobenzene; IEF, isoelectric focusing; and SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

† O'Dwyer PJ, LaCreta F, Nash S, Tinsley PW, Schilder R, Tew KD, Panting L, Litwin S, Comis RL and Ozols RF, Phase I trial of thiotepa in combination with the glutathione transferase inhibitor ethacrynic acid. Manuscript submitted for publication.

‡ Schisselbauer JC, Hogan WM, Buetow KA and Tew KD, Heterogeneity of glutathione *S*-transferase enzymes and gene expression in ovarian carcinoma. Manuscript submitted for publication.

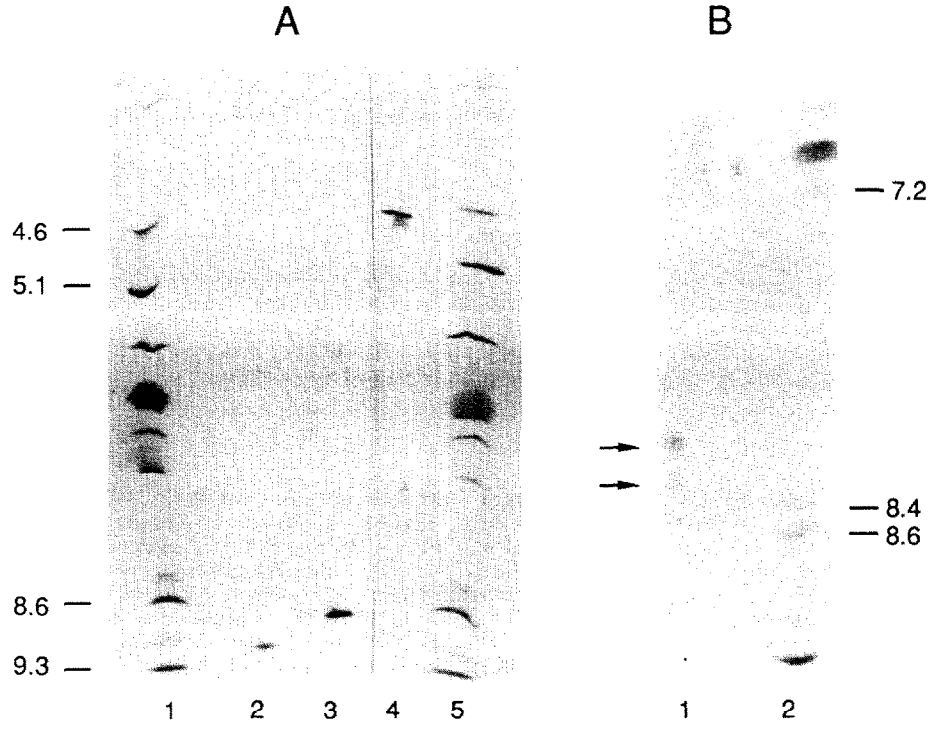

Fig. 1. One-dimensional IEF analysis of GST purified from adult human liver and adult human ovarian tumors. Protein per sample: 2.5 μg. Panel A (pH gradient 3.0–10.0): lanes 1 and 5, pI standards (Sigma), 3.6, 4.6, 5.1, 5.9, 6.6, 6.8, 7.2, 8.4, 8.6 and 9.3; lanes 2 and 3: human liver GSTα, pools I and II; and lane 4, human ovarian GSTπ, pool IV. Panel B: (pH gradient 6.5–9.0): lane 1, human liver GSTα, pool III; and lane 2, pI standards 6.6, 6.8, 7.2, 8.4, 8.6 and 9.3. Gel conditions: 1% IEF agarose (Pharmacia), 12% sorbitol, 6.3% ampholines (Pharmacia); run at 15 W, 1500 V for 1.5 hr; stained with Coomassie Blue

Incubations with purified GST and CMB. Reaction mixtures consisted of 100 μg/mL purified GST, 1 mM GSH (Sigma, St. Louis, MO), in 0.1 M potassium phosphate buffer, pH 6.5. After a 2-min preincubation at 37°, reactions were initiated by the addition of CMB (Sigma) in acetone solution and were permitted to run for 2.5 min. Reactions were stopped by the addition of 50 μL of concentrated perchloric acid/mL. When used, EA was added 30 sec before CMB. pH 6.5 was chosen because it permitted more sensitive differentiation between enzymatic and nonenzymatic contributions to monochloromonoglutathionyl chlorambucil (GS-CMB) (structural identity, Ref. 20 and 24) conjugation. Test reactions (nmol GS-CMB metabolite/min/0.1 mg protein ± SD) run at pH 7.0 (0.74 ± 0.06) and pH 7.4 (0.85 ± 0.11) with 100 μM CMB showed the enzymatic contributions to GS-CMB conjugation to be approximately equivalent to reactions at pH 6.5 (0.83 ± 0.12). Less than 10% of parent CMB was used in any given reaction. Production of the GS-CMB metabolite was found to be linear with time up to 2.5 min and was quantified by HPLC as described [21] with two modifications in the use of the mobile phase. The pH of the 0.1 M ammonium acetate buffer was 5.0 and, for kinetic studies without EA, a linear gradient of 30–90% methanol over 15 min was employed.

Figure 2 illustrates the total, nonenzymatic (spontaneous) and enzymatic (GSTα pool II) rates of formation of the GS-CMB metabolite with increasing CMB concentrations. The enzymatic rate was calculated by subtracting the spontaneous rate (without enzyme) from the total rate (with enzyme). The total and spontaneous rates of formation continued to increase linearly above 50 μM CMB, whereas the enzymatic contribution was saturable. To characterize functional similarities among GST classes and between human GSTα and mouse GSTα forms, kinetic analyses of the dependence of GS-CMB formation on CMB concentration were undertaken. Kinetic parameters calculated from double-reciprocal plots and regression analysis are listed in Table 1. All GST samples displayed approximately similar V_{max} values with human liver GSTα

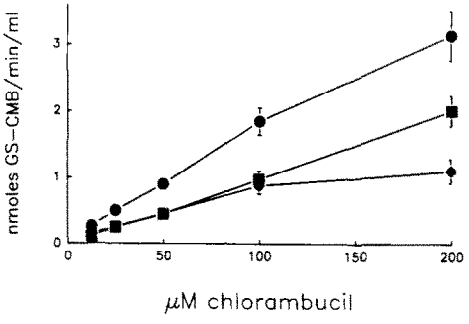

Fig. 2. Total (●), nonenzymatic (■) and enzymatic (◆) rates of GS-CMB formation with increasing CMB concentration. Human liver GSTα pool II (± 100 μg/mL) was incubated in 0.1 M potassium phosphate (pH 6.5) with 1 mM GSH and 12.5, 25, 50, 100 or 200 μM CMB for 2.5 min at 37° as described in Materials and Results. Each point is the mean (± SD) of assays performed in triplicate.

Table 1. Apparent kinetic constants for human and mouse GST-catalyzed formation of monochloromonoglutathionyl CMB metabolite.

Sample	Pool	V_{max} (nmol/min/0.1 mg)	K_m (μM)	Efficiency (V_{max}/K_m)
Human liver GSTα	I	0.8	19	0.042
	II	1.9	150	0.013
	III	3.4	220	0.016
Mouse liver GSTα	I	1.0	38	0.026
Human ovarian GSTπ	IV	1.6	830	0.002

Enzymatic assays were performed in duplicate (mouse GSTα and human ovarian π) or triplicate as described in the legend to Fig. 2 with minor differences [additional, higher concentrations of CMB were used for pools II and III (400 μM) and pool IV (400 and 600 μM)]. Kinetic parameters were calculated by linear regression analysis.

pool III being the highest. Apparent K_m values ranged from 19 μM (GSTα, pool I) to 830 μM (GSTπ, pool IV). Although the apparent K_m values of GSTα pools II and III were higher than that of pool I, the efficiency values were approximately similar among the three. Human ovarian GSTπ was the least efficient (V_{max}/K_m, by 6-fold) catalyst of this reaction despite a K_m value that was only four times that of GSTα pool III. The diuretic EA has been reported to cause maximal competitive inhibition of GSTα-catalyzed conjugation of GSH to CDNB [25]. The data listed in Table 2 show that it is a potent inhibitor of the *in vitro* enzymatic (GSTα) formation of the GS-CMB metabolite. The ability of EA to inhibit GST activity at low concentrations *in vitro* is consistent with its ability to sensitize resistant cell lines from both human and rat to CMB [26] and to sensitize human tumor xenografts to melphalan [27].

Discussion

Acquired resistance to alkylating agents is frequently associated with overexpression of GST isozymes. For this relationship to be relevant as a cause of resistance, it is necessary to show that drugs such as CMB are substrates for these isozymes. We have shown previously that CMB is metabolized by mouse GSTs [20]. It should be noted that the cyclic aziridinium ion intermediate derived from the parent mustard is likely to be the true substrate [20]. Melphalan metabolism both by purified mouse liver cytosolic GSTα isozymes (but not GSTμ and GSTπ isozymes) [28] and by Sepharose-bound monkey liver cytosolic protein containing GSTs [29] has been reported. However, metabolism studies on enzymes from other species cannot be extrapolated so readily to humans since markedly different substrate specificities have been observed with only a small number of amino acid substitutions [30], a phenomenon that in theory can occur with GSTs of the same class from different species [31]. Our results confirm that human GSTα, and to a lesser extent GSTπ, can enhance the conjugation of CMB to GSH. Despite some differences in amino acid composition, the kinetics of metabolite production by GSTα from mouse and human liver were similar. Although the maximal rates of catalysis were not high, the sharp slope of the concentration–response curve for alkylating agents predicates that the 2-fold enhancement of detoxification could have a significant impact on cell survival following CMB treatment. Similarly, the concentration-dependent inhibition of catalysis by EA (at concentrations which approximate clinically achievable serum values) supports the rationale for use of EA in conjunction with alkylating agents for treatment of certain cancers.

Table 2. Ethacrynic acid inhibition of human liver GSTα-catalyzed formation of GS-CMB

EA (μM)	GS-CMB (nmol/min/0.1 mg protein)	
	25 μM CMB	100 μM CMB
0	0.46	1.05
2.0	0.13	0.52
5.0	0.06	0.36
7.5	0.02	0.07

Analyses were performed in duplicate as described in Materials and Results and in Ref. 20.

Department of Pharmacology
Fox Chase Cancer Center
Philadelphia
PA 19111, U.S.A.

PAUL J. CIACCIO*
KENNETH D. TEW
FRANK P. LACRETA

* To whom correspondence should be addressed.

REFERENCES

1. Goldenberg GJ and Begleiter A, Alterations of drug transport. In: *Handbook of Experimental Pharmacology: Antitumor Drug Resistance* (Eds. Fox BW and Fox M), pp. 198–241. Springer, New York, 1988.
2. Suzukake K, Petro BJ and Vistica DT, Reduction in glutathione content of L-PAM resistant L1210 cells confers drug sensitivity. *Biochem Pharmacol* **31**: 121–124, 1982.
3. Wang AL and Tew KD, Increased glutathione *S*-transferase activity in a cell line with acquired resistance to nitrogen mustards. *Cancer Treat Rep* **69**: 677–682, 1985.
4. Buller AL, Clapper ML and Tew KD, Glutathione *S*-transferases in nitrogen mustard-resistant and sensitive cell lines. *Mol Pharmacol* **31**: 575–578, 1987.
5. Suzukake K, Vistica BP and Vistica DT, Dechlorination of L-phenylalanine mustard by sensitive and resistant tumor cells and its relationship to intracellular glutathione content. *Biochem Pharmacol* **32**: 165–167, 1983.
6. Mannervik B and Danielson VH, Glutathione trans-

ferase–Structure and catalytic activity. *CRC Crit Rev Biochem* **23**: 283–337, 1988.
7. Boyer TD, The glutathione *S*-transferases: An update. *Hepatology* **9**: 486–496, 1989.
8. Robson CN, Lewis AD, Wolf CR, Hayes JD, Hall A, Proctor SJ, Harris AL and Hickson ID, Reduced levels of drug-induced DNA cross-linking in nitrogen mustard-resistant Chinese hamster ovary cells expressing elevated glutathione *S*-transferase activity. *Cancer Res* **47**: 6022–6027, 1987.
9. McGown AT and Fox BW, A proposed mechanism of resistance to cyclophosphamide and phosphoramide mustard in a Yoshida cell line *in vitro*. *Cancer Chemother Pharmacol* **17**: 223–226, 1986.
10. Batist G, Tulpule A, Sinha BK, Katki AG, Myers CE and Cowan KH, Overexpression of a novel anionic glutathione S transferase in multidrug-resistant human breast cancer cells. *J Biol Chem* **261**: 15544–15549, 1986.
11. Lewis AD, Hayes JD and Wolf CR, Glutathione and glutathione-dependent enzymes in ovarian adenocarcinoma cell lines derived from a patient before and after the onset of drug resistance: Intrinsic differences and cell cycle effects. *Carcinogenesis* **9**: 1283–1287, 1988.
12. Cowan KH, Batist G, Tulpule A, Sinha BK and Myers CE, Similar biochemical changes associated with multidrug resistance in human breast cancer cells and carcinogen-induced resistance to xenobiotics in rats. *Proc Natl Acad Sci USA* **83**: 9328–9332, 1986.
13. Buchmann A, Kuhlmann W, Schwarz M, Kunz W, Wolf CR, Moll E, Friedberg T and Oesch F, Regulation and expression of four cytochrome P-450 isoenzymes, NADPH–cytochrome P-450 reductase, the glutathione transferases B and C and microsomal epoxide hydrolase in preneoplastic and neoplastic lesions in rat liver. *Carcinogenesis* **6**: 513–521, 1985.
14. Nakagawa K, Saijo N, Tsuchida S, Sakai M, Tsunohawa Y, Yohota J, Muramatsu M, Sato K, Terada M and Tew KD, Glutathione *S*-transferase π as a determinant of drug resistance in transfectant cell lines. *J Biol Chem* **265**: 4296–4301, 1990.
15. Moscow JA, Townsend AJ and Cowan KH, Elevation of π class glutathione *S*-transferase activity in human breast cancer cells by transfection of the GSTπ gene and its effect on sensitivity to toxins. *Mol Pharmacol* **36**: 22–28, 1989.
16. Black SM, Beggs JD, Hayes JD, Bartoszek A, Muramatsu M, Sakai M and Wolf CR, Expression of human glutathione *S*-transferases in *Saccharomyces cerevisiae* confers resistance to the anticancer drugs adriamycin and chlorambucil. *Biochem J* **268**: 309–315, 1990.
17. Puchalski RB and Fahl WE, Expression of recombinant glutathione *S*-transferase π, Ya, or Yb_1 confers resistance to alkylating agents. *Proc Natl Acad Sci USA* **87**: 2443–2447, 1990.
18. Leyland-Jones BR, Townsend AJ, Tu C-PD, Cowan KH and Goldsmith ME, Antineoplastic drug sensitivity of human MCF-7 breast cancer cells stably transfected with a human α class glutathione *S*-transferase gene. *Cancer Res* **51**: 587–594, 1991.
19. LaCreta FP, Olszewski JJ and Tew KD, Purification of glutathione *S*-transferase from rat liver and Walker 256 mammary carcinoma cells by high performance liquid chromatography and a glutathione affinity column. *J Chromatogr* **434**: 83–93, 1988.
20. Ciaccio PJ, Tew KD and LaCreta FP, The spontaneous and glutathione *S*-transferase-mediated reaction of chlorambucil with glutathione. *Cancer Commun* **2**: 279–286, 1990.
21. Singh SV, Dao DD, Partridge CA, Theodore C, Srivastava SK and Awasthi YC, Different forms of human liver glutathione *S*-transferases arise from dimeric combinations of at least four immunologically and functionally distinct subunits. *Biochem J* **232**: 781–790, 1985.
22. Kuzmich S, Vanderveer LA and Tew KD, Evidence for a glyconconjugate form of glutathione *S*-transferase pi. *Int J Pep Protein Res*, in press.
23. Soma Y, Satoh K and Sato K, Purification and subunit-structural and immunological characterization of five glutathione *S*-transferases in human liver, and the acidic form as a hepatic tumor marker. *Biochim Biophys Acta* **869**: 247–258, 1986.
24. Dulik DM, Colvin OM and Fenselau C, Characterization of glutathione conjugates of chlorambucil by fast atom bombardment and thermospray liquid chromatography/mass spectrometry. *Biomed Environ Mass Spectrom* **19**: 248–252, 1990.
25. Ploemen JHTM, Van Ommen B and van Bladeren PJ, Inhibition of rat and human glutathione *S*-transferase isoenzymes by ethacrynic acid and its glutathione conjugate. *Biochem Pharmacol* **40**: 1631–1635, 1990.
26. Tew KD, Bomber AM and Hoffman SJ, Ethacrynic acid and piriprost as enhancers of cytotoxicity in drug resistant and sensitive cell lines. *Cancer Res* **48**: 3622–3625, 1988.
27. Clapper ML, Hoffman SJ and Tew KD, Sensitization of human colon tumor xenografts to L-phenylalanine mustard using ethacrynic acid. *J Cell Pharmacol* **1**: 71–78, 1990.
28. Bolton MG, Colvin OM and Hilton J, Specificity of isozymes of murine hepatic glutathione *S*-transferase for the conjugation of glutathione with L-phenylalanine mustard. *Cancer Res* **51**: 2410–2415, 1991.
29. Dulik DM, Fenselau C and Hilton J, Characterization of melphalan-glutathione adducts whose formation is catalyzed by glutathione transferases. *Biochem Pharmacol* **35**: 3405–3409, 1986.
30. Burgess JR, Chow NI, Reddy CC and Tu CD, Amino acid substitutions in the human glutathione *S*-transferases confer different specificities in the prostaglandin endoperoxide conversion pathway. *Biochem Biophys Res Commun* **158**: 497–502, 1989.
31. Waxman DJ, Glutathione *S*-transferases: Role in alkylating agent resistance and possible target for modulation therapy—A review. *Cancer Res* **50**: 6449–6454, 1990.