Biochemical Pharmacology, Vol. 59, pp. 973–981, 2000.
© 2000 Elsevier Science Inc. All rights reserved.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00401-3

ELSEVIER

Phosphono Analogs of Glutathione: Inhibition of Glutathione Transferases, Metabolic Stability, and Uptake by Cancer Cells

Thomas Kunze and Stefan Heps*

PHARMAZEUTISCHES INSTITUT, CHRISTIAN-ALBRECHTS-UNIVERSITAT, D-24118 KIEL, GERMANY

ABSTRACT. Glutathione transferases (GSTs) have been shown to play an important role in multiple drug resistance in cancer chemotherapy. The inactivation of GST isoforms could lead to an enhanced activity of cytotoxic drugs. Thus, we have developed glutathione phosphono analogs [(*S*)-γ-glutamyl-(2*RS*)-($\pm$)-2-amino-(dialkoxyphosphinyl)-acetylglycines], which were previously shown to be inhibitors of GSTP1-1. In the present study, the inhibition characteristics of these analogs, including isoenzyme specificities, type of inhibition, and determination of K_i values, were determined. The inhibition of class alpha GSTs was competitive towards GSH. A mixed-type, non-competitive inhibition of class mu and pi GSTs was observed. The K_i values varied between 880 ± 210 and 0.45 ± 0.1 μM. The inhibitors were most effective towards class mu GSTs. In order to investigate the potential use of these GST inhibitors in intact cellular systems, two additional approaches were examined. Firstly, the metabolic stability was tested with purified γ-glutamyl transpeptidase and cell homogenates as well as during incubation of cell lines. No appreciable degradation was observed in any of the tested systems. Secondly, to facilitate cellular uptake, three derivatives were synthesized in which the glycine carboxylic group was esterified. Uptake and a possible intracellular cleavage to the corresponding free acids were monitored by HPLC analysis. The esters were effectively transported into HT29 (colon cancer) and EPG85-257P (gastric cancer) cells, respectively, and readily converted into the more active free acids. In conclusion, the tested inhibitors may be regarded as model compounds for the development of modulating agents in cancer chemotherapy. BIOCHEM PHARMACOL **59**;8:973–981, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. glutathione analog; glutathione transferase inhibitor; phosphonic acid; kinetic studies; neoplasm drug resistance; drug uptake

GSTs† (EC 2.5.1.18) are a group of proteins involved in the biotransformation of a broad range of reactive xenobiotics and endogenous compounds by initially catalyzing the addition of GSH to electrophilic functional groups [1]. Soluble GSTs from vertebrates were subdivided into species-independent gene classes, named alpha, mu, pi, kappa, sigma, and theta [2]. Additionally, several membrane-bound GSTs are known [3]. Elevated levels of class alpha, mu, or pi GSTs were observed in many types of cancer tissue compared to the corresponding healthy tissue [4]. The overexpression of GSTs is associated with increased resistance of tumors to a variety of antineoplastic agents [5, 6], particularly towards alkylating agents such as chlorambucil [7], melphalan [8], and nitrosourea [9]. The differences between tumor and healthy cells could be exploited to increase the activity of anticancer drugs by co-administration of selective GST inhibitors. This concept of modulation cancer therapy by GST inhibition was recently reviewed [10]. The isoenzyme-non-selective inhibitor ethacrynic acid showed such a sensitization in both cell culture [11] and in early phase I clinical trials [12, 13]. However, ethacrynic acid causes both a marked diuresis and an electrolyte imbalance *in vivo* in humans [12]. Therefore, the development of isoenzyme-specific inhibitors with fewer side effects is essential for the use of these compounds as adjuvant agents in chemotherapy of tumors. In a recent work [14], several phosphono analogs of glutathione were synthesized and found to be inhibitors of human placental GST. The tripeptides contain an $O{=}P(OR)_2$ moiety in place of the glutathione cysteinyl residue CH_2SH (Fig. 1). The three most potent inhibitors [14] were examined for their inhibition pattern using purified porcine GSTs [15, 16] and three recombinant human isoenzymes.

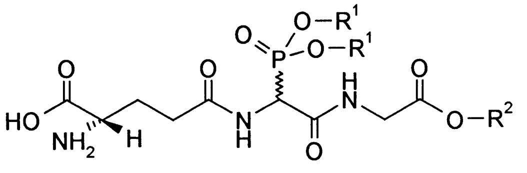
In the context of the general aim of the present study, the metabolic stability of the inhibitors was tested with

---

* Corresponding author: Dr. Thomas Kunze, Pharmazeutisches Institut, Christian-Albrechts-Universität Kiel, Gutenbergstr. 76, D-24118 Kiel, Germany. Tel. +49-431-880-1331; FAX +49-431-880-1352; E-mail: tkunze@pharmazie.uni-kiel.de

† *Abbreviations:* CDNB, 1-chloro-2,4-dinitrobenzene; DE-OH, (*S*)-γ-glutamyl-(2*RS*)-($\pm$)-2-amino-(diethoxyphosphinyl)-acetyl-glycine; DP-OH, (*S*)-γ-glutamyl-(2*RS*)-($\pm$)-2-amino-(diisopropoxyphosphinyl)-acetyl-glycine; DB-OH, (*S*)-γ-glutamyl-(2*RS*)-($\pm$)-2-amino-(di-*n*-butoxyphosphinyl)-acetyl-glycine; DE-CH_3, (*S*)-γ-glutamyl-(2*RS*)-($\pm$)-2-amino-(diethoxyphosphinyl)-acetyl-glycine methyl ester; DP-CH_3, (*S*)-γ-glutamyl-(2*RS*)-($\pm$)-2-amino-(diisopropoxyphosphinyl)-acetyl-glycine methyl ester; DB-CH_3, (*S*)-γ-glutamyl-(2*RS*)-($\pm$)-2-amino-(di-*n*-butoxyphosphinyl)-acetyl-glycine methyl ester; GST, glutathione transferase; and γ-GT, γ-glutamyl transpeptidase.

Received 12 May 1999; accepted 21 September 1999.



Abbr.	R1	R2
DE-OH	Et	H
DP-OH	*i*Pr	H
DB-OH	*n*Bu	H
DE-CH$_3$	Et	CH$_3$
DP-CH$_3$	*i*Pr	CH$_3$
DB-CH$_3$	*n*Bu	CH$_3$

FIG. 1. Structures of the inhibitors used in these studies.

various cellular and subcellular systems. Another aspect to be considered is the cellular uptake of GST inhibitors required for a potential synergetic activity in cancer chemotherapy. We expected that, in accord with the behavior of GSH itself [17], the glutathione analogs would only marginally permeate through the cellular membranes. Hence, we decided to increase the lipophilicity of the GST inhibitors by synthesizing the monomethyl esters of these analogs (Fig. 1). In the present report, the uptake into tumor cells and the conversion to the corresponding free acids is described.

MATERIALS AND METHODS

Chemicals and Materials

Buffers and salts were obtained from Merck and were of analytical or HPLC grade. Glutathione, γ-GT (type 1, bovine kidney), and CDNB were purchased from Sigma-Aldrich Chemical Co. Recombinant GST was obtained from Biotrin. DE-OH, DP-OH, and DB-OH were synthesized as described previously [14]. All other chemicals for the peptide synthesis were of reagent grade. Solvents were distilled before use. The human colon cancer cell line HT29 was purchased from Deutsches Krebsforschungszentrum. The human cell line EPG85-257P (gastric cancer) was a gift from Prof. M. Dietel [18].

Analytic Methods

Melting points: Büchi 510 apparatus (uncorr.); i.r. data: Perkin Elmer Fourier FT-IR 16PC spectrophotometer; ^{1}H and ^{13}C NMR spectra: Bruker ARX 300 and Bruker AM 400 spectrometer, solvent as indicated, internal standard tetramethylsilane (TMS). ^{13}C NMR spectra were ^{1}H-decoupled and assignment of signals was done with the assistance of DEPT135 and GATED experiments. Thermospray-MS: HP 5989A, solution of compounds in 0.1 M ammonium acetate/methanol 75:25 (v/v); elemental analysis: CHN Autoanalyzer, Hewlett Packard.

Synthesis of γ-Glutamyl-dialkoxyphosphinyl-glycyl-glycine Methyl Esters

The synthesis of γ-glutamyl-dialkoxyphosphinyl-glycyl-glycine methyl esters (DE-CH$_3$, DP-CH$_3$, and DB-CH$_3$) was essentially performed as described for the corresponding *tert* butyl esters [14] using methyl glycinate as C-terminal amino acid component.

(S)-γ-Glutamyl-(2RS)-(±)-2-amino-(diethoxyphosphinyl)-acetyl-glycine methyl ester. Colorless powder. i.r. (KBr): ν = 3223 cm^{-1}, 2989, 1745, 1661, 1515, 1357, 1238. ^{1}H-NMR (300.13 MHz, CDCl$_3$, diasteriometric ratio 50:50): δ = 1.28 ppm (br. t, J = 5.2 Hz, 6H, CH_2CH_3), 2.16 (m$_c$, 2H, Glu-$C_\beta H_2$), 2.58 (m$_c$, 2H, Glu-$C_\gamma H_2$), 3.68 (s, 3H, OCH_3), 3.74 (m$_c$, 2H, Gly-$C_\alpha H_2$), 4.13–4.19 ppm (m, 4H, CH_2CH_3), 5.32 (dd, J_1 = 8.6 Hz, J_2 = 21.5 Hz, 1H, CHP), 8.30 (m$_c$, NH, OH), 8.53 (m$_c$, NH, OH). ^{13}C-NMR (75.47 MHz, [D$_6$]DMSO): δ = 16.34 (CH_2CH_3), 26.8 (Glu-$C_\beta H_2$), 31.6 (Glu-$C_\gamma H_2$), 42.3 (Gly-$C_\alpha H_2$), 50.6 (d, $^1J_{C,P}$ = 148.4 Hz, CHP), 52.4 (OCH_3), 54.1 (Glu-C_αH), 64.0 (d, $^2J_{C,P}$ = 7.8 Hz, CH_2CH_3), 166.1, 173.2, 173.8 (CONH, COO), 169.7 ($COOCH_3$). MS (100 eV): *m/z* (%) = 412 (100) [M + H$^+$].

(S)-γ-Glutamyl-(2RS)-(±)-2-amino-(diisopropoxyphosphinyl)-acetyl-glycine methyl ester. Colorless amorphous powder. m.p. 135°. i.r. (KBr): ν = 3274 cm^{-1}, 2982, 2938, 1756, 1660, 1530, 1376, 1234. ^{1}H-NMR (300.13 MHz, CDCl$_3$, diasteriometric ratio 50:50): δ = 1.27–1.33 ppm (m, 12H, $CHCH_3$), 2.00 (m$_c$, 2H, Glu-$C_\beta H_2$), 2.57 (m$_c$ 2H, Glu-$C_\gamma H_2$), 3.69 (s, 3H, OCH_3), 3.73 (br. d, J = 5.8 Hz, 2H, Gly-$C_\alpha H_2$), 4.71–4.80 ppm (m, 2H, $CHCH_3$), 5.25 (dd, J_1 = 8.9 Hz, J_2 = 22.4 Hz, 1H, CHP), 8.09 (m$_c$, 2H, NH, OH), 8.32 (m$_c$, 1H, NH, OH), 8.42 (m$_c$, 1H, NH, OH). ^{13}C-NMR (75.47 MHz, CDCl$_3$): δ = 23.6, 23.8, (d, $^2J_{C,P}$ = 5.7 Hz, $CHCH_3$), 28.4 (Glu-$C_\beta H_2$), 31.9 (Glu-$C_\gamma H_2$), 41.7 (Gly-$C_\alpha H_2$), 50.7 (d, $^1J_{C,P}$ = 144.4 Hz, CHP), 52.3 (OCH_3), 53.6 (Glu-C_αH), 72.4 (d, $^2J_{C,P}$ = 6.9 Hz, $CHCH_3$), 165.9, 172.9, 173.3 (CONH, COO), 169.6 ($COOCH_3$). MS (100 eV): *m/z* (%) = 440 (100) [M + H$^+$].

*(S)-γ-Glutamyl-(2RS)-(±)-2-amino-(di-*n*-butoxyphosphinyl)-acetyl-glycine methyl ester*. Colorless powder. i.r. (KBr): ν = 3286 cm^{-1}, 2962, 1746, 1662, 1532, 1368, 1230. ^{1}H-NMR (300.13 MHz, CDCl$_3$, diasteriometric ratio 50:50): δ = 0.88 ppm (m$_c$, 6H, CH_2CH_3), 1.32 (m$_c$, 4H, CH_2CH_3), 1.58 (m$_c$, 4H, OCH_2CH_2), 1.97 (m$_c$, 2H, Glu-$C_\beta H_2$), 2.37 (m$_c$, 2H, Glu-$C_\gamma H_2$), 3.69 (s, 3H, OCH_3), 3.88 (m$_c$, 2H, Gly-$C_\alpha H_2$), 4.03 ppm (m$_c$, 4H, OCH_2CH_2), 4.37–4.44 (m, 1H, Glu-$C_\alpha H$), 5.08 (dd, J_1 = 8.9 Hz, J_2 = 21.7 Hz, 1H, CHP), 8.42 (m$_c$, 1H, NH), 8.58–8.69 (m, NH, OH). ^{13}C-NMR (75.47 MHz, [D$_6$]DMSO): δ = 13.6 (CH_2CH_3), 18.6 (CH_2CH_3), 26.6 (Glu-$C_\beta H_2$), 32.4 (Glu-$C_\gamma H_2$), 33.9 (OCH_2CH_2), 42.4 (Gly-$C_\alpha H_2$), 51.0 (d, $^1J_{C,P}$ = 148.9 Hz, CHP), 52.4

(OCH_3), 54.2 (Glu-$C_\alpha H$), 67.7 (OCH_2CH_2), 166.1, 173.1, 173.7 (CONH, COO), 169.8 $(COOCH_3)$. MS (100 eV): m/z (%) = 468 (100) $[M + H^+]$.

Purification of GSTs from Porcine Liver and Lung

GSTs were isolated by affinity chromatography starting from 100,000 *g* supernatant of liver homogenate and subsequent separation into the isoenzymes by ion-exchange HPLC as described earlier [15]. The isolation of the class pi GST pP1-1 was performed by the method of Dirr *et al.* [16]. Identity and purity of the isoenzyme was checked by SDS–PAGE, isoelectric focusing, and immunoblot analysis [15].

Determination of GST Activity

GST activity towards CDNB was measured at pH 6.5 and 25°C, containing max. 2% ethanol final conc. (v/v), and product formation was monitored at 340 nm ($\epsilon_{340} = 9.6$ mM^{-1} cm^{-1}) [19].

γ-GT Assay

γ-GT activity was assayed with L-γ-glutamyl-3-carboxy-4-nitroanilide and glycylglycine as substrates using a Unimate 3 GGT test kit purchased from Hoffmann–La Roche AG following the manufacturer's instructions [20].

Kinetics and Inhibition of GSTs

IC_{50} values were determined by measuring the initial rate in the presence and in the absence of inhibitor. The concentration of inhibitor giving 50% inhibition, the IC_{50} value, was determined by non-linear fits of the data to the hyperbolic function: $V_i/V_0 = IC_{50}/(IC_{50} + [I])$ [21], where V_0 is the observed activity without inhibitor and V_i is the activity in the presence of inhibitor. The program used was SigmaPlot from Jandel Scientific. Inhibitor solutions were diluted appropriately before addition to the assay solution in order to maintain a constant concentration of organic solvent (DMSO, 2.5% (v/v)) when the inhibitor concentration was varied. The determination of K_i values was performed at 6 concentrations of GSH (0.125–4 mM for class alpha isoenzymes and 0.025–1 mM for class mu and pi GSTs), const. 1 mM CDNB, and with or without inhibitor. In addition, K_i values at variable concentrations of CDNB (0.1–2.0 mM) and constant GSH (1 mM) were determined. Inhibitors (0.1–2000 μM) were dissolved in DMSO. Reactions were started by addition of enzyme preparation. Inhibition was analyzed by Lineweaver–Burk, Eadie–Hofstee, and Hanes plots using least-squares linear regression analysis and by non-linear regression of the V_0 versus [S] plots. K_i values were calculated from the corresponding slope and intercept replots. The reaction was started by addition of enzyme preparations.

HPLC Analyses of Glutathione Phosphono Analogs

DETECTION AND QUANTIFICATION. Detection and quantification was performed by HPLC analyses with Merck–Hitachi equipment. Method A for γ-glutamyl-dialkoxyphosphinyl-glycyl-glycine methyl esters (DE-CH_3, DP-CH_3, and DB-CH_3): UV/VIS detection at $\lambda = 436$ nm, column dimension 125 × 3 mm, stationary phase Nucleosil (Macharey 120-5 C18), eluent A acetate buffer (50 mM, pH 4.13)/acetonitrile 60:40 (v/v), eluent B acetonitrile, linear gradient from 0% B to 8% B, 8 min followed by isocratic 8% B for 20 min, flow rate 0.6 mL min^{-1}. Method B: HPLC analyses of GSH and γ-glutamyl-dialkoxyphosphinyl-glycyl-glycines (DE-OH, DP-OH, and DB-OH) were essentially identical to method A except for the following modifications: eluent A acetate buffer (50 mM, pH 4.13)/acetonitrile 80:20 (v/v), eluent B acetonitrile, linear gradient from 0% B to 30% B, 25 min, followed by isocratic 30% B for 5 min, flow rate 0.6 mL min^{-1}.

SAMPLE PREPARATION. Precolumn derivatization was accomplished by reaction of the analogs with dabsyl chloride as described previously by Drnevich and Vary [22]. Samples (100 μL) were diluted with 100 μL acetonitrile and centrifuged at 6000 *g* for 3 min to remove precipitated protein. Forty microliters of supernatant was pipetted into a vial containing 40 μL of 0.2 M sodium bicarbonate (pH 8.3), then 80 μL of a 4-mM solution of dabsyl chloride in acetonitrile was added, and the mixture was finally incubated at 65° for 15 min. The vials were cooled down to room temperature for 5 min and subsequently 440 μL of a mixture of acetonitrile and water (1:1, v/v) was added; 10 μL of this solution was injected via autosampler.

Cell Culture

HT29 (colon cancer) and EPG85-257P (gastric cancer) cells were cultured in Leibovitz L-15 medium at 37° as described before [23]. Cells were counted in a Rosenthal chamber.

Stability of Glutathione Phosphono Analogs

Stability of GSH and the peptides against γ-GT was studied by incubating 150 μM GSH, DB-CH_3, and DB-OH, respectively, with purified γ-GT (1 U/mL final activity; γ-GT activity was determined as described above) in the presence of 2 mM glycylglycine dissolved in 50 mM phosphate buffer at pH 8.0 and 37° (total volume, 150 μL). The reaction was stopped with an equal volume of acetonitrile, samples were centrifuged at 6000 *g* for 3 min, and supernatants were taken for derivatization and subsequent HPLC analyses as described above.

Degradation of GSH, DB-CH_3, and DB-OH by cellular enzymes was assayed by incubating 150 μM (final concentration) of each peptide with cell homogenates of the cell lines HT29 and EPG85-257P. Cells were trypsinated and washed with PBS, pottered, diluted with 50 mM Tris buffer to a final protein concentration of 10 mg/mL, and stored on ice until incubation. Five microliters of the peptide solutions was added to 45 μL cell homogenate and incubated at 37°. The reaction was stopped by adding 50 μL acetonitrile at various time intervals and analyzed by HPLC.

Stability of the GSH analogs during cell culture was studied by incubation of exponential growing cells with $DB\text{-}CH_3$ and DB-OH at 150 μM final concentration, respectively. Cells were subcultured 24 hr before treatment with $DB\text{-}CH_3$ and DB-OH. At selected time intervals, samples were taken from the medium and stored at −80° until derivatization and HPLC.

Determination of Intracellular Concentrations of Glutathione Phosphono Analogs

HT29 and EPG85-257P cells were treated for 48 hr with 0.15 mM $DE\text{-}CH_3$, $DP\text{-}CH_3$, $DB\text{-}CH_3$, DE-OH, DP-OH and DB-OH. After incubation, cells ($1\text{–}5 \times 10^6$) were suspended in 500 μL PBS and washed three times with PBS by repeated centrifugation at 500 *g* for 5 min and resuspension. Disruption of cell membranes was achieved by sonication for 3 10-sec bursts with 1-min cooling on ice between each burst. The obtained suspensions were centrifuged at 6000 *g* for 3 min and supernatants were analyzed by HPLC as described above. To calculate the intracellular concentrations of the synthetic peptides, the cell volume was obtained by weighing the sediment of the cell suspensions.

RESULTS

Inhibition of GST Activity by Glutathione Phosphono Analogs

The GSH analogs DE-OH, DP-OH, and DB-OH were tested for inhibition of the conjugation of CDNB with GSH catalyzed by several porcine GSTs and three recombinant human GSTs. The results are given in Table 1. A tendency for improved binding with increasing chain lengths of the phosphonic acid esters was observed during inhibition studies with human placental GST [14]. This was confirmed with GSTs purified from porcine tissues and recombinant human GSTP1-1 (Table 1). The class alpha isoenzyme GST pA1-1 was only weakly inhibited. A comparison between the inhibition of the recombinant human GSTs with the corresponding porcine enzymes shows no significant difference in the inhibition pattern, with the exception of GST hA1-1 and GST pA1-1 (Table 1). The observed high IC_{50} values of the porcine GST pA1-1 correlate with the respective kinetic constants of the co-substrate GSH. For the GSTs, the following K_m^{GSH} values were determined: GST pA1-1, 560 μM; pA2-3, 680 μM; pM1-1, 120 μM; pM1-2, 140 μM; pM3-3, 80 μM, and pM4-4, 130 μM. The known K_m^{GSH} values of the human GSTs A1-1, M1a-1a, and P1-1 were 81, 95, and 110 μM [24], respectively. The different porcine class mu GSTs showed only small differences in their inhibition characteristics. The glycine methyl ester derivatives $DE\text{-}CH_3$, $DP\text{-}CH_3$, and $DB\text{-}CH_3$ were also tested for their inhibitory properties (Table 1). As expected, all IC_{50} values were higher than the values obtained with the corresponding free acids DE-OH, DP-OH, and DB-OH. The loss of the glycine carboxylate at the C-terminal position decreased the affinity for each phosphono analog to a similar extent.

TABLE 1. Inhibition of isolated porcine and recombinant human glutathione transferases

	IC_{50} values (μM)*		
Isoenzyme†	**DE-OH**	**DP-OH**	**DB-OH**
pA1-1	880 ± 90	>1000	61 ± 10
Human A1-1‡	206 ± 44	320 ± 68	15 ± 3.2
pA2-3	187 ± 43	467 ± 118	7.4 ± 1.7
pM1-1	239 ± 30	243 ± 60	1.2 ± 0.1
Human M1-1‡	311 ± 33	290 ± 41	4.7 ± 1.6
pM1-2	255 ± 26	188 ± 51	2.2 ± 0.5
pM3-3	443 ± 82	98 ± 23	5.4 ± 0.9
pM4-4	243 ± 29	266 ± 42	1.0 ± 0.3
pP1-1	172 ± 32	50 ± 8	10 ± 0.8
Human P1-1‡	145 ± 22	61 ± 12	15 ± 4
	$DE\text{-}CH_3$	**$DP\text{-}CH_3$**	**$DB\text{-}CH_3$**
Human A1-1‡	750 ± 110	>1000	123 ± 32
Human M1-1‡	340 ± 85	252 ± 40	67 ± 13
Human P1-1‡	288 ± 56	201 ± 36	98 ± 12

* IC_{50} values were estimated in three independent *in vitro* experiments using CDNB as substrate and determined by non-linear curve fitting. Values represent the average ± SD.

† Class alpha and mu GSTs were isolated from porcine liver [15] and the class pi GST pP1-1 was purified from porcine lung according to the method of Dirr *et al.* [16]. Designation follows the nomenclature proposed by Mannervik *et al.* [29] and the adaptation to porcine GSTs [30].

‡ Recombinant human GST was purchased from Biotrin.

Inhibition Characteristics and K_i *Values*

Kinetic studies were performed to characterize the interaction between the phosphono analogs of GSH and the inhibited GSTs in more detail. The initial rates were determined at various GSH and CDNB concentrations. To exclude an irreversible inhibition, enzymes were incubated with 500 μM inhibitor for 30 min and subsequently dialyzed for 24 hr. No significant loss of activity was observed in comparison with a control enzyme preparation without inhibitor (data not shown). In addition, no time-dependent inactivation was observed (data not shown). Thus, it may be assumed that inhibition by phosphono analogs is of a reversible nature. Since the GST reaction is bimolecular, we made the simplifying assumption that, by employing one substrate at a fixed concentration above the apparent K_m value (1 mM of GSH or CDNB, respectively) while varying the second substrate, the conditions would approximate those of a unimolecular reaction. A competitive inhibition was observed for the isoenzyme pA2-3, whereas pM1-1 and pP1-1 showed a mixed, non-competi-

TABLE 2. Inhibition of porcine and recombinant human glutathione transferases: kinetic parameters of DE-OH, DP-OH, and DB-OH

Enzyme	Inhibitor	Type of inhibition*	K_{ic}*	K_{iu}*
Variable substrate: glutathione (CDNB 1 mM)				
pA1-1	DE-OH	Comp.	880 ± 210	—
pA1-1	DP-OH	ND†	>1000	—
pA1-1	DB-OH	Comp.	28 ± 2.4	—
Human A1-1‡	DB-OH	Mixed	8.0 ± 2.0	71 ± 23
Variable substrate: CDNB (glutathione 1 mM)				
pA1-1	DE-OH	Non-comp.	748 ± 90	733 ± 59
pA1-1	DP-OH	ND†	>1000	>1000
pA1-1	DB-OH	Non-comp.	25 ± 2.7	28.1 ± 3.6
Human A1-1‡	DB-OH	Mixed	6.4 ± 0.9	60 ± 29
Variable substrate: glutathione (CDNB 1 mM)				
pA2-3	DE-OH	Comp.	230 ± 25	—
pA2-3	DP-OH	Comp.	318 ± 31	—
pA2-3	DB-OH	Comp.	3.2 ± 0.6	—
Variable substrate: CDNB (glutathione 1 mM)				
pA2-3	DE-OH	Non-comp.	460 ± 67	452 ± 42
pA2-3	DP-OH	Non-comp.	810 ± 102	780 ± 58
pA2-3	DB-OH	Non-comp.	6.0 ± 0.7	7.1 ± 0.9
Variable substrate: glutathione (CDNB 1 mM)				
pM1-1	DE-OH	Comp.	260 ± 47	—
pM1-1	DP-OH	Comp.	14.4 ± 2.7	—
pM1-1	DB-OH	Mixed	0.45 ± 0.1	1.5 ± 0.1
Human M1-1‡	DB-OH	Mixed	2.3 ± 1.1	4.3 ± 2.8
Variable substrate: CDNB (glutathione 1 mM)				
pM1-1	DE-OH	Non-comp.	411 ± 45	477 ± 59
pM1-1	DP-OH	Non-comp.	29.0 ± 4.6	31.0 ± 5.3
pM1-1	DB-OH	Mixed	2.1 ± 0.5	4.0 ± 0.6
Human M1-1‡	DB-OH	Mixed	1.3 ± 0.38	6.9 ± 4.7
Variable substrate: glutathione (CDNB 1 mM)				
pP1-1	DE-OH	Comp.	710 ± 88	—
pP1-1	DP-OH	Mixed	10.3 ± 2.1	80.2 ± 16
pP1-1	DB-OH	Mixed	3.5 ± 0.6	24.2 ± 4.1
Human P1-1‡	DB-OH	Mixed	4.5 ± 1.2	27.3 ± 8.9
Variable substrate: CDNB (glutathione 1 mM)				
pP1-1	DE-OH	ND†	>1000	>1000
pP1-1	DP-OH	Mixed	26.3 ± 2.3	66.1 ± 19
pP1-1	DB-OH	Mixed	3.0 ± 0.8	7.8 ± 1.2
Human P1-1‡	DB-OH	Mixed	2.2 ± 1.0	39 ± 16

* The type of inhibition was determined by visualization of Lineweaver–Burk-, Eadie–Hofstee, and Hanes plots. The inhibition was determined with constant 1 mM CDNB and 0.125–4 mM GSH or constant 1 mM CDNB and 0.1–2 mM GSH, respectively. Calculation of K_{ic} (competitive inhibition constant) and K_{iu} (uncompetitive inhibition constant) values. K_{ic} and K_{iu} values are given as means of three independent experiments ± SD.

† ND = not detectable, as the inhibitor was not soluble at the high concentrations required to establish the K_{ic} and K_{iu} values.

‡ Recombinant human GST was purchased from Biotrin.

tive inhibition, characterized by decreasing $V'_{\max}$ values (increasing slope = $K_m/V_{\max}$) and concomitantly increasing K'_m values (negative intercepts on the x axis) with increasing inhibitor concentration. The dissociation constants K_{ic} and K_{iu} (competitive and uncompetitive inhibition constants) were determined via secondary replots of the intercepts or slopes, respectively. Replots were linear in each case, indicating dead-end inhibitor enzyme complexes (data not shown). Furthermore, an allosteric binding mode can be ruled out. The results of the kinetic studies with class alpha, mu, and pi isoenzymes are given in Table 2.

CLASS ALPHA GLUTATHIONE TRANSFERASES. The GSH analogs possess only a weak affinity to the tested class alpha GSTs and show competitive inhibition characteristics with respect to GSH and a non-competitive inhibition type versus the electrophilic substrate CDNB. It is assumed that these interactions are accomplished exclusively via the G-site of the transferases and the peptide backbone of the GSH analogs. A noteworthy competition between the electrophilic substrate CDNB and the inhibitor was only observed with the human GST hA1-1, resulting in a mixed-type inhibition (Table 2). The kinetic parameters of DP-OH could not be determined with the porcine isoenzyme GST pA1-1 because of its limited solubility and very low affinity. DP-OH is the poorest inhibitor of the class alpha isoenzymes in the diethyl, diisopropyl, and di-*n*-butyl series. Obviously, the bulky groups of the diisopropylphosphonic acid ester prevent a better binding.

CLASS MU GLUTATHIONE TRANSFERASES. The compounds DE-OH and DP-OH showed, in analogy to class alpha GSTs, a competitive inhibition with respect to GSH and a non-competitive type versus CDNB. A significant change in the inhibition characteristics was observed when the influence of DB-OH on CDNB conjugation was studied. A mixed non-competitive inhibition of GST pM1-1 was detected at constant CDNB concentrations (Table 2), indicating an incomplete competition between GSH and the GSH analog DB-OH. The inhibitory effect at high GSH concentrations could be explained as the possible simultaneous binding of GSH and DB-OH. This binding can only be rationalized by an interaction of the di-*n*-butyl ester residue with the hydrophobic binding site of this GST. This proposed mode of binding was confirmed by the results of the inhibition kinetics using GSH as the variable substrate (Table 2). The K_{ic} of DB-OH was significantly lower than the K_{iu}, indicating that the inhibition does indeed have a competitive component. The K_i values determined with GST pM1-1 were generally lower than those obtained for the class alpha enzymes pA1-1 and pA2-3. The difference is particularly pronounced for the diisopropyl derivative DP-OH. In the diethyl, diisopropyl, and di-*n*-butyl series, the diisopropyl compound showed a low binding affinity towards class alpha isoenzymes (Table 2). This was not the case for the other GST subfamilies. K_i values of the same series of compounds determined for the mu and pi class isoenzymes gave a good correlation with the lipophilicity of the compounds (Table 2). No significant differences were observed upon comparing human and porcine class mu GSTs.

CLASS PI GLUTATHIONE TRANSFERASES. The inhibition characteristics determined for hP1-1 and pP1-1 were quite similar to those of the class mu GSTs. Inhibition type and calculated K_i values of the GSH phosphono analogs DE-OH, DP-OH, and DB-OH are given in Table 2. Among the three compounds, only the diethyl ester DE-OH showed simple competitive inhibition characteristics for GSH metabolism, while the diisopropyl and di-*n*-butyl derivatives DP-OH and DB-OH were mixed-type, non-competitive inhibitors (Table 2). In analogy to the isoenzyme pM1-1, simultaneous binding of both GSH analog and GSH seems to be possible for the isoenzyme pP1-1. A correlation of the inhibitory effect on GST activity with the lipophilicity of the phosphonic acid esters was also demonstrated for the isoenzyme pP1-1 (Table 2).

Stability of Glutathione Phosphono Analogs

The di-*n*-butyl phosphonates DB-OH and DB-CH_3 were chosen for a detailed study of their stability, since these compounds were found to be the best inhibitors. Several test systems were used in order to elucidate the stability of the peptides. First, the most prominent structure for an enzymatic catalyzed cleavage would probably be the amide bond of the γ-glutamyl residue. DB-OH, DB-CH_3, and GSH were incubated in the presence of glycylglycine with bovine kidney γ-GT. The concentrations of the peptides were monitored by HPLC analysis. For DB-OH and DB-CH_3, no significant degradation was observed during an incubation period of 30 min, whereas GSH was almost completely diminished (Fig. 2A). To clarify a possible intracellular degradation, e.g. via ester hydrolysis or other metabolism pathways, the peptides were incubated with whole cell homogenates of the colon cancer cell line HT29. No decrease in the DB-OH concentration was established (Fig. 2B). DB-CH_3 was degraded moderately, possible due to ester hydrolysis. Similar results were obtained with homogenates of the gastric cancer cell line EPG85-257P (Fig. 2C). The third experimental set-up was a long-duration incubation during cell culture of the two cell lines HT29 and EPG85-257P. The aim of these studies was to examine the influence of tumor cells and culture media, respectively, on the stability of the GST inhibitors. Again, no significant degradation of DB-OH was detected, while DB-CH_3 was slightly less stable during an incubation period of 50 hr (Fig. 2D).

Cellular Uptake of Glutathione Phosphono Analogs

It was expected that the GSH phosphono analogs with a free C-terminal carboxylic acid function would not be taken up by cells. In order to use more lipophilic derivatives, three methyl esters (DE-CH_3, DP-CH_3, and DB-CH_3) were synthesized (Fig. 1). The cell lines HT29 and EPG85-257P were treated with these compounds. Both extracellular and intracellular concentrations of the methyl esters and the corresponding free acids were determined using HPLC analyses. The results are presented in Fig. 3. After 48 hr, the extracellular concentrations of all compounds were almost unchanged. The release of the corresponding free acids was either only marginal or was not observed. In contrast, high concentrations of these glycinates were detected in the cell homogenate of HT29 cells (Fig. 3). The absolute amounts of the methyl esters DE-CH_3, DP-CH_3, and DB-CH_3 converted into free acids were 101 ± 25 pmol/10^6 cells, 18.2 ± 8 pmol/10^6 cells, and 212 ± 26 pmol/10^6 cells, respectively. Similar results were obtained using EPG85-257P cells (81 ± 19 pmol/10^6 cells, 12.2 ± 5 pmol/10^6 cells, and 163 ± 33 pmol/10^6 cells, respectively). In addition, the cell lines were incubated with DE-OH, DP-OH, and DB-OH, respectively. No transport into the cells was detected (data not shown).

DISCUSSION

The phosphono analogs of GSH tested in the present study were synthesized for use as model compounds for the modulation of cancer chemotherapy. The rationale of this approach is to increase the efficiency of antineoplastic

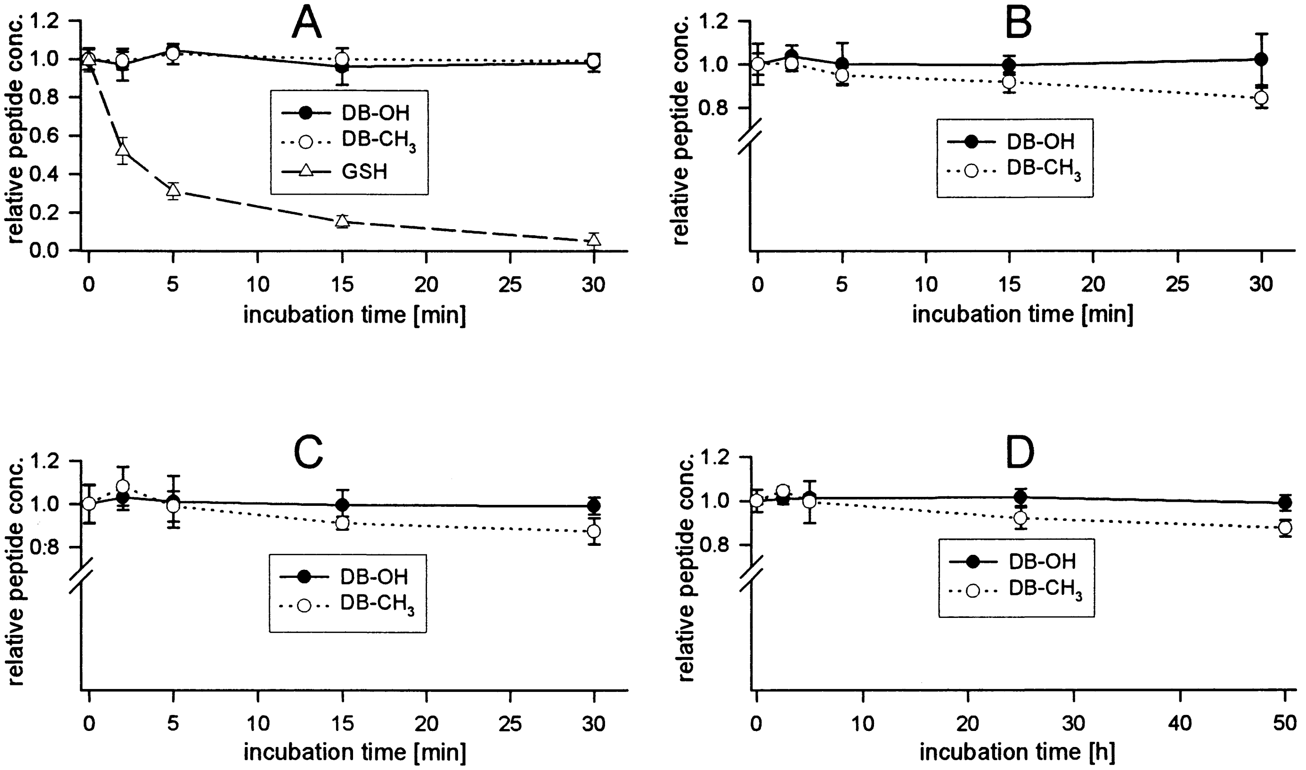


FIG. 2. Metabolic stability of DB-OH and DB-CH_3. (A) Compounds were incubated at pH 7.4 and 37° with 1 U/mL γ-GT (final conc.). (B) Compounds were incubated at pH 7.4 and 37° with whole cell homogenate of HT29 cells and (C) with whole cell homogenate of EPG85-257P cells, 10 mg prot/mL final concentration, respectively. (D) Compounds (150 μM final concentration) were dissolved in medium and incubated with growing HT29 cells. The time–course of concentrations in medium was monitored by HPLC analysis. Points and bars, means ± SD (N = 3–6 individual experiments).

drugs by inhibiting their inactivation catalyzed by GSTs in the cancer cell. Since GSTs are important enzymes for the detoxification of alkylating drugs in tumor cells as well as in normal tissues, any effective inhibition of all isoenzymes is likely to cause adverse side effects for the whole organism. However, a selective inhibition of GST isoenzymes that are overexpressed in a particular tumor could lead to an improved therapeutic index with diminished side effects. Consequently, a selective inhibition of certain GST isoenzymes seems to be a prerequisite for the application of such modulators in chemotherapy.

In the present study, the selectivity profile of a series of

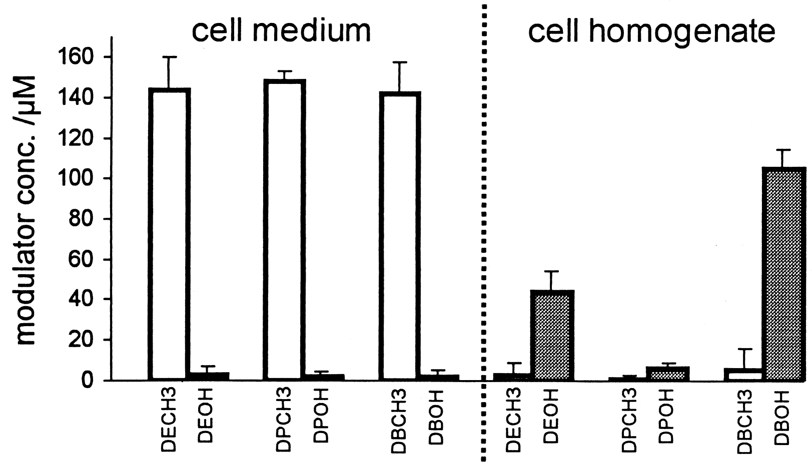


FIG. 3. Extracellular and intracellular concentrations of glutathione phosphono analogs after 48-hr incubation of the methyl esters DE-CH_3, DP-CH_3, and DB-CH_3 with exponential growing HT29 cells. The starting cell medium concentration of each compound was 150 μM. Data represent the means ± SD of 6 independent experiments.

synthesized GSH phosphono analogs was investigated with several GST isoenzymes. In general, class mu GSTs were most efficiently inhibited, followed by classes pi and alpha. In addition, a good correlation between the lipophilicity of the phosphonic acid ester alkyl chain and the inhibitory effect of a given GSH analog was observed. These two trends are in good agreement with previous studies on *S-n*-alkyl GSH derivatives [25]. The only exception to this pattern is the remarkably weak inhibition of class alpha GSTs by DP-OH. This discrepancy may be explained by the differences in the tertiary structure of class alpha GSTs as compared to other GSTs [26]. The C-terminal α-helix of the isoenzyme hA1-1 forms a lid over the active site. Compounds with bulky side chains, such as the diisopropyl phosphonic ester group of DP-OH, are likely to possess steric hindrance leading to a decreased binding affinity.

For the use of a GST inhibitor as a chemosensitizer, at least two other prerequisites have to be taken in account: 1) stability in biological systems and 2) drug uptake. As expected, the uptake of DB-OH into cells of two different tumor cell lines was very low. The highly charged molecule did not pass through the cell membranes via diffusion, and the presence of active transport systems can probably be excluded. Considering the structures of the GST inhibitors, a hydrolytic breakdown catalyzed by γ-GT could be a major metabolic pathway. A significant breakdown using various systems was not observed. γ-GT is mainly selective for the γ-glutamyl residue, but activity also depends on the amino acid to which the γ-Glu is bond [27]. The phosphonic acid ester group of the central amino acid likely protects the critical peptide bond against cleavage by γ-GT. The stability of the tested GST inhibitors encouraged us to synthesize the more lipophilic esters DE-CH_3, DP-CH_3, and DB-CH_3 in order to create potential prodrugs. The uptake into HT29 and EPG85-257P cells and the subsequent intracellular release of the corresponding acids showed that these compounds could have an influence on the principal GST activity in tumor cells. Hence, DB-CH_3, the prodrug of the most potent GST inhibitor, represents an auspicious starting compound in the development of *in vivo* chemosensitizers. The specific advantages of the new type of phosphono analog of GSH presented in this study are: 1) no potential release of GSH compared to *S*-alkyl GSH analogs and 2) a greater structural variability due to the two "arms" of the phosphonoic acid ester group. Future cell culture tests and *in vivo* studies for a putative modulation of anticancer drug resistance would seem to be especially promising for tumor cell lines showing high levels of class mu GST expression. Overexpression of class mu GSTs has been reported for HeLa cells [28] and for non-small cell lung cancer cells [9].

Currently, the palette of available phosphono analogs is being extended by the synthesis of additional compounds in the authors' laboratory. The extended compound palette is expected to provide more detailed insights into the molecular basis of GST inhibition in the near future. In addition, molecular modeling studies are planned to verify the proposed mode of binding.

The authors wish to thank Monika Henke for technical assistance.

References

1. Ketterer B, Meyer DJ and Clark AG, Soluble glutathione transferase isozymes. In: *Glutathione Conjugation* (Eds. Ketterer B and Sies H), pp. 73–135. Academic Press, London, 1988.
2. Armstrong RN, Structure, catalytic mechanism, and evolution of the glutathione transferases. *Chem Res Toxicol* **10:** 2–18, 1997.
3. Andersson C, Mosialou E, Weinander R and Morgenstern R, Enzymology of microsomal glutathione *S*-transferase. *Adv Pharmacol* **27:** 19–35, 1994.
4. Howie AF, Forrester LM, Glancey MJ, Schlager JJ, Powis G, Beckett GJ, Hayes JD and Wolf RC, Glutathione *S*-transferase and glutathione peroxidase expression in normal and tumour human tissues. *Carcinogenesis* **11:** 451–458, 1990.
5. Mulders TM, Keizer HJ, Breimer DD and Mulder GJ, *In vivo* characterization and modulation of the glutathione/glutathione *S*-transferase system in cancer patients. *Drug Metab Rev* **27:** 191–229, 1995.
6. Tew KD, Glutathione-associated enzymes in anticancer drug resistance. *Cancer Res* **54:** 4313–4320, 1994.
7. Morgan AS, Ciaccio PJ, Tew KD and Kauvar LM, Isozyme-specific glutathione *S*-transferase inhibitors potentiate drug sensitivity in cultured human tumor cell lines. *Cancer Chemother Pharmacol* **37:** 363–370, 1996.
8. Hall AG, Matheson E, Hickson ID, Foster SA and Hogarth L, Purification of an α class glutathione *S*-transferase from melphalan-resistant Chinese hamster ovary cells and demonstration of its ability to catalyze melphalan–glutathione adduct formation. *Cancer Res* **54:** 3369–3372, 1994.
9. Berhane K, Hao XY, Egyhazi S, Hansson J, Ringborg U and Mannervik B, Contribution of glutathione transferase M3–3 to 1,3-bis(2-chloroethyl)-1-nitrosourea resistance in a human non-small cell lung cancer cell line. *Cancer Res* **53:** 4257–4261, 1993.
10. Mulder GJ and Ouwerkerk-Mahadevan S, Modulation of glutathione conjugation *in vivo*: How to decrease glutathione conjugation *in vivo* or in intact cellular systems *in vitro*. *Chem Biol Interact* **105:** 17–34, 1997.
11. Hansson J, Berhane K, Castro VM, Jungnelius U, Mannervik B and Ringborg U, Sensitization of human melanoma cells to the cytotoxic effect of melphalan by glutathione transferase inhibitor ethacrynic acid. *Cancer Res* **51:** 94–98, 1991.
12. O'Dwyer PJ, LaCreta F, Nash S, Tinsley PW, Schilder R, Clapper ML, Tew KD, Panting L, Litwin S, Comis RL and Ozols RF, Phase I study of thiotepa in combination with the glutathione transferase inhibitor ethacrynic acid. *Cancer Res* **51:** 6059–6065, 1991.
13. Petrini M, Conte A, Caracciolo F, Sabbatini A, Grassi B and Ronca G, Reversing of chlorambucil resistance by ethacrynic acid in a B-CLL patient. *Br J Haematol* **85:** 409–411, 1993.
14. Kunze T, Phosphono analogues of glutathione as new inhibitors of glutathione *S*-transferases. *Arch Pharm (Weinheim)* **329:** 503–509, 1996.
15. Kunze T, Purification and characterization of class alpha and mu glutathione *S*-transferases from porcine liver. *Comp Biochem Physiol B Biochem Mol Biol* **116:** 397–406, 1997.
16. Dirr HW, Mann K, Huber R, Ladenstein R and Reinemer P, Class π glutathione *S*-transferase from pig lung. *Eur J Biochem* **196:** 693–698, 1991.

17. Anderson ME, Powrie F, Puri RN and Meister A, Glutathione monoethyl ester: Preparation, uptake by tissues, and conversion to glutathione. *Arch Biochem Biophys* **239:** 538–548, 1985.
18. Dietel M, Arps H, Lage H and Niendorf A, Membrane vesicle formation due to acquired mitoxantrone resistance in human gastric carcinoma cell line EPG85–257. *Cancer Res* **50:** 6100–6106, 1990.
19. Habig WH, Pabst MJ and Jakoby WB, Glutathione *S*-transferases. The first enzymatic step in mercapturic acid formation. *J Biol Chem* **249:** 7130–7139, 1974.
20. Szasz G, γ-Glutamyltranspeptidase. In: *Methoden der Enzymatischen Analyse* (Ed. Bergmeyer HU), pp. 757–762. Verlag Chemie, Weinheim, 1974.
21. Tahir MK and Mannervik B, Simple inhibition studies for distinction between homodimeric and heterodimeric isoenzymes of glutathione transferase. *J Biol Chem* **261:** 1048–1051, 1986.
22. Drnevich D and Vary TC, Analysis of physiological amino acids using dabsyl derivatization and reversed-phase liquid chromatography. *J Chromatogr* **613:** 137–144, 1993.
23. Leibovitz A, The growth and maintenance of tissue-cell cultures in free gas exchange with the atmosphere. *Amer J Hyg* **78:** 173–180, 1963.
24. Meyer DJ, Significance of an unusually low K_m for glutathione in glutathione transferases of the α, μ and π classes. *Xenobiotica* **23:** 823–834, 1993.
25. Flatgaard JE, Bauer KE and Kauvar LM, Isoenzyme specificity of novel glutathione *S*-transferase inhibitors. *Cancer Chemother Pharmacol* **33:** 63–70, 1993.
26. Cameron AD, Sinning I, L'Hermite G, Olin B, Board PG, Mannervik B and Jones TA, Structural analysis of human alpha-class glutathione transferase A1–1 in the apo-form and in complexes with ethacrynic acid and its glutathione conjugate. *Structure* **3:** 717–727, 1995.
27. Tate SS and Meister A, Interaction of gamma-glutamyl transpeptidase with amino acids, dipeptides, and derivatives and analogs of glutathione. *J Biol Chem* **249:** 7593–7602, 1974.
28. Hao XY, Widersten M, Ridderström M, Hellman U and Mannervik B, Co-variation of glutathione transferase expression and cytostatic drug resistance in HeLa cells: Establishment of class Mu glutathione transferase M3–3 as the dominating isoenzyme. *Biochem J* **297:** 59–67, 1994.
29. Mannervik B, Awasthi YC, Board PG, Hayes JD, Di Ilio C, Ketterer B, Listowsky I, Morgenstern R, Muramatsu M, Pearson WR, Pickett CB, Sato K, Widersten M and Wolf CR, Nomenclature for human glutathione transferases. *Biochem J* **282:** 305–306, 1992.
30. Dirr H, Reinemer P and Huber R, Refined crystal structure of porcine class Pi glutathione *S*-transferase (pGST P1–1) at 2.1 Å resolution. *J Mol Biol* **243:** 72–92, 1994.