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Interactions of glutathione *S*-transferase-π with ethacrynic acid and its glutathione conjugate

Sanjay Awasthi [a], Sanjay K. Srivastava [b], Faiyaz Ahmad [a], Hassan Ahmad [b] and G.A.S. Ansari [b,c]

[a] *Division of Hematology and Oncology, University of Texas Medical Branch, Galveston, TX (USA),* [b] *Department of Human Biological Chemistry and Genetics, University of Texas Medical Branch, Galveston, TX (USA) and* [c] *Department of Pathology, University of Texas Medical Branch, Galveston, TX (USA)*

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Ethacrynic acid, a diuretic drug known to be an inhibitor of glutathione *S*-transferases (GSTs), has been shown to enhance the cytotoxicity of the alkylating agent class of chemotherapeutic drugs in cultured cancer cells resistant to alkylating agents. This action of ethacrynic acid is presumably mediated by inhibition of GSTs which are implicated in detoxification of alkylating agents. In addition to being an inhibitor of GSTs, ethacrynic acid also interacts with GSTs as a substrate for conjugation with GSH to yield an ethacrynic acid-GSH conjugate. This conjugate is formed both enzymatically and non-enzymatically and itself is a GST inhibitor. Since ethacrynic acid-GSH conjugate is itself likely to be able to mediate reversal of alkylating agents through GST inhibition, we have synthesized and purified the ethacrynic acid-GSH conjugate, studied the kinetics of inhibition of human lung π-class GST by ethacrynic acid and the conjugate, and compared the kinetics of the enzymatic and non-enzymatic formation of the conjugate using an HPLC method. Results of our studies showed that the ethacrynic acid-GSH conjugate was a more potent inhibitor of human lung GST-π than ethacrynic acid (K_i = 1.5 vs. 11.5 μM, respectively) and that their mechanisms for GST inhibition were distinct (competitive and non-competitive, respectively). Comparison of enzymatic and non-enzymatic rates of conjugate formation in vitro indicated that GST-π catalyzed a rapid conjugation of ethacrynic acid with GSH at a concentration of ethacrynic acid an order of magnitude above that required to nearly completely inhibit GST catalyzed conjugation of 1-chloro-2,4-dinitrobenzene. However, because of the rapid non-enzymatic reaction, and the inhibition of GST-π with the accumulation of the conjugate in the reaction mixture, the overall quantity of the conjugate formed after 150 min was nearly identical in the presence or absence of GST-π. Results of these studies suggest that inhibition of GSTs by ethacrynic acid-GSH conjugate may be the main mechanism through which ethacrynic acid reverses alkylating agent resistance.

Introduction

Ethacrynic acid ((2,3-dichloro-4-(2-methylenebutyryl)phenoxy)acetic acid), a sulfhydryl-reactive loop diuretic [1,2], has been demonstrated to enhance the cytotoxicity of the alkylating agent class of chemotherapeutic drugs towards cultured cancer cells otherwise resistant to alkylating agents [3–5]. This activity of ethacrynic acid (EA) has been suggested to be due to its inhibitory effect [3,6,7] on glutathione *S*-transferases (EC 2.5.1.18, GSTs) which have been implicated in alkylating agent resistance of cancer cells [8–13]. The mechanisms through which EA inhibits GSTs are complicated [14] and incompletely understood. EA interacts with GST as a substrate [15,16], as a non-substrate ligand [17], and the conjugate (EA-SG) of EA and glutathione (GSH) which can be formed either spontaneously [18] or through a GST-catalyzed reaction [19], inhibits GSTs [7]. Detailed studies to elucidate the interactions of EA with GSTs are therefore necessary not only for a complete understanding of the mechanisms through which EA enhances alkylating-agent cytotoxicity but also to understand as yet incompletely explained observations such as the failure of EA to enhance alkylating agent cytotoxicity in some alkylating-agent-resistant cells [5,20] and the unpredictable effects on enhancement of cytotoxicity with variations in timing and duration of exposure of EA and alkylating agents [5]. Since EA-SG is the major metabolite of EA and because it may itself enhance alkylating-agent cytotoxicity by inhibiting GSTs, variable time-depen-

Correspondence to: S. Awasthi, Division of Hematology and Oncology, 4.164 Old John Sealy, Route E-65, University of Texas Medical Branch Galveston, TX 77555-0565, USA.

dence between EA exposure and enhancement of alkylating-agent toxicity may be related in part to differences in intracellular accumulation of EA-SG. Therefore, in order to determine the importance of EA-SG formation in the enhancement of alkylating-agent cytotoxicity by EA, we have synthesized and purified EA-SG and after establishing its authenticity have developed an HPLC method to quantify this compound. Using this method, we have compared the rates of non-enzymatic and GST-π-catalyzed reaction between GSH and EA by direct determination of EA-SG. In addition, we have studied the kinetics of inhibition of human lung GST-π by EA and EA-SG. The results of these studies indicated that spectrophotometric measurements [15] significantly underestimated the enzyme-catalyzed formation of EA-SG and that GST-π was able to accelerate the initial reaction between EA and GSH. However, with the accumulation of EA-SG, the GST activity was soon abrogated. Inhibition studies showed that EA-SG was a relatively more potent inhibitor of GST-π than EA, and the mechanisms of GST inhibition by EA-SG and EA were distinct. In light of these results, the possible role of EA-SG in mediating the EA-associated reversal of alkylating agent resistance is discussed.

Materials and Methods

Reagents. EA, GSH, 1-chloro-2,4-dinitrobenzene (CDNB), 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), and other reagent were purchased from Sigma (St. Louis, MO, USA). Solvents for HPLC were purchased from Pierce (Rockford, IL, USA). Antibodies against the α, μ and π class GSTs were same as those used in previous studies [21].

Synthesis and purification of EA-SG. Freshly prepared solutions of EA (50 mg/ml in absolute ethanol) and GSH (50 mg/ml in 10 mM potassium phosphate buffer (pH 7.0)) were mixed in 1:1 ratio and allowed to incubate at room temperature for 2 h. The unreacted GSH, and EA were separated from the conjugate (EA-SG) by preparative TLC on silica gel G plates developed in acetonitrile/water (7:2 (v/v)). Further purification of the conjugate was achieved by HPLC using a reverse-phase C_{18} column (25 × 0.46 cm I.D., pore size 5 μ) in a Beckman HPLC system. The mobile phase flow rate was 1.5 ml/min and it consisted of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B). A step-wise gradient of solvent B from 0% to 20% over the first 10 min, followed by 20% to 70% over the next 20 min was used for elution. Absorbance of the effluent was monitored at 230 and 270 nm. The conjugate eluted at 19.0 min was collected and lyophilized to give a white powder which was reinjected to HPLC to check the purity.

Spectral analysis of EA-SG. Fast atom bombardment (FAB) mass spectra of the conjugate were obtained on a Kratos MS50RF mass spectrometer fitted with an Iontech saddle-field ion gun operated at 6–8 kV with xenon as the FAB gas. GSH, EA and the HPLC purified conjugate were dissolved separately in ethanol and the FAB matrix (glycerol/oxalate, 40 mM oxalic acid in 90% glycerol) before subjecting them to FAB mass spectrometry. The data were collected and analyzed on the Kratos Mach III data system. The absorbance spectra of EA and conjugate between 200 to 400 nm was determined using a Gilford Response spectrophotometer.

Inhibition of GST with EA and EA-SG. GST-π was purified from human lung using our previously described protocol [22]. GST activity using CDNB as the electrophilic substrate was determined as previously described [15]. For determination of the concentration of inhibitor at which 50% enzyme activity was retained (I_{50}), the reaction mixture was prepared containing 830 μl potassium phosphate buffer (100 mM (pH 6.5)), 20 μl purified human lung GST-π (4 U/ml), and 25 μl EA (0.4 to 200 μM) or EA-SG (0.4 to 40 μM) in absolute ethanol and incubated at room temperature for 5 min. The CDNB conjugation reaction was initiated by addition of 25 μl CDNB (40 mM in absolute ethanol) and 100 μl GSH (10 mM). The blanks contained all ingredients except for GST. Percent of control activity for each concentration of inhibitor was plotted against the inhibitor concentration and I_{50} was determined from the plot. In order to determine type of inhibition and for determination of K_i, concentrations of CDNB and GSH were varied between 0.2 and 1.0 mM.

Quantifying EA-SG by HPLC. An HPLC separation method was developed to give baseline separation of EA-SG from GSH and EA using a Pharmacia PepRPC-C_{18} reverse-phase column (10 × 1 cm I.D., pore size 15 μ) in the Pharmacia FPLC® system. The mobile phase consisted of solvent A and B as described above. A solvent gradient from 0% B to 100% B was developed over 18 ml. Flow rate was kept constant at 3 ml per min. Before each separation, the column was washed sequentially with 5 ml 100% solvent B and then re-equilibrated with 10 ml of 100% solvent A. Absorption of the column eluate was monitored at 230 and 270 nm using an LKB-Bromma 2141 variable wavelength and recorded on chart paper using a Pharmacia chart recorder at 0.5 cm/ml. Varying amounts of the authentic EA-SG ranging from 0.1 μM to 200 μM were injected into the FPLC column in a constant volume of 100 μl, and concentration of EA-SG was plotted against the area under the curve for the EA-SG peak in order to generate a standard curve of EA-SG.

Comparison of enzymatic and non-enzymatic rates of EA-SG formation. For comparison of the enzymatic

and non-enzymatic rate of reaction between EA and GSH, a 950-μl reaction mixture was prepared by addition of 750 μl potassium phosphate buffer 100 mM (pH 6.5), 100 μl GSH 2.5 mM, and 100 μl potassium phosphate buffer 10 mM (pH 7.0) with or without human GST-π. 1 U of enzymatic activity was defined as consumption of substrate at 1 μmol/min at 25°C. The final concentration of GST-π was 22 μg/ml which had specific activity of 18.2 U/mg protein towards CDNB and 4.8 U/mg protein towards EA by Habig's method [15]. The conjugation reaction was initiated by addition of 50 μl freshly prepared solution of 4 mM EA in absolute ethanol (final concentration 200 μM). 100 μl of this reaction mixture was injected into the HPLC column after incubation intervals ranging from 1 to 150 min. EA-SG formed in the reaction mixture was quantified using the standard curve of area under the EA-SG peak vs. nmol of EA-SG in the reaction mixture. Non-enzymatic and enzymatic rates of EA-SG formation were also measured at pH 6.0 and 7.4.

Results

Synthesis and characterization of EA-SG

From the reaction mixture, the reactants (EA and GSH) and product (EA-SG) could be clearly separated by TLC. EA moved close to the solvent front ($R_f = 0.95$) while GSH stayed near the origin ($R_f = 0.15$). A ninhydrin positive spot ($R_f = 0.30$) distinct from GSH was also observed on TLC and was identified as EA-SG as described below. HPLC analysis of the reaction mixture revealed the presence of two peaks when monitored at 270 nm, one at retention time (R_t) 26.0 min, corresponding to authentic EA and another at R_t 19.0 min. The peak at R_t 19.0 min was collected and when reinjected into the column gave a single symmetrical peak at R_t 19.0 min and it could not be further resolved into additional peaks when several different solvents and elution programs were used (data not shown).

The structure of the compound with R_t 19.0 min from HPLC was confirmed by FAB-mass spectrometry. The adducted molecular ions at m/z 632/634/636 $[M + Na^+]$ and 610/612/614 $[M + H^+]$ were consistent with the proposed structure of EA-SG. Both adducted molecular ions showed a two chlorine pattern (10:6:1). The ion m/z 576/578 showed a one chlorine pattern (3:1) which resulted through loss of HCl from the protonated molecular ion (data not shown). These results indicated the conjugate to be a product of a Michael addition of the sulfhydryl group of GSH with β carbon of the α,β-unsaturated ketone moiety of EA. Visible and UV absorption spectra in the region of 200 to 400 nm of 100 μM EA and EA-SG were compared. An absorption peak of EA-SG was seen at approx. 270 nm with an extinction coefficient at 270 nm (ϵ_{270}) of 5.7 mM^{-1} cm^{-1}, similar to that previously reported [15]. The ϵ_{270} of EA was found to be 3.2 mM^{-1} cm^{-1}.

Inhibition of GST-π with EA and EA-SG

GST-π was chosen for these studies because it has been reported to be the least sensitive of the three major classes of GSTs to inhibition by EA [19]. Apparently homogenous GST-π from human lung was obtained following the protocol described before [22]. This preparation showed a single protein band on SDS-PAGE gels. In Western blot analysis against antibodies specific for human α, μ, and π-class GSTs, this band recognized only the antibodies against human GST-π (data not shown), indicating that the GST-π preparation used in these studies was free of other classes of GSTs present in the human lung. At the time-inhibition experiments were performed, the specific activities of this enzyme as determined by the method of Habig et al. were 18.1 and 4.8 μmol/min per mg protein towards CDNB and EA, respectively. The human lung GST-π was incubated with EA or EA-SG at room temperature for periods ranging from 1 to 10 min and its activity toward CDNB was measured as described by Habig et al. [15]. These studies indicated that maximal degree of inhibition was reached within 1 min of incubation of the enzyme with the inhibitor. All subsequent inhibition studies were therefore performed by incubating the enzyme with the inhibitor in absence of substrates for a convenient time of 5 min prior to starting the reaction by adding both substrates. Comparison of the inhibitory effect of EA and EA-SG on the activity of GST-π towards CDNB

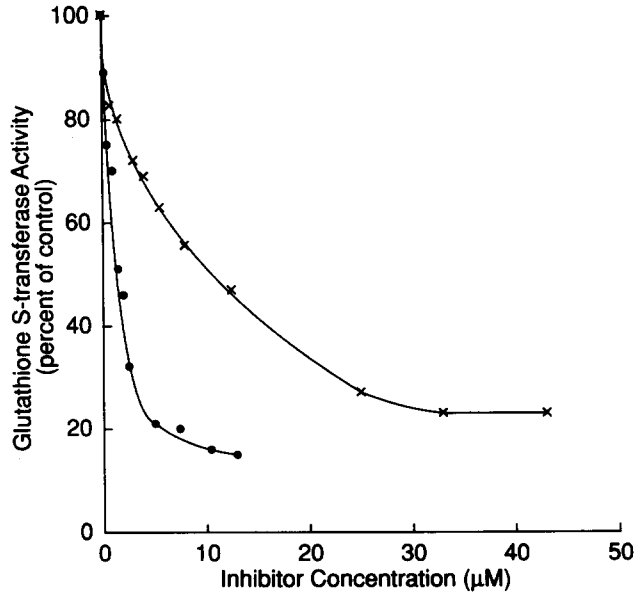

Fig. 1. Percent inhibition of human lung GST-π by ethacrynic acid-glutathione conjugate (•) compared to ethacrynic acid (×). Details are presented in the text.

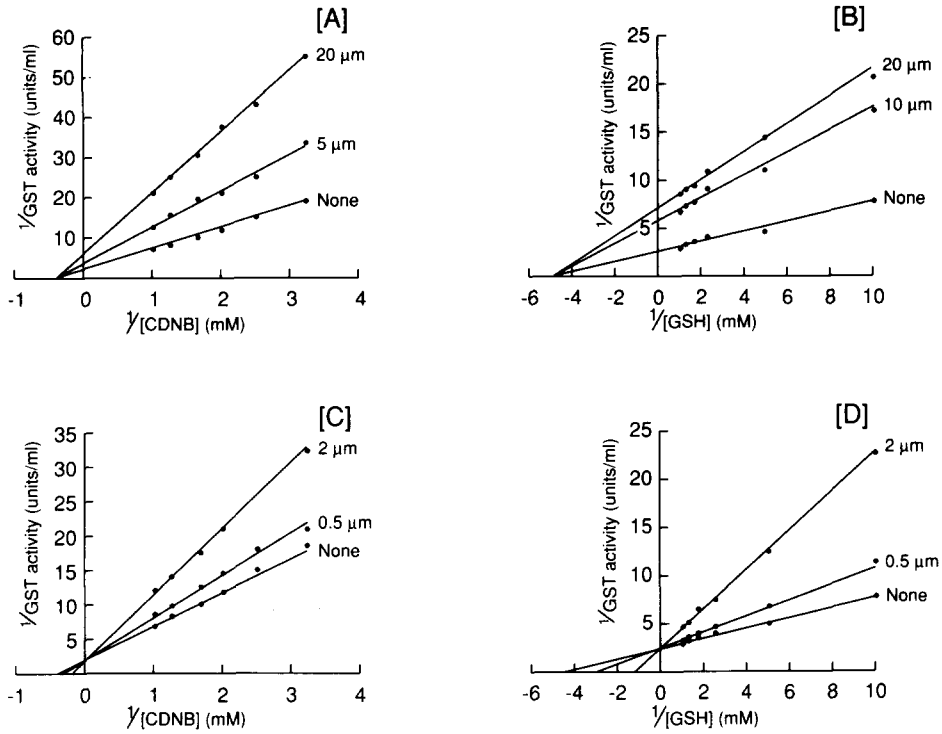

Fig. 2. Lineweaver-Burk plots of the kinetics of inhibition of human lung GST-π by ethacrynic acid with respect to CDNB (A) and GSH (B) and of inhibition by the ethacrynic acid-glutathione conjugate with respect to CDNB (C) and GSH (D). Details are presented in the text.

(Fig. 1) revealed that the I_{50} of EA was approx. 7-fold higher than that of EA-SG. Kinetic studies were performed using three concentrations of either EA or EA-SG and varying concentrations of GSH and CDNB (Fig. 2). In contrast to EA, which was a non-competitive inhibitor, EA-SG was found to be a competitive inhibitor of GST-π with respect to both GSH and CDNB. Inhibition constants of EA and EA-SG, 11.5 and 1.5 μM, respectively, further confirmed the results of I_{50} values of EA and EA-SG showing that as compared to EA, EA-SG was a 7-fold more potent inhibitor of CDNB conjugation by GST-π.

Comparison of enzymatic and non-enzymatic rates of EA-SG formation

For quantifying EA-SG, a baseline separation of EA-SG from GSH and EA was achieved by HPLC on the Pharmacia FPLC® system (Fig. 3). Varying amounts of purified EA-SG injected in the HPLC column yielded a linear calibration curve which passed through the origin (data not shown). The detection limit of EA-SG was found to be approx. 0.2 nmol. The time-course of EA-SG formation at pH 6.5 in absence or presence of GST-π is shown in Fig 4. The initial rate of EA-SG formation was accelerated by about 2.8-fold (10.0 vs. 3.6 nmol/min per ml reaction mixture) in the presence of GST-π. The specific activity of GST-π towards EA as determined by the HPLC method was 7.7 U/mg protein, approx. 1.6-fold higher than 4.8 U/mg protein determined spectrophotometrically us-

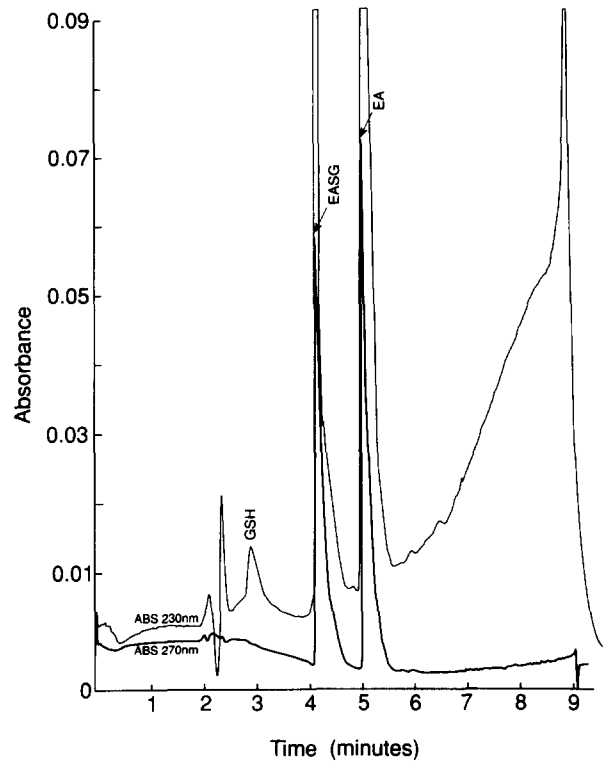

Fig. 3. Absorption tracing at 230 (———) and 270 (——) nm during HPLC on the Pharmacia FPLC® system of the reaction mixture containing glutathione (250 μM), ethacrynic acid (200 μM) and GST-π (0.4 U) after 15 min incubation at room temperature shows clear separation of glutathione (R_t 2.4 min) from ethacrynic acid (R_t 5.4 min) and the ethacrynic acid-glutathione conjugate (R_t 4.3 min).

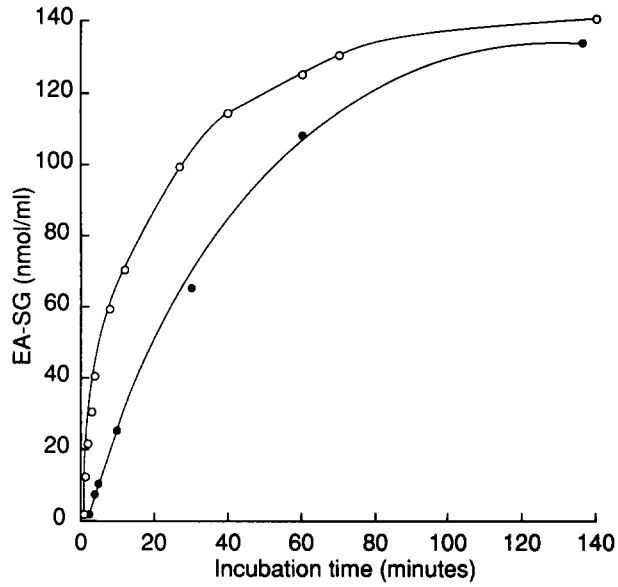

Fig. 4. Formation of the ethacrynic acid-glutathione conjugate in a reaction mixture described in the methods section with (○) or without (●) 0.5 U purified human lung GST-π/ml.

ing 5.7 mM^{-1} cm^{-1} as the extinction coefficient for calculation. The rate of EA-SG formation in the reaction mixture containing enzyme gradually decreased, so that after about 10 min the reaction rates with or without enzyme were comparable. After 150 min there was only a small difference in the total amount of EA-SG formed in the reaction mixtures with or without GST-π. The nonenzymatic rate at pH 7.4 was about 2.5-fold higher than at pH 6.5. Whereas GST enhanced the non-enzymatic rate of EA-SG formation 2.8-fold at pH 6.5, the rate was increased only 1.1-fold at pH 7.4 (data not shown).

Discussion

Present studies indicate that the reaction between EA and GSH resulted in formation of a GSH-adduct of EA as the principal reaction product. Mass spectrometric data indicated that this adduct (EA-SG) was a product of a thermodynamically favorable Michael addition reaction resulting in addition of GSH at the β-carbon of the α,β-unsaturated ketone moiety of EA. We noted a relatively small (less than two-fold) difference in the ϵ_{270} between EA and EA-SG. This implied that calculating enzymatic activity using the ϵ_{270} of EA [15] rather than difference between ϵ_{270} of EA and EA-SG may result in an underestimation of the enzyme-catalyzed rate of EA-SG formation. Indeed, our results show that when enzymatic reaction is quantified by directly measuring the formation of EA-SG, the enzymatic rate is about 1.6-fold higher than that determined by the spectrophotometric method [15].

The results of present studies show that EA is conjugated with GSH rapidly even without an enzymatic catalyst. This non-enzymatic reaction is accelerated at more alkaline pH. This is consistent with previous studies [15] demonstrating an increased non-enzymatic rate of electrophile-GSH conjugation at higher pH. The rate of EA-SG formation at pH 6.5 was accelerated by GST-π even in presence of 200 μM EA, a concentration of EA sufficient to nearly completely inhibit GST-π-catalyzed conjugation of CDNB with GSH. Although GST-π accelerated formation of EA-SG, it was a relatively weak catalyst for this reaction at pH 6.5, and even weaker at physiologic pH. Furthermore, the catalytic activity of GST-π for EA-GSH conjugation was rapidly abrogated under present conditions in less than 10 min and the total amount of EA-SG formed after 150 min was relatively independent of presence of GST-π. Results of studies on the inhibition of GST-π by EA-SG indicated that sufficient concentration of EA-SG may accumulate in the reaction mixture to result in the observed time dependent inhibition. GST-π was able to catalyze conjugation of EA with GSH at a concentration of EA sufficient to nearly completely inhibit its activity towards CDNB. The reason for this differential inhibition of CDNB conjugation compared to EA conjugation may be that relatively less GST activity was required to catalyze the more thermodynamically favorable Michael addition reaction of GSH with EA as compared to the less favorable addition-substitution reaction of CDNB with GSH. Alternatively, these results may imply a separate active-site for GST-catalyzed conjugation of GSH to α,β-unsaturated ketones than that is required for CDNB. Differential inhibition of GST-π also suggests that detoxification of all electrophilic compounds by GST-π is not similarly affected by EA or EA-SG.

The results of our studies comparing the kinetics of inhibition of human lung GST-π indicated not only that EA-SG was a more potent inhibitor than EA, but also that the mechanisms of inhibition for these compounds were distinct. We found that EA was a non-competitive inhibitor of purified human lung GST-π with respect to both CDNB and GSH. Our findings differ from a previous report by Ploemen et al. [7] who found that inhibition of the rat α-class GST isozyme (1-1) by EA was non-competitive with respect to GSH but competitive with respect to CDNB. This apparent anomaly could be due to the differences in the nature of human GST-π and rat α-class GST (1-1). It should be pointed out that our results also differ from those of another study [14] which reported mixed type of inhibition of GSTs by EA [14]. It is possible that in those studies [14], pre-incubation of EA and GSH in the reaction mixture prior to addition of GST resulted in formation of significant quantities of EA-SG, leading to mixed inhibitory effects of EA and EA-SG. During

the present studies, we have avoided significant formation of EA-SG in inhibition studies of GST-π by incubating EA with the enzyme in absence of GSH prior to starting the reaction by addition of GSH and CDNB. In contrast to the non-competitive inhibition of GST-π by EA, the purified EA-SG was a competitive inhibitor. More potent inhibition of GST by the product (EA-SG) of GSH-electrophile conjugation reaction as seen during the present study is consistent with other studies which show that the GSH-electrophile conjugates were more potent inhibitor of GST than the respective parent compounds [24–27]. We are not able to explain the results of previous studies [7] showing EA to be a more potent inhibitor of human placental GST-π as compared to EA-SG. It can be speculated that this may be due to spontaneous formation of EA-SG or different sources of the enzymes.

Results of our studies show that at physiologic pH EA is rapidly converted into EA-SG which is a significantly more potent inhibitor of GST-π than EA and the non-enzymatic formation of EA-SG is likely to continue unabated even after inhibition of GST-π activity towards EA. Inhibition of GSTs by EA-SG accompanied by depletion of GSH may therefore, play a significant role in EA-mediated enhancement of alkylating agent cytotoxicity. Detailed in-vitro and in-vivo studies into the kinetics of EA-SG accumulation and disposition may be helpful in predicting the duration of susceptibility towards alkylating agents in malignant and normal tissues.

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