

ABSTRACT
Elimination of hydrogen sulfide from glutathione (GSH) converts a well-known cellular nucleophile to an electrophilic species, γ-glutamyldehydroalanine-glycine (EdAG). We have found that a sulfonium metabolite formed from GSH and busulphan undergoes a facile β-elimination reaction to give EdAG, which is an α,β-unsaturated dehydroalanine analog of GSH. EdAG was identified as a metabolite of busulphan in a human liver cytosol fraction. EdAG condenses with GSH in a Michael addition reaction to produce a lanthionine thioether [(2-amino-5-[3-[2-[[4-amino-5-hydroxy-5-oxopentanoyl]amino]-3-(carboxymethylamino)-3-oxopropyl]sulfanyl-1-(carboxymethylamino)-1-oxopentanoyl]amino]-3-(carboxymethylamino)-3-oxopropyl]sulfanyl-1-(carboxymethylamino)-1-oxopentanoic acid). LC, liquid chromatography; HVOD, hepatic venoocclusive disease.

Glutathione (GSH) is the most prevalent low molecular weight cellular thiol in mammalian cells. In addition to having a major cellular role in oxidative status, there is an important function of GSH in sequestering toxic, electrophilic xenobiotics (Boyland and Chasseaud, 1969; Chasseaud, 1976). Nucleophilic reactivity of GSH is based on conjugation of its thiol group with electrophilic compounds (Commandeur et al., 1995). β-Elimination of hydrogen sulfide from a cysteine residue forms a dehydroalanine residue. Dehydroalanine-containing peptides react as Michael acceptors that link cysteine residues with dehydroalanine groups to generate nonreducible thiocarbamates (Friedman et al., 1977; Dufour et al., 2007; Willey and van der Donk, 2007).

The dehydroalanine analog of GSH, γ-glutamyldehydroalanine-glycine (EdAG) is shown in Fig. 1. To accomplish the β-elimination reaction on GSH, strongly basic conditions are needed to dissociate the relatively nonacidic α-proton that has a pK\text{a} projected to be above 21 (Murkin and Tanner, 2002). Lysis of glutathione disulfide with strong alkali produces EdAG as one of the identified products (Jones et al., 2006). For example, EdAG can be readily synthesized from a dinitrophenyl derivative of GSH (Asquith and Carthew, 1972). More recently, Munter et al. (2003) identified EdAG as a base-catalyzed product formed from a chloroprene-GSH adduct.

Conjugation with GSH, forming an unstable S-glutathione sulfonium conjugate [γ-glutamyl-β-(S-tetrahydrothiophenyl)alanylglycine; GS-THT] (Fig. 1), is the major elimination

ABBREVIATIONS: GSH, glutathione; EdAG, γ-glutamyldehydroalanine-glycine; GS-THT, γ-glutamyl-β-(S-tetrahydrothiophenyl)alanylglycine; GST, glutathione S-transferase; DMSO, dimethyl sulfoxide; MS, mass spectrometry; MS/MS, tandem mass spectrometry; GSG, (2-amino-5-[[3-[[2-[[4-amino-5-hydroxy-5-oxopentanoyl]amino]-3-(carboxymethylamino)-3-oxopropyl]sulfanyl-1-(carboxymethylamino)-1-oxopropan-2-yl]amino]-5-oxopentanonic acid; LC, liquid chromatography; HVOD, hepatic venoocclusive disease.
pathway of the anticancer drug busulfan (Roberts and Warwick, 1961; Czerwinski et al., 1996; Ritter et al., 1999). Tetrahydrothiophene is released from GS' THT under alkaline conditions (Roberts and Warwick, 1961) or through involvement of the mercapturic acid pathway and β-lyases (Onkenhout et al., 1986; Marchand et al., 1988; Cooper et al., 2008).

In this study, we report that the other product formed in the β-elimination reaction of GS' THT is EdAG, the dehydroalanine analog of GSH. We have shown that EdAG reacts as an electrophile in the formation of nonreducible thioether adducts with sulfhydryl-containing compounds, and it binds to the glutathione S-transferase (GST) isoform responsible for GS' THT formation.

Materials and Methods

Busulfan, l-cysteine, GSH, 1-bromo-4-chlorobutane, deuterium oxide, formic acid, butanol, Dowex-50W, dimethyl sulfoxide (DMSO), d6-DMSO, ammonium hydroxide, 1-chloro-2,4-dinitrobenzene, and GST-Agarose were obtained from Sigma-Aldrich (St. Louis, MO). Acetic acid, sodium bicarbonate, dibasic potassium phosphate, monobasic potassium phosphate, sodium hydroxide, hydrochloric acid, acetonitrile (optima), methanol (high-performance liquid chromatography grade), sodium chloride, acetone, Tris, Tween 20, and 2-mercaptoethanol were purchased from Thermo Fisher Scientific (Waltham, MA). Pooled human liver cytosol was obtained from BD Gentest (Woburn, MA). Trichloroacetic acid was obtained from Sigma-Aldrich. Human GSTA1-1 was obtained from Invitrogen (Carlsbad, CA). Electrophoresis reagents polyacrylamide, SDS, ammonium persulfate, bromphenol blue, and TEMED were obtained from Bio-Rad (Hercules, CA). Coomassie Brilliant Blue 250 was obtained from Life Technologies. GS' THT was prepared by a modified method of Marchand et al., (1988). Purification was performed by semipreparative high-performance liquid chromatography on an Econosphere C8 semipreparative column (10 × 250 mm) (Alltech, Deerfield, IL) and a UV/visible detector set (DU640; Beckman Coulter, Somerset, NJ) at a wavelength of 210 nm. Isocratic elution was with 50:50 methanol/water (v/v) at a flow rate of 3.0 ml/min. Solvents were removed under vacuum on a rotary evaporator.

EdAG was prepared by the elimination of 2,4-dinitrothiophenolate from S-(2,4-dinitrophenyl)glutathione (Sokolovsky et al., 1964) as described by Asquith and Carteth (1972). Positive ionization electrospray MS: $m/z$ 274 (MH$^+$), MS/MS: $m/z$ 145 (MH$^+$ pyroglutamic acid); $^1$H NMR (600 MHz, D$_2$O) δ$_H$ 2.08 (2H, m), 2.48 (2H, m), 3.46 (1H, m), 3.93 (2H, s), 5.71 (1H, s), and 5.79 (1H, s) ppm.

The stability of GS' THT at pH 7.4 and 8.0 was determined by measuring rates of its disappearance. Stock solutions were made in distilled water (2 mg/ml) and further diluted in 100 mM potassium phosphate buffer (pH 7.4 or pH 8.0) to produce a 0.83 mM final concentration.
concentration of GS THT. Solutions were incubated in a shaking water bath at 37°C. Aliquots (6 μl) removed at 0, 0.25, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 5, and 6 h were diluted with 1.5 ml of distilled water, and 50-μl aliquots were injected into a Waters Alliance 2695 separation module equipped with a Waters 996 Photodiode Array Detector and coupled to a Waters Micromass ZMD mass spectrometer with electrospray ionization (Waters, Milford, MA). The photodiode array detector was programmed to scan between 200 and 300 nm, and a mass spectrometer was programmed to use electrospray ionization in a positive-ion mode with selected ion recording of the m/z 362 ion. The mobile phase consisted of methanol/water (50:50, v/v) pumped at 0.4 ml/min through an Agilent Zorbax SB-NC C18, 5 μm reversed-phase column (Agilent Technologies, Santa Clara, CA).

Identification of EdAG after Incubation of Busulfan with Human Liver Cytosol. Busulfan (0–5 mM) was incubated with human liver cytosol (total protein, 1.0 mg) in the presence of 1.0 mM GSH in 100 mM Tris (Trizma) buffer (pH 7.4, total volume 1.0 ml). Busulfan was dissolved in DMSO, and the total DMSO concentration was less than 1.0% v/v. The mixture was preincubated for 5 min in a shaking water bath maintained at 37°C, and reactions were started by the addition of busulfan. The formation of GS THT, EdAG, and a nonreducible lanthionine thioether, GSG, were monitored by liquid chromatography (LC-MS) using a Thermo Hypersil column (150 × 2.1 mm, 5 μm; Thermo Fisher Scientific). A gradient of mobile phase A (0.5% formic acid in 90/10, v/v, water/acetonitrile) and mobile phase B (0.1% formic acid in 50/50, v/v, isopropanol/methanol) at 0.2 ml/min was run 0 to 5 min, 100% A; 5 to 7 min, 0–50% B; 7 to 9 min, 50–100% B; and 9 to 15 min, 100% A. A Finnigan LCQ DECA mass spectrometer (Thermo Fisher Scientific) was operated in the positive-ion electrospray ionization mode with full scan mode monitoring m/z ions. The mixture was mixed with a solution containing 0.1% w/v Coomassie Brilliant Blue, 46% v/v methanol, and 7.5% w/v acetic acid and destained with a solution containing 5% w/v methanol and 7.5% w/v acetic acid in water.

Cytotoxicity of EdAG In Vitro. C6 rat glioma cells obtained from the American Type Culture Collection (Manassas, VA) were maintained in Dulbecco’s modified Eagle’s medium-F12 (1:1) (Invitrogen) supplemented with 1% antibiotic-antimycotic mixture (Invitrogen) and 10% fetal calf serum (Sigma-Aldrich) at 37°C under an atmosphere of 5% CO2-95% air. For toxicity experiments, the cells were seeded into 12-well Primaria cluster dishes (Becton Dickinson Labware, San Jose, CA) at a density of 5 × 104 cells/well and incubated in 0.5 ml of Dulbecco’s modified Eagle’s medium-F12/fetal calf serum overnight. Subsequently, the cells were exposed to busulfan and EdAG for 24 h. Busulfan was added in DMSO (final concentration in the culture medium of DMSO was <1%), and EdAG was added as an aqueous solution. Sister cultures incubated without any additions or with DMSO served as controls. Cell viability was assessed by the trypan blue exclusion test. In brief, the medium was removed and the adhering cells were trypsinized and stained with trypan blue. Viable, dye excluding, cells were counted in a hemocytometer.

Results

Conversion of Busulfan to EdAG. Busulfan was incubated with human liver cytosol in 100 mM Tris buffer, pH 7.4, at 37°C in the presence of GSH. EdAG was identified as a metabolite of busulfan (Fig. 2A) based on LC/MS data. EdAG was trapped as a glutathione conjugate, GSG (Fig. 2B). Figure 1 shows the proposed pathway of EdAG and GSG formation from busulfan, GSH, and GSTs. The proposed intermediate in the conversion of busulfan to EdAG, sulfonium GS THT, was detected in the incubation mixture by LC/MS (Cooper et al., 2008). GS THT was proposed to be an intermediate in the reaction because incubation of GS THT in 100 mM potassium phosphate buffer (pH 7.4) for 6 h at 37°C resulted in a significant loss of GS THT. These findings are consistent with the report by Roberts and Warwick (1961), showing that GS THT is unstable at pH 7.4 based on the detection of tetrahydrothiophene as a degradation product. The most likely pathway to the formation of tetrahydrothiophene from GS THT is through a β-elimination reaction proposed in Fig. 1, showing the production of tetrahydrothiophene and EdAG. To confirm the identity of EdAG in the reaction, GS THT (57 mM) in deuterated 100 mM phosphate buffer (pD ~8.0) was incubated for 12 h at 37°C. The formation of EdAG from GS THT was detected by monitoring the vinylic proton signals of EdAG in the 1H NMR spectrum.
Fig. 2. Busulfan metabolism by human liver cytosolic fractions. MS/MS mass chromatograms of EdAG (A) and GSG (B) at 4-h incubation time. The proposed MS/MS fragmentation patterns for EdAG and GSG are shown in the insets. A, neutral losses from the EdAG MH⁺ ion (m/z 274) are consistent with loss of water (m/z 256), glycine (m/z 199), and pyroglutamic acid (m/z 145). B, neutral losses from the MH⁺ ion (m/z 581) are consistent with loss of water (m/z 563), loss of pyroglutamic acid (m/z 452), and loss of two molecules of pyroglutamic acid (m/z 323).
Reactivity of EdAG toward the Nucleophiles, Cysteine and GSH. EdAG contains an α,β-unsaturated carbonyl system that adds nucleophiles, such as sulfhydryl anions, to form a conjugate or adduct. Condensation of EdAG with cellular nucleophiles, GSH and cysteine, under physiological conditions yielded a glutathione analog with a lanthionine cross-linkage (GSG) and S-(β-alanyl)glutathione, respectively. Mass spectral evidence in support of the assigned structure of GSG is shown in Fig. 2B, which produced the same MS/MS spectrum as the GSG formed in the busulfan and human liver cytosol incubation. Because dehydroalanine is achiral, the addition of GSH or cysteine to EdAG forms a new chiral center, and there is the probability that GSG consists of a pair of diastereomers. The stereochemistry of synthesized GSG and S-(β-alanyl)glutathione was not determined.

Analysis of Binding of EdAG to GSTA1-1. An analysis of the capacity of EdAG to bind to GST was facilitated by the use of GSH-Agarose. As shown in Fig. 3, human GSTA1-1 bound to GSH-Agarose was eluted with 10 mM GSH (Fig. 3, lane 8) or 10 mM EdAG (Fig. 3, lane 9). The band at approximately 70 kDa in Fig. 3, lane 7, is most likely aggregated GSTA1.1. GST species present in pooled human liver cytosol and binding to GSH-Agarose were also eluted by 10 mM GSH (Fig. 3, lane 4) as well as by 10 mM EdAG (Fig. 3, lane 6). This observation confirms that GST can recognize EdAG, and it further establishes that EdAG can effectively compete with GSH for binding to GST.

The occurrence of a single band with $M_c \approx 24,000$ in Fig. 3, lanes 4 and 6, corroborates the absence of additional proteins in the liver cytosol capable of binding to GSH-Agarose that can be eluted with GSH or EdAG, respectively. Our method does not test for possible EdAG binding within the liver cytosol, although it is conceivable that EdAG can bind to the sulfhydryl moieties of other proteins, potentially irreversibly forming a mixed sulfide lanthionine linkage.

Fig. 3. Analysis of binding of EdAG GST. Experimental samples subjected to polyacrylamide gel electrophoresis as described under Materials and Methods. Molecular weight standards (lane 1). Human liver cytosol (lane 2), unbound material from liver cytosol after incubation with GSH-Agarose (lanes 3 and 5), liver protein eluted from GSH-Agarose with GSH (lane 4), and EdAG (lane 6). Purified human GSTA1-1 (lane 7), unbound material after incubation with GSH-Agarose (lane 10), and human GSTA1-1 eluted from GSH-Agarose with GSH (lane 8) and with EdAG (lane 9).

In Vitro Cytotoxicity of EdAG. The exposure of C6 cells to busulfan for 24 h profoundly decreases the number of viable cells. The concentrations of busulfan fall within the concentration ranges recently used by Lanvers-Kaminsky et al. (2006) who found a great variation in cellular susceptibility to this anticancer drug. For example, whereas growth of leukemia cell lines was inhibited by micromolar concentrations, millimolar concentrations were required to inhibit growth of osteosarcoma cell lines. We found the LD50 value for C6 cells to be approximately 460 μM (Fig. 4), indicating that these cells are modestly resistant to the toxic effects of busulfan. EdAG showed a similar cytotoxicity profile. However, twice as high concentrations were required to show the same level of toxicity exhibited by busulfan. The LD50 for EdAG was approximately 880 μM.

**Discussion**

There is extensive evidence that GSH is a cellular trapping agent of electrophilic compounds, such as alkylating anticancer agents. In the case of busulfan, conjugation with GSH leads initially to the formation of the conjugate GS−THT that is further converted by elimination of tetrahydrothiophene to form the dehydroalanine analog, EdAG. Whereas GS−THT formation is catalyzed in part by GST enzymes, it is not known whether the formation of EdAG from GS−THT is an enzyme-catalyzed process or occurs solely in a nonenzymatic β-elimination reaction. Nevertheless, under physiological conditions (pH 7.4 and 37°C), GS−THT spontaneously dissociates to tetrahydrothiophene and EdAG. This instability of GS−THT leads to a GSH analog in which the cysteine residue is converted to an electrophilic dehydroalanine moiety. Addition of a second molecule of GSH provides GSG, which is a glutathione adduct of EdAG.

The detection of both EdAG and GSG by LC-MS and LC-MS/MS in incubations of busulfan with human liver cytosol fortified with GSH suggests that EdAG is a metabolite of busulfan and that GSG is a conjugate of EdAG. The relevance of this busulfan metabolism pathway to medical practice has not been determined, although it is important to note that EdAG is weakly cytotoxic and can be categorized as a reac-
tive metabolite toward cellular nucleophiles. EdAG formed in liver-soluble fraction preparations is trapped by GSH. Addition of a sulfhydryl to the double bond of a dehydroalanine residue can occur readily forming a thioether or lanthionine linkage (Asquith and Carthew, 1972), and there are examples of lantibiotic antimicrobial agents that contain lanthionine residues (Chatterjee et al., 2005). Lantibiotics consist of cross-linked or cyclized peptides that are formed though Michael addition of a cysteine group to dehydroalanine residues. Lanthionine residues are associated with aging of the human lens and cataractogenesis (Linetsyk and LeGrand, 2005). These lanthionines include nonreducible mixed thioethers with glutathione. Lanthionine thioether linkages are not reducible in comparison with disulfide linkages, such as those found in reversible mixed-disulfide S-glutathionylation of proteins (Reddy et al., 2000). Dehydroalanine residues have been observed as artifacts in proteome analysis (Herbert et al., 2003), as common post-translational modifications in human serum albumin (Bar-Or et al., 2008), and after loss of selenium following oxidation of selenocysteine moieties from selenoproteins (Ma et al., 2003). Heat and alkaline treatment of foods results in the formation of dehydroalanyl residues and resulting cross-linked amino acids in proteins, as well as the epimerization of L-amino acid stereoisomers (Friedman, 1999). Electrophilic addition at the α,β-unsatuated moiety of dehydroalanine residues by a variety of nucleophiles is an entry to functionalized proteins (Snow et al., 1976; Friedman et al., 1977; Bernardes et al., 2008). Lanthionine bridges can also form in proteins during derivatization steps before mass spectral analysis, presumably through intramolecular condensation of a cysteine residue with a dehydroalanine (Amini and Nilsson, 2008).

The electrophilic reactivity of EdAG may be the cause of certain effects observed in busulfan therapy. Conditioning therapy with cytotoxic drugs, such as busulfan and cyclophosphamide, in patients undergoing bone marrow transplant is the most important factor leading to the development of hepatic veno-occlusive disease (HVOD) (Kalayoglu-Besisik et al., 2005). HVOD is caused by the destruction of sinusoidal endothelial cells and the surrounding centrilobular hepatocytes (Wadleigh et al., 2003). It was proposed that HVOD associated with conditioning regimens of busulfan in bone transplant patients is related to the metabolites of busulfan rather than to the parent compound (Srivastava et al., 2004). DeLeve and Wang (2000) suggested that this complication can be caused either directly through oxidative stress or indirectly through GSH depletion. We hypothesize that these conditions could be related to the nonenzymatic decomposition of GS\(^\text{S}\)THT to EdAG. Interestingly, it has recently been shown that busulfan treatment of mice resulted in up-regulation of glutathione synthesis and increased toxicity of busulfan (Bouligand et al., 2007). We suggest that higher GSH levels lead to higher concentrations of GS\(^\text{S}\)THT. An increase in GS\(^\text{S}\)THT would lead to increased nonenzymatic formation of EdAG, which, in turn, would lead to nonreducible glutathionylation of proteins associated with the circulatory system. Thus, increased nonreducible glutathionylation may contribute to busulfan-induced HVOD. Future studies are needed to explore the suggestion that GST catalyzes the formation of GSG by binding both GSH and EdAG.

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References


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