Journal of Medicinal Chemistry

Subscriber access provided by American Chemical Society

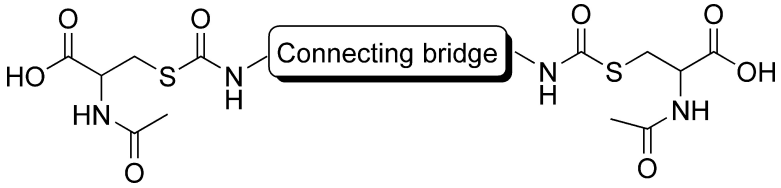
Article

2-Acetylamino-3-[4-(2-acetylamino-2-carboxyethylsulfanylcarbonylamino)-phenylcarbamoylsulfanyl]propionic Acid and Its Derivatives as a Novel Class of Glutathione Reductase Inhibitors

Teresa Seefeldt, Chandradhar Dwivedi, Greg Peitz, Jocqueline Herman, Laura Carlson, Zhiling Zhang, and Xiangming Guan

J. Med. Chem., **2005**, 48 (16), 5224-5231• DOI: 10.1021/jm050030i • Publication Date (Web): 15 July 2005

Downloaded from http://pubs.acs.org on March 28, 2009



More About This Article

Additional resources and features associated with this article are available within the HTML version:

- Supporting Information
- Access to high resolution figures
- Links to articles and content related to this article
- Copyright permission to reproduce figures and/or text from this article

View the Full Text HTML

ACS Publications
High quality. High impact.

Journal of Medicinal Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

2-Acetylamino-3-[4-(2-acetylamino-2-carboxyethylsulfanylcarbonylamino)-phenylcarbamoylsulfanyl]propionic Acid and Its Derivatives as a Novel Class of Glutathione Reductase Inhibitors

Teresa Seefeldt, Chandradhar Dwivedi, Greg Peitz, Jocqueline Herman, Laura Carlson, Zhiling Zhang, and Xiangming Guan*

Department of Pharmaceutical Sciences, College of Pharmacy, South Dakota State University, Brookings, South Dakota 57007

Received January 12, 2005

Glutathione reductase (GR) catalyzes the reduction of oxidized glutathione to reduced glutathione. The enzyme is an attractive target for the development of antimalarial agents, agents to decrease malarial drug resistance and anticancer agents. In addition, inhibition of the enzyme has been employed as a tool in research for various purposes. In this paper, we present a rational design of 2-acetylamino-3-[4-(2-acetylamino-2-carboxyethylsulfanylcarbonylamino)phenylcarbamoylsulfanyl]propionic acid and its derivatives as irreversible GR inhibitors. The K_i and k_{inact} values of 2-acetylamino-3-[4-(2-acetylamino-2-carboxyethylsulfanylcarbonylamino)phenylcarbamoylsulfanyl]propionic acid, the most potent derivative of the series, are 88 μM and 0.1 min^{-1}, respectively. Although the K_i value of the inhibitor is in the micromolar range, it is more potent than *N*,*N*-bis(2-chloroethyl)-*N*-nitrosourea, which is currently the most commonly employed irreversible GR inhibitor with a reported IC_{50} value of 646 μM. Additional attractive features of the inhibitor include its ready availability through a one-step synthesis and good solubility in both organic and aqueous solutions.

Introduction

Glutathione reductase (GR) (EC 1.6.4.2), a dimeric FAD-containing enzyme with a redox-active disulfide at its active site, catalyzes the reduction of oxidized glutathione (GSSG) to reduced glutathione (GSH) using NADPH as the source of reducing equivalents (Scheme 1).[1] The enzyme is responsible for maintaining a high intracellular ratio of GSH/GSSG and, therefore, plays a key role in the cell's defense mechanism against oxidative stress. From a drug development perspective, inhibition of the enzyme has been an attractive approach for the development of antimalarial agents,[2−7] agents to decrease malarial drug resistance,[2,5,6,8−11] and anticancer agents.[3] In addition, inhibition of the enzyme has also been employed as a tool in research for various purposes.[12−23] Different classes of GR inhibitors have been reported that include *N*,*N*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) and 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea,[24] 6-hydroxy-3-oxo-3*H*-xanthene-9-propionic acid,[25] 10-arylisoalloxazines,[4] flavin analogues,[9] methylene blue dye,[26] *S*-nitrosoglutathione,[27] 2,4-dihydroxylbenzylamine,[28] dinitrosyl−iron−dithiolate complex,[29] ajoene,[30] 1,4-naphthoquinone alcanoic acids and their derivatives,[7,8] various isocyanate conjugates of cysteine,[31,32] glutathione,[31−34] and mercapturic acids.[34] For various reasons, BCNU, an anticancer alkylating agent and irreversible GR inhibitor with a reported IC_{50} value of 646 μM,[28] remains the most commonly used GR inhibitor in research.[12−23] However, the toxicity caused by nonspecific interactions as well as the inhibition of DNA synthesis by BCNU complicates the use of the compound as a GR inhibitor.[28,35]

In the present paper, we present 2-acetylamino-3-[4-(2-acetylamino-2-carboxyethylsulfanylcarbonylamino)-phenylcarbamoylsulfanyl]propionic acid (**2**) and its derivatives (**3**−**8**) as a novel class of irreversible and selective GR inhibitors (Figure 1). By use of a reported irreversible GR inhibitor *N*-acetyl-*S*-(*p*-chlorophenylcarbamoyl)cysteine (**1**)[34] as a lead, seven compounds (Figure 1, **2**−**8**) were designed as GR inhibitors based on the structures of GSSG and the enzyme binding site of GSSG. All seven compounds exhibited GR inhibitory activity. Compound **2**, the most potent inhibitor of the derivatives, exhibited much more potent activity than the lead compound and was more potent than BCNU, suggesting that the general strategy employed for the design of GR inhibitors is a rational one.

Results

Synthesis. Compounds **2**−**8** were synthesized through a one-step synthesis by reacting *N*-acetyl-L-cysteine with a corresponding isocyanate. The yields ranged from 6% to 35%.

Comparison of Glutathione Reductase Inhibitory Activity. To assess the GR inhibitory activities of the designed compounds, we decided to employ the lead compound (**1**) and BCNU, the most commonly used irreversible GR inhibitor, as reference compounds. Compound **2** was the first analogue synthesized in the series. Figure 2 shows that at 0.5 mM, compound **2** was much more potent than the lead compound and more potent than BCNU, indicating that the design strategy could lead to a more potent GR inhibitor. Following the synthesis of compound **2**, compounds **3**−**8** were prepared and tested for their GR inhibitory activity. All derivatives were more potent than the lead compound at 0.5 mM (data not shown). Table 1 lists the IC_{50} values of compounds **2**−**8** and BCNU. Interestingly, compound **2** remained as the most potent GR inhibitor of the series (Table 1).

* To whom correspondence should be addressed. Phone: (605) 688-5314. Fax: (605) 688-5993. E-mail: Xiangming.Guan@sdstate.edu.

10.1021/jm050030i CCC: $30.25 © 2005 American Chemical Society
Published on Web 07/15/2005

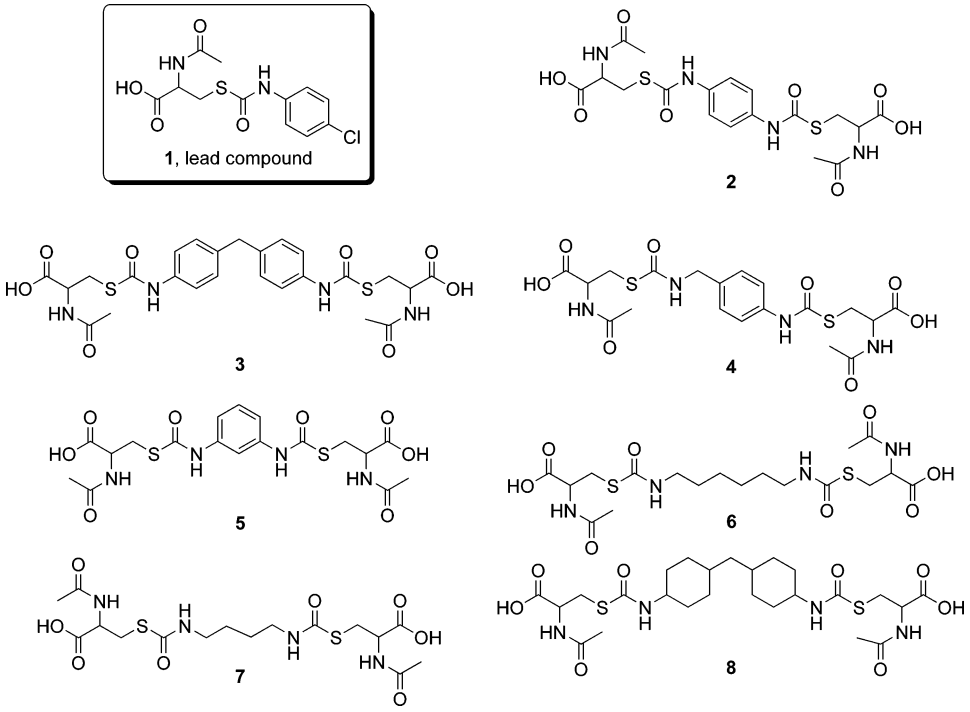
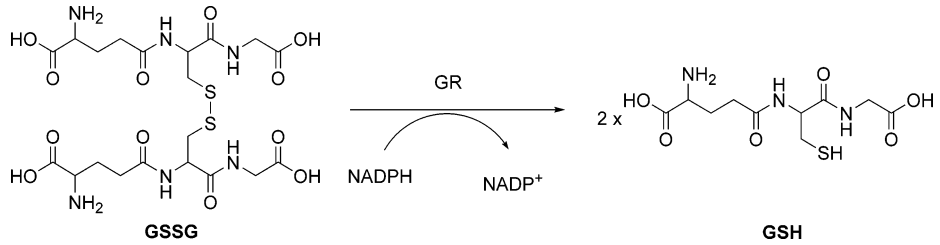


Figure 1. Structures of the lead compound and compounds **2**−**8** designed as GR inhibitors.

Scheme 1. GR-Catalyzed GSSG Reduction



Effect of Compound 2 on Glutathione Peroxidase and Glutathione *S*-Transferase. Once compound **2** was identified as the most potent GR inhibitor of the series, the compound was evaluated further with respect to its selectivity. We tested its effects on glutathione peroxidase (GP) and glutathione *S*-transferase (GST), two enzymes involved in GSH metabolism. At 0.1 mM, compound **2** produced no inhibition of GP. A 13 ± 3% inhibition was observed for GST. These data confirm that compound **2** is a selective GR inhibitor.

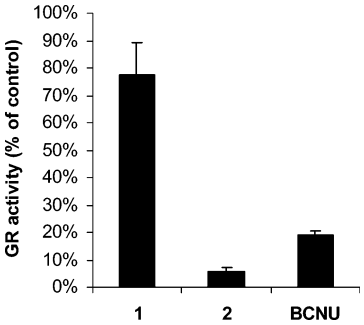


Figure 2. Inhibition of GR by the lead compound (**1**), compound **2**, and BCNU. GR (~0.02 units/mL) was incubated with an inhibitor (0.5 mM) in the presence of NADPH (0.2 mM) and BSA (1 mg/mL) in phosphate buffer (pH 7.4, 0.1 M) at 25 °C for 30 min. The enzyme activity was determined as described in the GR assay. The results are presented as the mean ± SE of triplicate experiments.

Table 1. IC_{50} of Compounds **2**−**8** and BCNU Derived from the Inhibition of Yeast GR[a]

compd	IC_{50} [b] (μM)
2	50 ± 6
3	85 ± 3
4	117 ± 14
5	149 ± 5
6	455 ± 25
7	515 ± 49
8	477 ± 20
BCNU	441 ± 15

[a] Yeast GR (0.9 unit/mL) was incubated with an inhibitor at various concentrations in phosphate buffer (0.1 M, pH 7.4) containing BSA (1 mg/mL) and NADPH (0.2 mM) at 25 °C for 30 min. Aliquots were withdrawn and the remaining GR activity was determined as described in the GR assay. An identical incubation in the absence of the inhibitor was conducted as control. The results are presented as the mean ± SE of triplicate experiments. [b] IC_{50} is the concentration of a compound producing 50% reduction in enzyme activity.

Determination of the Irreversibility of the Inhibition. Determination of whether the inhibition was reversible or irreversible was achieved by extensive dialysis.[36] GR was first inhibited completely by 1 mM compound **2** followed by extensive dialysis in phosphate buffer. No GR activity was recovered over the 4 h dialysis period, revealing that the inhibition was irreversible (Figure 3). In contrast, significant enzyme activity was observed for the control in which no

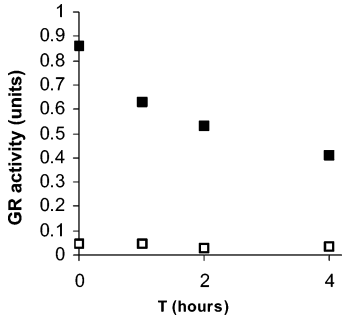


Figure 3. Dialysis of the **2**-inactivated GR. Yeast GR (0.9 units/mL) was incubated at 25 °C with compound **2** (1 mM) in the presence of NADPH (0.2 mM) and BSA (1 mg/mL) in phosphate buffer (pH 7.4, 0.1 M) for 60 min. The inactivated enzyme was dialyzed in phosphate buffer, and aliquots were withdrawn to determine GR activity as described in the Experimental Section. The open and solid squares show the GR activity derived from aliquots of dialysis of the **2**-treated enzyme and a control, respectively. The data were from a representative of duplicate experiments.

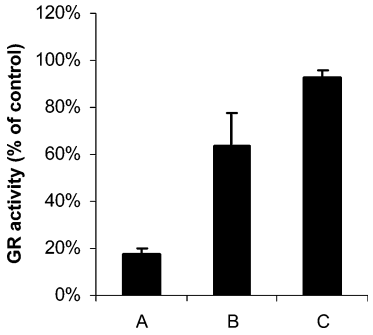


Figure 4. GSSG protection of GR against inhibition by compound **2**. Yeast GR (0.7 unit/mL) was incubated at 25 °C with compound **2** (0.2 mM) and NADPH (0.2 mM) in the presence [(B) 0.05 mM, (C) 0.25 mM)] or absence (A) of GSSG for 15 min. An aliquot was withdrawn and tested for GR activity as described in the GR assay. The results are presented as the mean ± SE of triplicate experiments.

compound **2** was added during the incubation period (Figure 3).

Substrate Protection against Glutathione Reductase Inhibition by Compound 2. To determine whether compound **2** inactivated the enzyme at or near the active site, a substrate protection experiment was conducted. Figure 4 demonstrates that GSSG protected the enzyme from inhibition in a concentration-dependent manner. When the concentration of GSSG was raised to 0.25 mM, the inhibition of GR by compound **2** was completely prevented, suggesting that the inactivation occurred likely at or near the active site of the enzyme.[36]

NADPH-Dependent Glutathione Reductase Inhibition. During the GR-catalyzed GSSG reduction, NADPH reduces the disulfide bond between Cys-45 and Cys-50 of yeast GR (corresponding to Cys-58 and Cys-63 in human GR)[37] to two thiols. The thiols then reduce GSSG to GSH.[1] On the basis of the finding that compound **2** irreversibly inhibited GR and earlier reports that most of the irreversible GR inhibitors produced inactivation through a covalent binding with the thiol functional groups,[24,28−31,38] an exploration was conducted to determine whether the thiols of Cys-45 and/or Cys-50 were involved in the irreversible inhibition. Inhibitory experiments were carried out in the absence or presence of NADPH. In the absence of NADPH, the disulfide bond between Cys-45 and Cys-50 would remain unchanged. As shown in Figure 5, inhibition only occurred in the presence of NADPH. No inhibition was observed in the absence of NADPH, suggesting that the irreversible inhibition required the presence of the thiols derived from the disulfide bond.

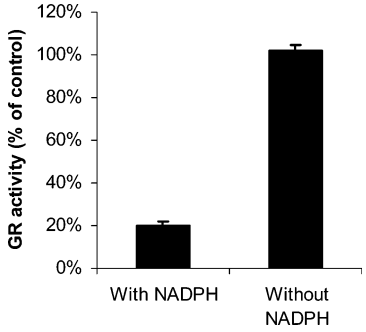


Figure 5. NADPH-dependent inhibition of GR by compound **2**. Yeast GR (0.7 unit/mL) was incubated at 25 °C with compound **2** (0.2 mM) in the presence (0.2 mM) or absence of NADPH for 15 min. An aliquot was withdrawn and tested for GR activity as described in the GR assay. The results are presented as the mean ± SE of triplicate experiments.

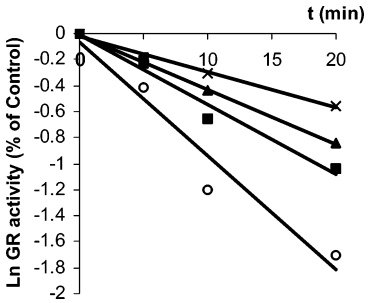


Figure 6. Kinetics of the GR inhibition by compound **2**. The figure is a plot of the natural logarithm of the percent GR activity versus time when treated with compound **2**. Yeast GR (0.3 units/mL) was incubated with different concentrations of compound **2** [32 (×), 63 (▲), 125 (■), 250 (○) μM] in the presence of BSA (1 mg/mL) and NADPH (0.2 mM). Aliquots were withdrawn and tested for residual GR activity at different time intervals (0, 5, 10, 20 min) as described in the Experimental Section. The slopes of the lines representing apparent rate constants of inhibition (k_{app}) were determined by linear regression analysis. The data were derived from a representative of triplicate experiments.

***K*$_i$ and *k*$_{inact}$ Determination.** The inhibitory parameters K_i and k_{inact} of compound **2** were determined according to the method of Kitz and Wilson.[39] Figure 6 is derived from the results when GR was incubated with compound **2** at different concentrations over a 20 min period. The data show that the enzyme activity decreased with time (time-dependent inhibition) and concentration (concentration-dependent inhibition). These data further confirm that the GR inhibition by compound **2** was irreversible.[36,39] Figure 6 also demonstrates a linear correlation between the natural logarithm of the percent enzyme activity and incubation time. A replotting of the reciprocal of apparent rate constants of inhibition (K_{app}) (slopes of Figure 6) versus the reciprocal of inhibitor concentrations [I] showed a linear correlation (Figure 7), from which the inactivation rate

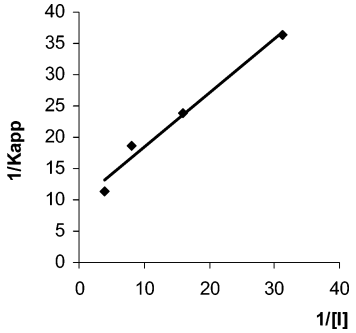


Figure 7. Kitz and Wilson replotting of GR inhibition by compound **2**: double-reciprocal plot of k_{app} vs compound **2** concentrations [I]. The dissociation constant K_i of the reversible enzyme−inhibitor complex and the inactivation rate constant k_{inact} were derived from the intercept and slope of the line determined by linear regression analysis based on the formula presented in the text.

constant k_{inact} (0.1 min^{-1}) and inhibitory constant K_i (88 μM) were derived on the basis of the following equations where it is assumed that [I] ≫ [E_0].

$$1 + E \overset{K_i}{\rightleftharpoons} EI \xrightarrow{k_{inact}} E^*$$

$$\frac{1}{k_{app}} = \frac{1}{k_{inact}} + \frac{K_i}{k_{inact}} \frac{1}{[I]}$$

[E_0] is the concentration of GR at time 0, EI is the enzyme inhibitor complex, and E* is the inactivated enzyme.[39]

Discussion

GR has been an attractive target for the development of antimalarial agents, anticancer agents, and agents to reverse drug resistance of malaria. In this paper, we report 2-acetylamino-3-[4-(2-acetylamino-2-carboxyethylsulfanylcarbonylamino)phenylcarbamoylsulfanyl]-propionic acid (**2**) and its derivatives (**3**−**8**) as a novel class of GR inhibitors (Figure 1). The design of this class of GR inhibitors was based on the structures of a lead compound, GSSG, and the enzyme binding site of GSSG (Figure 8). The lead compound **1** has been identified as an irreversible GR inhibitor, and the *S*-carbamoyl structure (*S*-C(O)N−) (indicated by an arrow in Figure 8) is required for the carbamoylation of the thiols at the active site (unpublished results from this group). An examination of the structures of GSSG (Figure 8) and GSSG binding site[1] led us to propose a general strategy for the design of this novel class of GR inhibitors. The rationale for this strategy is that by connecting two part A moieties (Figure 8) of the lead compound together, which results in a molecule with one carboxylic acid functional group on each end of the molecule (Figure 8), the compound will be structurally more similar to the natural substrate GSSG and also will retain the required carbamoylating functional group (Figure 8). Further, the carboxylic acid functional groups on both ends of the molecule can mimic the electrostatic interactions between the anionic carboxylic acid functional groups of GSSG and the cationic functional groups at the GSSG binding site of the enzyme.[1] It is also hoped that the inhibitory activity may be enhanced by the two carbamoylating groups in the molecule. Each of the two carbamoylating groups may be able to form a covalent bond with one of the two thiols or other nucleophiles present at the active site of the enzyme (double carbamoylation) (Scheme 2). The connecting bridge can be optimized by varying substitutes with different length, hydrophobicity, and electron density. Figure 1 shows the structures of the seven proposed compounds that were synthesized and tested for their effects on GR.

Our results demonstrate that all designed compounds were GR inhibitors with different inhibitory potency. These inhibitors were more potent than the lead compound, confirming that the design strategy is a rational one. Compound **2**, the most potent derivative, exhibited more potent GR inhibitory activity than BCNU (Figures 2 and Table 1). Evaluation of the effects of compound **2** on GP and GST revealed that compound **2** is a selective GR inhibitor. Dialysis of the **2**-inactivated GR showed that the inhibition was irreversible (Figure 3). The irreversibility was further confirmed by the time- and concentration-dependent inactivation of the enzyme by the inhibitor (Figure 6). Our data also show that the inactivation was prevented by the substrate GSSG (Figure 4), indicating that the inactivation occurred likely at or near the active site of the enzyme. By removing NADPH from the incubation, we demonstrated that the inactivation required the thiols to be derived from the disulfide of Cys-45 and Cys-50 at the active site of yeast GR. This finding is in agreement with the inactivation mechanism of a number of earlier reported irreversible GR inhibitors such as 2,4-dihydroxybenzylamine,[28] dinitrosyl−iron−dithiolate,[29] BCNU,[24,38] ajoene,[30] and isocyanate conjugates of cysteine.[31] These irreversible inhibitors were shown to produce their irreversible inhibition through a covalent binding with the thiols. Finally, the inhibitory parameters K_i and k_{inact} were determined to be 88 μM and 0.1 min^{-1}, respectively, on the basis of the method of Kitz and Wilson.[39]

Because of the limited number of compounds tested, conclusive determination of the structure−activity relationship (SAR) regarding lipophilic, electronic, and steric requirements of this class of GR inhibitors may, at this point, not be appropriate. However, the compounds with a connecting bridge containing an aromatic system (compounds **2**−**5**) were shown to be more potent than those with an aliphatic chain (compounds **6**−**8**). The inhibitory activity improvement by an aromatic system was further demonstrated by the fact that compound **3** was almost 6 times more potent than compound **8**, although the lipophilicity of these two compounds are similar based on their HPLC properties in two different mobile systems. The retention time for compounds **3** and **8** were 24.1 and 24.2 min in the water/acetonitrile system and were 34.7 and 35.5 min in water/methanol system, respectively (see Supporting Information). However, more study is needed to further understand the SAR of these inhibitors.

On the basis of the obtained evidence, a proposed mechanism of the GR inactivation by compound **2** is outlined in Figure 9. The inactivation likely occurred when the disulfide bond between Cys-45 and Cys-50 of yeast GR was reduced by NADPH to two thiol functional groups. The thiols, which are excellent nucleophiles, attacked the carbamoyl group of the inhibitor, leading to covalent bond formation (carbamoylation) and inac-

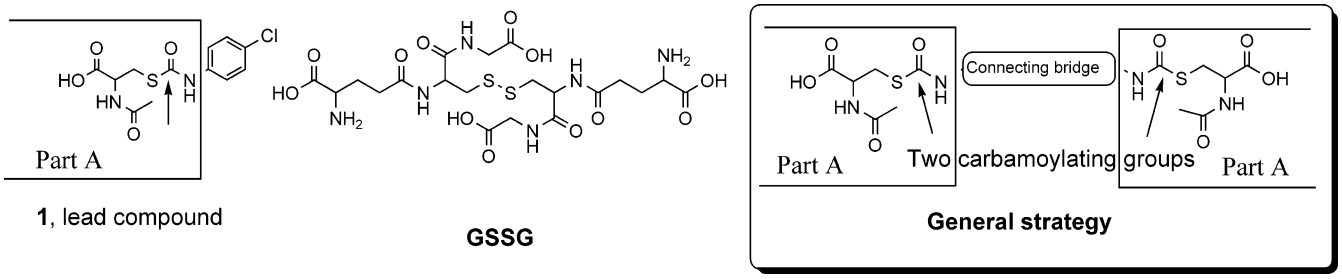


Figure 8. Chemical structures of the lead compound, GSSG, and the proposed general strategy for the design of GR inhibitors.

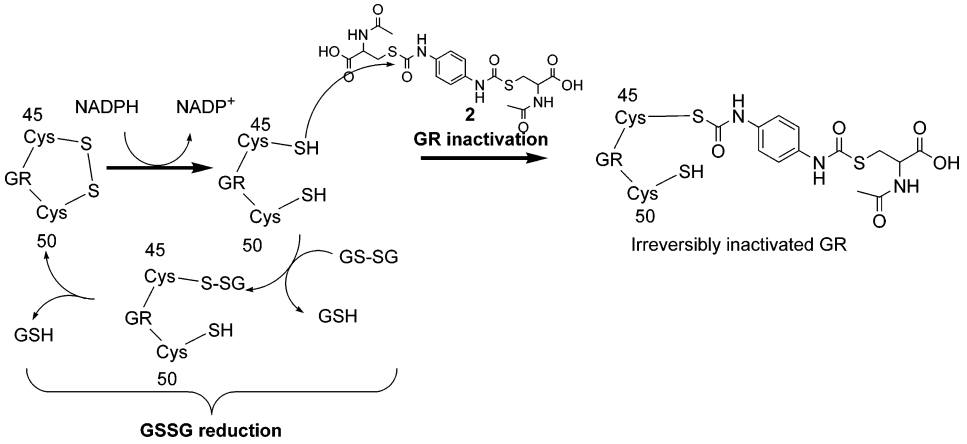
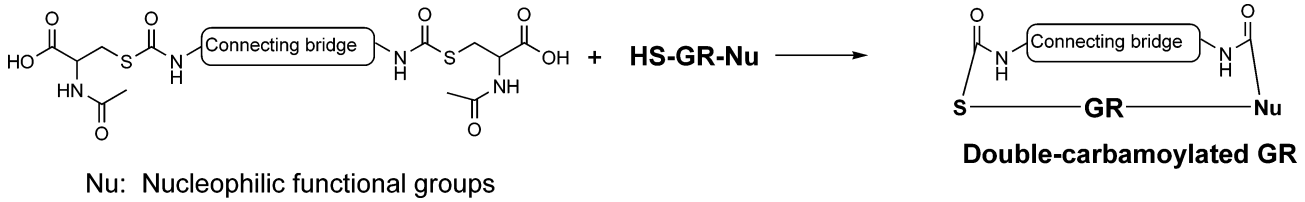


Figure 9. GR-catalyzed GSSG reduction and the proposed mechanism for the inactivation of GR by compound **2**.

Scheme 2. Proposed Covalent Bond Formation between a Proposed Double Carbamoylating GR Inhibitor and Thiols or Other Nucleophiles Present in GR



tivation of the enzyme. Since Cys-58 of human GR was the thiol demonstrated to form a covalent bond with an irreversible inhibitor because of its location at the entrance of the active site,[24,27,29,30] we believe that it was the corresponding Cys-45 of yeast GR that formed a covalent bond with compound **2** (Figure 9). At this point, we have no data to show whether one or both of the carbamoylating groups of the inhibitor participated in the inactivation.

In summary, we have identified 2-acetylamino-3-[4-(2-acetylamino-2-carboxyethylsulfanylcarbonylamino)-phenylcarbamoylsulfanyl]propionic acid (**2**) and its derivatives as a novel class of irreversible GR inhibitors. One of the advantages of this class of compounds is that they are readily available through a one-step synthesis from commercially available starting materials. Compound **2** was found to be the most potent inhibitor of the series. Although, the K_i value of compound **2** is in the micromolar range, it is more potent than BCNU, the most commonly used irreversible GR inhibitor. Further, the compound has good solubility in both organic and aqueous solutions, which is an additional attractive feature.

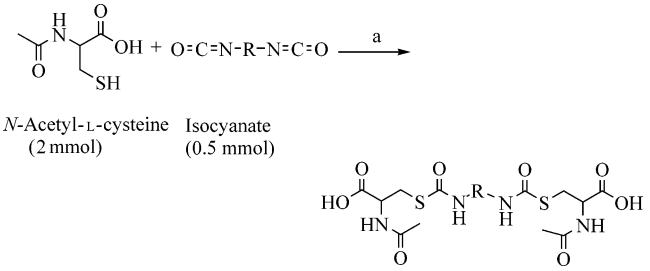
Experimental Section

^{1}H and ^{13}C nuclear magnetic resonance spectra were recorded on a Bruker 400 MHz NMR spectrometer and are reported in parts per million. UV absorption was obtained on an HP 8452A diode array spectrophotometer. Mass spectral data were obtained with the Finnigan MAT Navigator HPLC/MS mass detector.

Yeast GR, bovine liver GST, bovine erythrocyte GP, bovine serum albumin (BSA), GSH, GSSG, *N*-acetyl-L-cysteine, and NADPH were purchased from Sigma Chemical Co. (St. Louis, MO). Isocyanates were obtained from Aldrich Chemical Co. (Milwaukee, WI). BCNU was provided generously by Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ). The 3% H_2O_2 was purchased from Wal-Mart. All other solvents and chemicals were of HPLC or reagent grade and were used as received. Phenyl solid extraction cartridges (6 mL × 100 mg) were purchased from J&W Scientific (Folsom, CA). Disposable dialyzers (DisPoDialyzers) (molecular weight cutoff of 8000) were purchased from Spectrum (Laguna Hills, California).

Synthesis of 2-Acetylamino-3-[4-(2-acetylamino-2-carboxyethylsulfanylcarbonylamino)phenylcarbamoylsulfanyl]propionic Acid and Its Derivatives. A general one-step procedure was employed (Scheme 3). A solution of an isocyanate (0.5 mmol) in a tetrahydrofuran (THF)/water mixture (2:1) (30 mL) was introduced dropwise to a solution of *N*-acetyl-L-cysteine (2 mmol) in saturated sodium bicarbonate (10 mL) under argon with stirring at room temperature. The solution was allowed to stir for an additional 15 min. THF was removed in vacuo. The remaining aqueous solution was acidified to pH 2 with HCl (2 M) and loaded onto a phenyl solid extraction cartridge that was preconditioned with 80% acetonitrile in water containing 0.1% trifluoroacetic acid (TFA). The column product was eluted with acetonitrile and water

Scheme 3. General Procedure for the Synthesis of the Designed GR Inhibitors[a]



[a] Reagents and conditions: (a) argon, 25 °C, THF/H_2O (2:1), $NaHCO_3$, 15 min.

containing 0.1% TFA. Analytical thin-layer chromatography (TLC) was performed on Sigma-Aldrich glass-backed silica gel 60 F254 plates. TLC visualization was accomplished with a UV lamp (compounds **2**−**5**) or in an iodine chamber (compounds **6**−**8**). The fractions containing the product were pooled and freeze-dried to give the product.

The purity of the products was checked by high-pressure liquid chromatography (HPLC), which was performed on a Beckmann system equipped with a UV detector set at 254 nm for compounds **2**−**5** and at 220 nm for compounds **6**−**8**. The HPLC employed an Adsorbosil C_{18} column (250 mm × 3.2 mm i.d., 5 μm) (Alltech, Deerfield, IL) with a flow rate of 0.5 mL/min. Compound purity was assessed by two HPLC methods. Method A utilized a gradient of 0% to 100% acetonitrile in water containing 0.1% (v/v) TFA in 40 min, and method B used a gradient of 0% to 100% methanol in water containing 0.1% (v/v) TFA in 40 min. All the final products had 95% or greater purity (see Supporting Information).

2-Acetylamino-3-[4-(2-acetylamino-2-carboxyethylsulfanylcarbonylamino)phenylcarbamoylsulfanyl]propionic Acid (2). Yield: 35%. Mp: 185 °C (dec), 99% purity by HPLC. ^{1}H NMR (DMSO-d_6): δ 1.84 (s, 6 H, 2 × CH_3), 3.04 (dd, J = 8.8, 13.6 Hz, 2H, 2 × SCH), 3.38 (dd, J = 5.0, 13.6 Hz, 2H, 2 × SCH), 4.34−4.41 (m, 2H, 2 × NCH), 7.41 (s, 4H, phenyl), 8.24 [d, J = 8.4 Hz, 2H, 2 × C(O)NH−CH], 10.24 [s, 2H, 2 × C(O)NH-phenyl]. ^{13}C NMR (DMSO-d_6): δ 172.22, 169.6, 164.1, 134.8, 120.0, 52.5, 31.0, 22.7. MS, *m/z*: 487 (M + H)$^+$, 469, 427, 382, 358, 324.

2-Acetylamino-3-{4-[4-(2-acetylamino-2-carboxyethylsulfanylcarbonylamino)benzyl]phenylcarbamoylsulfanyl}propionic Acid (3). Yield: 18%. Mp 152−154 °C, 99% purity by HPLC. ^{1}H NMR (DMSO-d_6): δ 1.84 (s, 6 H, 2 × CH_3), 3.03 (dd, J = 8.8, 14.0 Hz, 2H, 2 × SCH), 3.37 (dd, J = 4.8, 14.0 Hz, 2H, 2 × SCH), 3.82 (s, 2H, CH_2-phenyl), 4.32−4.40 (m, 2H, 2 × NCH), 6.95 (d, 4H, J = 11.6 Hz, phenyl), 7.10 (d, 4H, J = 11.6 Hz, phenyl), 8.29 (d, 2H, J = 8.6 Hz, 2 × C(O)NH−CH], 10.34 [s, 2H, 2 × C(O)NH-phenyl]. ^{13}C NMR (DMSO-d_6): δ 172.2, 169.1, 164.1, 137.1, 136.8, 129.4, 119.5, 52.6, 30.9, 22.7. ESI-MS, *m/z*: 577 (M + 1)$^+$.

2-Acetylamino-3-{4-[(2-acetylamino-2-carboxyethylsulfanylcarbonylamino)methyl]phenylcarbamoylsulfanyl}propionic Acid (4). Yield: 13%. Mp: 151−153 °C, 99% purity by HPLC. ^{1}H NMR (DMSO-d_6): δ 1.82 (s, 3 H, CH_3), 1.84 (s, 3 H, CH_3), 2.88−3.01 (m, 4H, 2 × SCH_2), 4.05−4.43 [m, 4H, −(NCH)$_2$−, NCH_2Ph)], 7.18 (d, J = 8.4 Hz, 2H, phenyl), 7.43 (d, J = 8.4 Hz, 2H, phenyl), 8.22 (d, J = 8.0 Hz, 1H, NH), 8.29 (d, J = 8.0 Hz, 1H, NH), 8.68 (t, J = 5.6 Hz, 1H, NH), 10.35 (s, 1H, NH). ^{13}C NMR (DMSO-d_6): δ 172.3, 172.2, 169.6, 169.6, 165.6, 164.3, 138.0, 134.3, 128.2, 119.3, 52.7, 52.5, 43.94, 30.9, 30.8, 22.7. ESI-MS, *m/z*: 501 (M + 1)$^+$.

2-Acetylamino-3-[3-(2-acetylamino-2-carboxyethylsulfanylcarbonylamino)phenylcarbamoylsulfanyl]propionic Acid (5). Yield: 33%. Mp: 167−170 °C, >96% purity by HPLC. ^{1}H NMR (acetone-d_6): δ 1.84 (s, 6 H, 2 × CH_3), 3.16 (dd, J = 8.0, 14.4 Hz, 2H, 2 × SCH), 3.45 (dd, J = 4.8, 14.4 Hz, 2H, 2 × SCH), 4.55−4.63 (m, 2H, 2 × NCH), 7.09−7.22 (m, 4H, phenyl), 7.78 [s, 2H, 2 × C(O)NH−CH], 9.25 [s, 2H, 2 × C(O)NH-phenyl]. ^{13}C NMR (DMSO-d_6): δ 172.2, 170.7, 165.7, 140.7, 130.6, 115.5, 111.0, 53.7, 32.2, 23.1. ESI-MS, *m/z*: 487 (M + 1)$^+$.

2-Acetylamino-3-[6-(2-acetylamino-2-carboxyethylsulfanylcarbonylamino)hexylcarbamoylsulfanyl]propionic Acid (6). Yield: 32%. Mp: 168−174 °C (dec), >95% purity by HPLC. ^{1}H NMR (DMSO-d_6): δ 1.34−1.76 [m, 8 H, −(CH$_2$)$_4$−], 2.02 (s, 6 H, 2 × CH_3), 3.18 (dd, J = 8.8, 13.6 Hz, 2H, 2 × SCH), 3.24−3.39 (m, 4H, −(NCH$_2$)$_2$−], 3.52 (dd, J = 5.0, 13.6 Hz, 2H, 2 × SCH), 4.40−4.56 (m, 2H, 2 × NCH), 8.39 [t, J = 5.4 Hz, 2H, 2 × C(O)NH−CH$_2$], 8.45 [d, J = 8.0 Hz, 2H, 2 × C(O)NH−CH. ^{13}C NMR (DMSO-d_6): δ 172.3, 169.6, 165.0, 52.8, 41.0, 30.68, 29.3, 26.3, 22.7. ESI-MS, *m/z*: 495 (M + 1)$^+$.

2-Acetylamino-3-[4-(2-acetylamino-2-carboxyethylsulfanylcarbonylamino)butylcarbamoylsulfanyl]propionic Acid (7). Yield: 6%. Mp: 144−150 °C, >98% purity by HPLC. ^{1}H NMR (DMSO-d_6): δ 1.3−1.45 (m, 8 H, −(CH$_2$)$_2$], 1.82 (s, 6 H, 2 × CH_3), 2.97 (dd, J = 5.0, 14.8 Hz, 2H, 2 × SCH), 3.02−3.18 (m, 4H, −(NCH$_2$)$_2$−], 3.29 (dd, J = 5.0, 13.6 Hz, 2H, 2 × SCH), 4.24−4.32 (m, 2H, 2 × NCH), 8.17 [t, J = 5.4 Hz, 2H, 2 × C(O)NH−CH$_2$], 8.21 [d, J = 8.0 Hz, 2H, 2 × C(O)NH−CH]. ^{13}C NMR (DMSO-d_6): δ 172.3, 169.6, 165.1, 52.8, 40.8, 30.7, 26.8, 22.7. ESI-MS, *m/z*: 467 (M + 1)$^+$.

2-Acetylamino-3-{4-[4-(2-acetylamino-2-carboxyethylsulfanylcarbonylamino)cyclohexylmethyl]cyclohexylcarbamoylsulfanyl}propionic Acid (8). Yield: 29%. Mp: 153−154 °C, 95% purity by HPLC. ^{1}H NMR (DMSO-d_6): δ 0.74−1.84 [m, 20 H, cyclohexyl, −CH$_2$−], 1.82 (s, 6 H, 2 × CH_3), 2.93 (dd, J = 8.8, 13.6 Hz, 2H, 2 × SCH), 3.27 (dd, J = 5.2, 13.6 Hz, 2H, 2 × SCH), 3.34−3.49 (m, 2H, −(NCH)$_2$−], 4.19−4.29 (m, 2H, 2 × NCH), 7.96−8.09 [m, 2H, 2 × C(O)-NH−CH$_2$], 8.19 [d, J = 8.0 Hz, 2H, 2 × C(O)NH−CH]. ^{13}C NMR (DMSO-d_6): δ 172.4, 169.5, 164.6, 53.0, 50.9, 33.6, 32.6, 32.0, 30.8, 29.0, 27.9. ESI-MS, *m/z*: 588 (M + 1)$^+$.

Glutathione Reductase Assay. All GR assays were carried out in GR assay phosphate buffer (pH 7.4, 0.1 M), which contains BSA (1 mg/mL) and NADPH (0.2 mM) at 25 °C in a total volume of 1 mL. The reaction was initiated by adding GSSG (0.52 mM). Determination of the GR activity was achieved by measurement of the initial rates of disappearance of NADPH determined spectrophotometrically at λ = 340 nm.

Evaluation of Glutathione Reductase Inhibitory Potency and IC_{50} Determination. Yeast GR (0.9 units/mL) was incubated with an inhibitor at various concentrations in phosphate buffer (0.1 M, pH 7.4) containing BSA (1 mg/mL) and NADPH (0.2 mM) at 25 °C for 30 min. Aliquots were withdrawn, and the remaining GR activity was determined as described in the GR assay. An identical incubation in the absence of the inhibitor was conducted as control.

Determination of the Irreversibility of the Inhibition. Yeast GR (0.9 units/mL) was incubated at 25 °C with compound **2** (1 mM) in the presence of NADPH (0.2 mM) and BSA (1 mg/mL) with a total volume of 0.5 mL for 60 min. No GR activity was detected at the end of 60 min. The mixture was transferred to a 1 mL DisPoDialyzer with a molecular weight cutoff of 8000 and dialyzed in phosphate buffer (800 mL, pH 7.4, 0.1M). The phosphate buffer was replaced with fresh buffer every hour, and the dialysis continued for 4 h. Aliquots were withdrawn at different time intervals and tested for GR activity as described in the GR assay. An identical incubation in the absence of the inhibitor was conducted as control.

Substrate Protection against Glutathione Reductase Inhibition by Compound 2. Yeast GR (0.7 units/mL) was incubated at 25 °C with compound **2** (0.2 mM) and NADPH (0.2 mM) in the presence (0.05 and 0.25 mM) or absence of GSSG for 15 min. Aliquots were withdrawn, and the remaining GR activity was determined as described in the GR assay. A control in the absence of compound **2** and GSSG was conducted in parallel.

NADPH-Dependent Glutathione Reductase Inhibition. Yeast GR (0.7 units/mL) was incubated at 25 °C with compound **2** (0.2 mM) in the presence (0.2 mM) or absence of NADPH for 15 min. Aliquots were withdrawn, and the remaining GR activity was determined as described in the GR

assay. An identical incubation in the absence of the inhibitor was conducted as control.

Effect of Compound 2 on Glutathione *S*-Transferase. Bovine liver GST (0.02 units/mL) was incubated at 25 °C with compound **2** (0.1 mM) for 30 min in a total volume of 1 mL. Determination of the remaining GST activity was achieved by following a GST assay protocol from Sigma (St. Louis, MO). An identical incubation in the absence of the inhibitor was conducted as a control.

Effect of Compound 2 on Glutathione Peroxidase. Bovine erythrocyte GP (0.02 units/mL) was incubated at 25 °C with compound **2** (0.1 mM) for 30 min in a total volume of 1 mL. Determination of the remaining GP activity was achieved by following a GP assay protocol from Sigma (St. Louis, MO). An identical incubation in the absence of the inhibitor was conducted as a control.

Kinetics of Irreversible Inhibition. Yeast GR (0.3 units/mL) was incubated at 25 °C with compound **2** at different concentrations (32, 63, 125, 250 μM) in the presence of BSA (1 mg/mL) and NADPH (0.2 mM). Aliquots were withdrawn and tested for residual GR activity at different time intervals (0, 5, 10, 20 min) as described in the GR assay. An identical incubation in the absence of the inhibitor was conducted as a control. Irreversible kinetic parameters were obtained according to the method of Kitz and Wilson.[39]

Acknowledgment. This work was supported by Grant CA079540-01 from the National Institutes of Health, a South Dakota State University Research Support Grant, and a Merck Research Scholar Award. BCNU was provided generously by Bristol-Myers Squibb Pharmaceutical Research Institute (Princeton, NJ).

Appendix

Abbreviations. BCNU, *N,N*-bis(2-chloroethyl)-*N*-nitrosourea; BSA, bovine serum albumin; FAD, flavin adenine dinucleotide; GP, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione *S*-transferase; NADPH, nicotinamide adenine dinucleotide phosphate; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

Supporting Information Available: Purity of compounds **2**−**8** were determined by two HPLC methods. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Schirmer, R. H.; Krauth-Siegel, R. L. Glutathione Reductase. In *Glutathione, Chemical, Biochemical, and Medical Aspects*; Dolphin, D., Poulson, R., Avramovic, O., Eds.; Wiley: New York, 1989; Vol. III, Part A, pp 553−596.

(2) Zhang, Y. A.; Hempelmann, E.; Schirmer, R. H. Glutathione reductase inhibitors as potential antimalarial drugs. Effects of nitrosoureas on *Plasmodium falciparum* in vitro. *Biochem. Pharmacol*. **1988**, *37*, 855−860.

(3) Schirmer, R. H.; Müller, J. G.; Krauth-Siegel, R. L. Disulfide reductase inhibitors as chemotherapeutic agents: the design of drugs for trypanosomiasis and malaria. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 141−154.

(4) Schonleben-Janas, A.; Kirsch, P.; Mittl, P. R.; Schirmer, R. H.; Krauth-Siegel, R. L. Inhibition of human glutathione reductase by 10-arylisoalloxazines: crystalline, kinetic, and electrochemical studies. *J. Med. Chem.* **1996**, *39*, 1549−1554.

(5) Sarma, G. N.; Savvides, S. N.; Becker, K.; Schirmer, M.; Schirmer, R. H.; Karplus, P. A. Glutathione reductase of the malarial parasite *Plasmodium falciparum*: crystal structure and inhibitor development. *J. Mol. Biol*. **2003**, *328*, 893−907.

(6) Schirmer, R. H.; Coulibaly, B.; Stich, A.; Scheiwein, M.; Merkle, H.; Eubel, J.; Becker, K.; Becher, H.; Muller, O.; Zich, T.; Schiek, W.; Kouyate, B. Methylene blue as an antimalarial agent. *Redox Rep*. **2003**, *8*, 272−275.

(7) Biot, C.; Bauer, H.; Heiner Schirmer, R. H.; Davioud-Charvet, E. 5-Substituted tetrazoles as bioisosteres of carboxylic acids. Bioisosterism and mechanistic studies on glutathione reductase inhibitors as antimalarials. *J. Med. Chem.* **2004**, *47,* 5972−5983.

(8) Davioud-Charvet, E.; Delarue, S.; Biot, C.; Schwobel, B.; Boehme, C. C.; Mussigbrodt, A.; Maes, L.; Sergheraert, C.; Grellier, P.; Schirmer, R. H.; Becker, K. A prodrug form of a *Plasmodium falciparum* glutathione reductase inhibitor conjugated with a 4-anilinoquinoline. *J. Med. Chem.* **2001**, *44*, 4268−4276.

(9) Becker, K.; Christopherson, R. I.; Cowden, W. B.; Hunt, N. H.; Schirmer, R. H. Flavin analogs with antimalarial activity as glutathione reductase inhibitors. *Biochem. Pharmacol*. **1990**, *39*, 59−65.

(10) Becker, K.; Rahlfs, S.; Nickel, C.; Schirmer, R. H. Glutathione—functions and metabolism in the malarial parasite *Plasmodium falciparum*. *Biol. Chem*. **2003**, *384*, 551−566.

(11) Biot, C.; Dessolin, J.; Grellier, P.; Davioud-Charvet, E. Double-drug development against antioxidant enzymes from *Plasmodium falciparum*. *Redox Rep*. **2003**, *8*, 280−283.

(12) Tew, K. D.; Kyle, G.; Johnson, A.; Wang, A. L. Carbamoylation of glutathione reductase and changes in cellular and chromosome morphology in a rat cell line resistant to nitrogen mustards but collaterally sensitive to nitrosoureas. *Cancer Res*. **1985**, *45*, 2326−2333.

(13) Vanhoefer, U.; Yin, M. B.; Harstrick, A.; Seeber, S.; Rustum, Y. M. Carbamoylation of glutathione reductase by *N*,*N*-bis(2-chloroethyl)-*N*-nitrosourea associated with inhibition of multidrug resistance protein (MRP) function. *Biochem. Pharmcol*. **1997**, *53,* 801−809.

(14) Romero-Ramos, M.; Venero, J. L.; Garcia-Rodriguez, S.; Ayala, A.; Machado, A.; Cano, J. Semichronic inhibition of glutathione reductase promotes oxidative damage to proteins and induces both transcription and translation of tyrosine hydroxylase in the nigrostriatal system. *Free Radical Res*. **2003**, *37*, 1003−1012.

(15) Li, S.; Li, X.; Rozanski, G. J. Regulation of glutathione in cardiac myocytes. *J. Mol. Cell. Cardiol.* **2003**, *35*, 1145−1152.

(16) Komatsu, H.; Hoshino, A.; Funayama, M.; Kawahara, K.; Obata, F.; Oxidative modulation of the glutathione-redox couple enhances lipopolysaccharide-induced interleukin 12 P40 production by a mouse macrophage cell line, J774A.1. *Free Radical Res*. **2003**, *37*, 293−299.

(17) Vexler, Z. S.; Wong, A.; Francisco, C.; Manabat, C.; Christen, S.; Tauber, M.; Ferriero, D. M.; Gregory, G. Fructose-1,6-bisphosphate preserves intracellular glutathione and protects cortical neurons against oxidative stress. *Brain Res*. **2003**, *960*, 90−98.

(18) Hojo, Y.; Saito, Y.; Tanimoto, T.; Hoefen, R. J.; Baines, C. P.; Yamamoto, K.; Haendeler, J.; Asmis, R.; Berk, B. C. Fluid shear stress attenuates hydrogen peroxide-induced c-Jun NH2-terminal kinase activation via a glutathione reductase-mediated mechanism. *Circ. Res*. **2002**, *91*, 712−718.

(19) Kaneko, T.; Iuchi, Y.; Kobayashi, T.; Fujii, T.; Saito, H.; Kurachi, H.; Fujii, J. The expression of glutathione reductase in the male reproductive system of rats supports the enzymatic basis of glutathione function in spermatogenesis. *Eur. J. Biochem*. **2002**, *269*, 1570−1578.

(20) Jopperi-Davis, K. S.; Park, M. S.; Rogers, L. K.; Backes, C. H., Jr.; Lim, I. K.; Smith, C. V.; Compartmental inhibition of hepatic glutathione reductase activities by 1,3-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) in Sprague-Dawley and Fischer-344 rats. *Toxicol. Lett*. **2004**, *147*, 219−228.

(21) Gunaratnam, M.; Grant, M. H. The role of glutathione reductase in the cytotoxicity of chromium(VI) in isolated rat hepatocytes. *Chem.-Biol. Interact*. **2001**, *134*, 191−202.

(22) Nikulina, M. A.; Andersen, H. U.; Karlsen, A. E.; Darville, M. I.; Eizirik, D. L.; Mandrup-Poulsen, T. Glutathione depletion inhibits IL-1 beta-stimulated nitric oxide production by reducing inducible nitric oxide synthase gene expression. *Cytokine* **2000**, *12*, 1391−1394.

(23) Sakurai, T.; Kojima, C.; Ochiai, M.; Ohta, T.; Sakurai, M. H.; Waalkes, M. P.; Fujiwara, K. Cellular glutathione prevents cytolethality of monomethylarsonic acid. *Toxicol. Appl. Pharmacol*. **2004**, *195*, 129−141.

(24) Karplus, P. A.; Krauth-Siegel, R. L.; Schirmer, R. H.; Schulz, G. E. Inhibition of human glutathione reductase by the nitrosourea drugs 1,3-bis(2-chloroethyl)-1-nitrosourea and 1-(2-chloroethyl)-3-(2-hydroxyethyl)-1-nitrosourea. A crystallographic analysis. *Eur. J. Biochem*. **1988**, *171*, 193−198.

(25) Savvides, S. N.; Karplus, P. A. Kinetics and crystallographic analysis of human glutathione reductase in complex with a xanthene inhibitor. *J. Biol. Chem.* **1996**, *271*, 8101−8107.

(26) Farber, P. M.; Arscott, L. D.; Williams, C. H., Jr, Becker, K.; Schirmer, R. H. Recombinant *Plasmodium falciparum* glutathione reductase is inhibited by the antimalarial dye methylene blue. *FEBS Lett.* **1998**, *422,* 311−314.

(27) Becker, K.; Gui, M.; Schirmer, R. H. Inhibition of human glutathione reductase by *S*-nitrosoglutathione. *Eur. J. Biochem*. **1995**, *234*, 472−478.

(28) FitzGerald, G. B.; Bauman, C.; Hussoin, M. S.; Wick, M. M. 2,4-Dihydroxybenzylamine: a specific inhibitor of glutathione reductase. *Biochem. Pharmacol*. **1991**, *41*, 185−190.

(29) Boese, M.; Keese, M. A.; Becker, K.; Busse, R.; Mulsch, A. Inhibition of glutathione reductase by dinitrosyl−iron−dithiolate complex. *J. Biol. Chem*. **1997**, *272*, 21767−21773.
(30) Gallwitz, H.; Bonse, S.; Martinez-Cruz, A.; Schlichting, I.; Schumacher, K.; Krauth-Siegel, R. L. Ajoene is an inhibitor and subversive substrate of human glutathione reductase and *Trypanosoma cruzi* trypanothione reductase: crystallographic, kinetic, and spectroscopic studies. *J. Med. Chem*. **1999**, *42*, 364−372.
(31) Jochheim, C. M.; Baillie, T. A. Selective and irreversible inhibition of glutathione reductase in vitro by carbamate thioester conjugates of methyl isocyanate. *Biochem. Pharmacol*. **1994**, *47*, 1197−1206.
(32) Kassahun, K.; Jochheim, C. M.; Baillie, T. A. Effect of carbamate thioester derivatives of methyl- and 2-chloroethyl isocyanate on glutathione levels and glutathione reductase activity in isolated rat hepatocytes. *Biochem. Pharmcol*. **1994**, *48*, 587−594.
(33) Guan, X.; Davis, M. R.; Tang, C.; Jochheim, C. M.; Jin, L.; Baillie, T. A. Identification of *S*-(*n*-butylcarbamoyl)glutathione, a reactive metabolite of tolbutamide in the rat, and evaluation of its inhibitory effects on glutathione reductase in vitro. *Chem. Res. Toxicol*. **1999**, *12*, 1138−1143.
(34) Guan, X.; Hoffman, B. N.; McFarland, D. C.; Gilkerson, K. K.; Dwivedi, C.; Erickson, A. K.; Bebensee, S.; Pellegrini, J. Glutathione and mercapturic acid conjugates of sulofenur and their activity against a human colon cancer cell line. *Drug Metab. Dispos*. **2002**, *30*, 331−335.
(35) Kann, H. E., Jr.; Schott, M. A.; Petkas, A. Effects of structure and chemical activity on the ability of nitrosoureas to inhibit DNA repair. *Cancer Res*. **1980**, *40*, 50−55.
(36) Silverman, R. B. *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*; CRC Press: Boca Raton, FL, 1988; Vol. I, pp 3−30.
(37) Arscott, L. D.; Veine, D. M.; Williams, C. H., Jr. Mixed disulfide with glutathione as an intermediate in the reaction catalyzed by glutathione reductase from yeast and as a major form of the enzyme in the cell. *Biochemistry* **2000**, *39*, 4711−4721.
(38) Schallreuter, K. U.; Gleason, F. K.; Wood, J. M. The mechanism of action of the nitrosourea anti-tumor drugs on thioredoxin reductase, glutathione reductase and ribonucleotide reductase. *Biochim. Biophys. Acta* **1990**, *1054*, 14−20.
(39) Kitz, R.; Wilson, I. B. Esters of methanesulfonic acid as irreversible inhibitors of acetylcholinesterase. *J. Biol. Chem*. **1962**, *237*, 3245−3249.

JM050030I