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The Myeloablative Drug Busulfan Converts Cysteine to Dehydroalanine and Lanthionine in Redoxins

Michele Scian[†], Miklos Guttman[†], Samantha D. Bouldin[‡], Caryn E. Outten[‡], and William M. Atkins^{†,*}

[†]Department of Medicinal Chemistry, University of Washington, Box 357610, Seattle, Washington 98195-7610, United States

[‡]Department of Chemistry and Biochemistry, University of South Carolina, Columbia, South Carolina 29208, United States

Abstract

The myeloablative agent busulfan (1,4-butane-diol dimethanesulfonate) is an old drug that is used routinely to eliminate cancerous bone marrow prior to hematopoietic stem cell transplant. The myeloablative activity and systemic toxicity of busulfan have been ascribed to its ability to cross-link DNA. In contrast, here we demonstrate that incubation of busulfan with the thiol redox proteins glutaredoxin or thioredoxin at pH 7.4 and 37 °C results in the formation of putative *S*-tetrahydrothiophenium adducts at their catalytic Cys residues, followed by β -elimination to yield dehydroalanine. Both proteins contain a second Cys, in their catalytic C-X-X-C motif, which reacts with the dehydroalanine, the initial Cys adduct with busulfan, or the *S*-tetrahydrothiophenium, to form novel intramolecular cross-links. The reactivity of the dehydroalanine (DHA) formed is further demonstrated by adduction with glutathione to yield a lanthionine and by a novel reaction with the reducing agent tris(2-carboxyethyl)phosphine (TCEP), which yields a phosphine adduct via Michael addition to the DHA. Formation of a second quaternary organophosphonium salt via nucleophilic substitution with TCEP on the initial busulfan-protein adduct or on the THT⁺-Redoxin species is also observed. These results reveal a rich potential for reactions of busulfan with proteins *in vitro*, and likely *in vivo*. It is striking that several of the chemically altered protein products retain none of the atoms of busulfan, in contrast to typical drug-protein adducts or traditional protein modification reagents. In particular, the ability of a clinically used drug to convert Cys to dehydroalanine in intact proteins, and its subsequent reaction with biological thiols, is unprecedented.

Graphical Abstract

*Corresponding Author: wink@u.washington.edu. Fax: 206 685 0379.

Notes

The authors declare no competing financial interest.

Supporting Information

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MS/MS spectra of DHA-Thx, THT⁺-Thx, Cyclo-Thx, and CL-Thx, theoretical and observed masses for peptides with modifications (Table S1), and a plot of $\ln([B][A_0]/[B_0][A])$ versus time for reactions of busulfan with Grx-1 (Figure S3) (PDF)

The myeloablative drug busulfan is used widely to precondition patients with leukemia, lymphoma, or other hematologic disorders prior to hematopoietic stem cell transplant (HSCT).^{1–3} Although busulfan has been a “drug of choice” for HSCT protocols for many years, it has a narrow therapeutic index and is associated with severe hepatic toxicity and other side effects.^{4–8} Busulfan is historically considered to be a DNA alkylating agent that disrupts DNA repair and replication.³ Specifically, the N⁷ position of guanine has been shown to react with busulfan, and DNA cross-links have been identified in cells treated with busulfan.^{9–12} Interestingly, not all alkylating agents are equally reactive with DNA, and busulfan-induced protein–DNA cross-links may be more toxic than DNA cross-links, based on comparison to hepsulfam in some cell models.¹⁰ In fact, the mechanism of cytotoxicity of busulfan in target cells could include its reaction with proteins, but its reactivity with proteins has not been characterized. Interestingly, it has been suggested that busulfan could cause conversion of Cys residues in proteins to dehydroalanine, which occurs with the reaction of busulfan with glutathione (GSH), but this has not been demonstrated experimentally. Hypothetically, busulfan would react most readily with Cys residues that participate in catalytic functions and are most nucleophilic at pH 7.4. Here, we demonstrate the ability of busulfan to convert Cys residues of two thiol-reactive redox enzymes to dehydroalanine (DHA), and their subsequent reaction with thiol nucleophiles such as glutathione (GSH). Furthermore, the busulfan-dependent generation of DHA in these proteins allowed incorporation of the phosphine tris(2-carboxyethyl)phosphine (TCEP; MW = 250.19 Da) to yield stable protein-phosphine adducts, demonstrating the utility of DHA for expanding the available range of accessible protein modifications. Although there has been significant effort aimed to generate DHAs in proteins for purposes of protein engineering,^{13–16} the ability of a clinically used drug to mediate such reactions is unprecedented. The results have significant implications for the therapeutic and toxic mechanisms of busulfan and for potential applications in protein engineering. The range of busulfan-mediated chemical reactions observed with these redoxins suggests that its therapeutic and toxic effects may not be limited to DNA alkylation.

EXPERIMENTAL PROCEDURES

Chemicals

All chemicals were purchased from Sigma-Aldrich with the exception of TCEP (Hampton Research). Human thioredoxin-1 (Thrx-1) was purchased from Thermo-Fisher Scientific [~57% Met1-cleaved, >95% pure as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), 1 mg/mL in 20 mM HEPES (pH 7.5), specific activity of

8.15 units/mg based on an insulin reduction assay, $\epsilon_{280} = 6970 \text{ M}^{-1} \text{ cm}^{-1}$], while human glutaredoxin-1 [Grx-1, 100% Met1-cleaved, >90% pure as determined by SDS-PAGE, 16.3 mg/mL in 50 mM Tris-HCl and 5 mM TCEP (pH 7.5), specific activity of 220 units/mg based on an HED assay, $\epsilon_{280} = 3160 \text{ M}^{-1} \text{ cm}^{-1}$] and the C26S mutant [100% Met1-cleaved, >90% pure as determined by SDS-PAGE, 12.7 mg/mL in 50 mM Tris-HCl and 5 mM TCEP (pH 7.5), $\epsilon_{280} = 3040 \text{ M}^{-1} \text{ cm}^{-1}$] were expressed in *Escherichia coli* and purified as previously reported.^{17,18}

Incubation of Grx-1 and Thrx-1 with Busulfan

Incubations of purified protein were performed at 37 °C for varying times up to 24 h in phosphate-buffered saline (PBS) containing 250 μM TCEP and 2% (v/v) DMSO, at pH 7.4, and under agitation at 160 rpm. The initial busulfan:protein molar ratio was invariably set to 20 (i.e., 50 μM protein and 1 mM busulfan). In some instances, the pH of Grx-1 incubation mixtures at 24 h was adjusted to 8.5 with 1.0 M NaOH and the mixtures were incubated for an additional 24 h at 37 °C to favor β -elimination from residual THT⁺-protein adducts (non-CL-Redoxin). For the reaction time course experiments, aliquots were taken at variable time points and immediately stored at -80 °C. Each aliquot was then thawed immediately before mass spectrometric analysis. To generate busulfan-mediated Grx-1-TCEP covalent adducts, incubations were conducted as described above but in the presence of 5 mM TCEP (1:20:100 Grx-1:busulfan:TCEP).

Incubation of DHA-Grx-1 with Glutathione

Grx-1 solutions previously incubated for 24 h with busulfan in the presence of 250 μM TCEP were first buffer exchanged [PBS and 250 μM DTT (pH 7.4)] with a Zeba Spin desalting column (7 kDa MWCO) to remove busulfan and subsequently incubated with 5 mM GSH at 37 °C and 160 rpm for 24 h. Finally, the mixture was treated with 10 mM DTT for 1 h at RT to reduce possible disulfide bonds. The product was directly analyzed by LC-MS (intact protein analysis) or by LC-MS/MS after trypsin digestion.

Alkylation Experiments with Grx-1 and Grx-1-TCEP Adducts

Grx-1 solutions previously incubated for 24 h with busulfan in the presence of 5 mM TCEP were directly treated with 20 mM iodoacetamide (IAM) at pH 8.0 for 1 h at RT in the dark. Alternatively, Grx-1 solutions incubated for 24 h with busulfan in the presence of 250 μM TCEP were first buffer exchanged [50 mM Tris-HCl and 250 μM TCEP (pH 7.0)] with a Zeba Spin desalting column (7 kDa MWCO, Thermo-Fisher Scientific) to remove busulfan and subsequently alkylated with 20 mM IAM (pH 7.0) at RT for 4 h in the dark.

Grx-1 and Thrx-1 Tryptic Digestion

Grx-1, Thrx-1, and their busulfan-modified products were proteolyzed with sequencing-grade modified trypsin (100 $\mu\text{g/mL}$ in 50 mM acetic acid, Promega) for mass spectrometry analysis. Wherever applicable, protein samples were previously depleted of unreacted busulfan (or GSH) by buffer exchange (Zeba Spin desalting column, 7 kDa MWCO) with PBS (pH 7.4).

Briefly, 10 μL aliquots of an $\sim 500 \mu\text{g/mL}$ protein solution ($\sim 5 \mu\text{g}$ of total protein) were denatured at RT with 8 M urea. After addition of Tris-HCl (pH 8.0) to a final concentration of 50 mM, dithiothreitol (DTT) was added to a final concentration of 10 mM. The resulting mixture was left at RT for 1 h. Subsequently, IAM was added to a final concentration of 20 mM, and the mixture was incubated at RT in the dark for 1 h, after which fresh DTT was added to a final concentration of 10 mM to quench the unreacted IAM. The resulting mixture ($\sim 20 \mu\text{L}$) was then diluted 8-fold ($\sim 160 \text{ L}$ final volume) with 50 mM ammonium bicarbonate and 1 mM CaCl_2 (pH 8.0), supplemented with sequencing-grade modified trypsin to a 1:20 trypsin:protein mass ratio, and incubated overnight at 37°C . Acetonitrile (ACN) was then added to a final concentration of 5% (v/v), and the pH dropped to ~ 2.5 after the addition of formic acid (LC-MS-grade, Thermo-Fisher Scientific) to quench the residual trypsin activity.

Alternatively, reduction with DTT (10 mM for 2 h), IAM alkylation (20 mM for 4 h), quenching with DTT (10 mM), and overnight trypsin digestion were conducted at pH 7.0 in 50 mM Tris-HCl (with 1 mM CaCl_2 for trypsin digestion) to minimize cyclization (i.e., cyclo-Grx1 and/or CL-Grx1 formation) during sample processing.

Mass Spectrometry

Samples were analyzed on a SYNAPT G2-Si quadrupole time-of-flight mass spectrometer (Waters, Milford, MA). To ensure high mass accuracy throughout each analysis, a lock mass (leucine enkephalin, $[\text{M} + \text{H}]^+ = 556.2771 \text{ Da}$) was sampled every 60 s during the run.

For the intact protein LC-MS analysis, a total of $\sim 1 \mu\text{g}$ was loaded on a POROS-R1 column ($150 \text{ mm} \times 2.1 \text{ mm}$, $10 \mu\text{m}$ particle size, Applied Biosystems) and resolved with a binary mobile-phase linear gradient [A being 0.1% (v/v) formic acid and B being ACN with 0.1% (v/v) formic acid] from 10 to 95% B over the course of 17 min, at a flow rate of 0.3 mL/min. MS spectra were recorded in positive mode, scanning through an m/z range of 200–3000 Da.

In all instances where it was necessary to estimate the fractional content of modified versus unmodified protein in a protein/busulfan reaction mixture, smoothed peaks of identical charge states were integrated and the fractional area was calculated [e.g., $X_A = A^{+X}/(A^{+X} + B^{+X} + C^{+X})$, where A^{+X} , B^{+X} , and C^{+X} are the computed integrals of DHA-bound protein, THT⁺-bound protein, and unmodified protein, respectively]. This approach, however, is based on the assumption that both the native and modified protein species shared equivalent ionization efficiencies. Moreover, because the isobaric species DHA-Redoxin and Cyclo-Redoxin cannot be distinguished by ESI-MS, they were integrated and considered as a single product. A similar approach was applied to THT⁺-Redoxin and CL-Redoxin.

For the LC-MS/MS analysis of Grx-1 tryptic digests, $\sim 750 \text{ ng}$ of total protein digest was resolved on a UPLC BEH C18 column ($100 \text{ mm} \times 1.0 \text{ mm}$, $1.7 \mu\text{m}$ particle size, Waters) and subjected to a linear gradient [A being 0.1% (v/v) formic acid and B being ACN with 0.1% (v/v) formic acid] from 5 to 50% B over the course of 24 min, at a flow rate of 0.08 mL/min. Identical conditions were used for Thrx-1 tryptic digests, with a UPLC BEH C8 column ($150 \text{ mm} \times 1.0 \text{ mm}$, $1.7 \mu\text{m}$ particle size, Waters).

The MS spectra were recorded in data-dependent acquisition (DDA) mode, with a survey scan of 0.2 s through an m/z range of 300–2000 Da, and a subsequent MS/MS scan from 50 to 2000 Da for 2 s with the trap collision energy set to 30 eV.

Targeted analyses with identical LC–MS runs were also performed to characterize the low-abundance species, DHA-peptide and THT⁺-peptide. The selected ions were $[M + 3H]^{3+}$ and $[M + 3H]^{4+}$ for Grx-1 and $[M + 2H]^{2+}$ and $[M + 2H]^{3+}$ for Thrx-1. Similarly, the $[M + 3H]^{3+}$ and $[M + 4H]^{4+}$ ions were selected for targeted analysis of the GSH adduct with the DHA-bound peptide from Grx-1.

Data acquisition, processing, and visualization were performed using MassLynx (Waters). Peptides were manually assigned using exact mass and MS/MS spectra with the aid of Protein Prospector (prospector.ucsf.edu). In all instances, the fragment mass error was found to be 10 ppm.

RESULTS AND DISCUSSION

Whole Protein Analyses

By analogy to the proposed *in vivo* metabolism of busulfan,^{19,20} which results in γ -glutamyl-dehydroalanyl-glycine (EdAG) formation via an initial conjugation with the Cys residue of GSH presumably followed by β -elimination, we speculated that reactive and accessible Cys residues of fully folded proteins would undergo a similar reaction with busulfan under physiological conditions. To test this hypothesis, we explored the reactivity of busulfan with human cytosolic glutaredoxin (Grx-1) and human thioredoxin (Thrx-1). We have recently shown that Grx-1^{17,18} and Thrx-1 (data not published) react with the busulfan metabolite EdAG forming a nonreducible lanthionine adduct. Because these redoxins contain a conserved C-X-X-C motif in which the N-terminal Cys residue displays an unusually low pK_a (~3.5 and ~6.3 for Grx-1 and Thrx-1, respectively),^{21,22} they could plausibly function as additional busulfan targets *in vivo* or reveal new chemical reactions that could occur with other proteins containing reactive Cys residues.

On the basis of the reaction with GSH, anticipated products of the reaction of busulfan with redoxins included the *S*-tetrahydrothiophenium adduct (THT⁺-Redoxin) and the DHA-Redoxin resulting from the subsequent base-catalyzed β -elimination (Scheme 1). Incubations with either Grx-1 or Thrx-1 were conducted at 37 °C with 50 μ M redoxin in PBS (pH 7.4) containing 250 μ M TCEP, 1.0 mM busulfan, and 2% (v/v) DMSO. Under these conditions, species with masses of $[MH - 34]^+$ and $[M + 55]^+$ compatible with the expected products were detected by intact protein LC–MS and their formation and relative abundance monitored throughout a 24 h reaction time course (Figure 1). Note that a fraction (~57%) of the Thrx-1 used lacked the N-terminal methionine, and this produced heterogeneity for the initial protein as well as after reaction with busulfan as shown in Figure 1b. However, both Thrx-1 starting species yielded the THT⁺-Redoxin and the DHA-Redoxin at comparable rates. For both Grx-1 and Thrx-1, the DHA-Redoxin is readily observed. Also, in Figure 1, the putative species corresponding to THT⁺-Redoxin is labeled $[MH + 54]^+/[M + 55]^+$ for reasons described below. It is important to emphasize that, as initially predicted, only products derived from reaction of busulfan with one Cys residue per redoxin

molecule were detected at a significant level. It should also be noted that the initial busulfan-redoxin adduct (Bu-Redoxin, $[MH + 150]^+$), which is the likely first intermediate along the DHA-Redoxin formation pathway (Scheme 1), was never detected, hence suggesting a relatively fast conversion to THT^+ -Redoxin.

Analysis of the reaction time course of both redoxins revealed incomplete conversion of the protein to the expected products due to competition with slow oxidation of the catalytic cysteines, despite the presence of an initial 5-fold molar excess of TCEP/redoxin. This phenomenon, which appeared to be more severe for Thrx-1, was not observed in samples incubated under anaerobic conditions (data not shown). Moreover, when a 100-fold molar excess of TCEP (i.e., 5 mM) was used in an attempt to prevent protein oxidation, two new species were detected, with a 250 Da mass increase compared to the masses of THT^+ -Redoxin and DHA-Redoxin (Figure 2). These species were identified as TCEP adducts likely formed via one or both of the reaction mechanisms proposed in Scheme 1. In particular, Phospho-DHA-Redoxin could be formed by a phospho-Michael addition^{23,24} of the phosphine (TCEP) to DHA-Redoxin or by nucleophilic attack of TCEP (via SN_2) at C2 of THT^+ -Redoxin with concomitant elimination of THT. Similarly, the Phospho- THT^+ -Redoxin could result from the nucleophilic attack of TCEP on the initial Bu-Redoxin adduct with concomitant elimination of the mesylate leaving group ($CH_3SO_3^-$), or from TCEP nucleophilic attack at C2 of the sulfonium ion ring in THT^+ -Redoxin. Regardless of the mechanism, the stable adduction by TCEP clearly demonstrates the incorporation of electrophilic sites into the redoxins as a result of reaction with busulfan.

From the reaction time course (Figure 1), it was also observed that the relative amounts of the putative THT^+ -Redoxin and DHA-Redoxin species stopped changing at long incubation times. This behavior was unexpected because, in the absence of further redoxin depletion, THT^+ -Redoxin was expected to convert monoexponentially to DHA-Redoxin (first-order reaction). The relative THT^+ -Redoxin and DHA-Redoxin content (as found at a 24 h incubation time) did not change significantly after dialysis for 6 h in PBS at pH 7.4 and 4 °C, or after a further 24 h incubation at pH 8.5 and 37 °C. The higher pH would favor β -elimination from putative THT^+ -Redoxin. The lack of β -elimination suggested the possibility of alternative reaction pathways and prompted us to further investigate the $[M + 55]^+$ species.

The presence of two Cys residues in the proximity of each other in the active site of Grx-1 and Thrx-1 (C-X-X-C motif) and the knowledge of their role in catalysis lead to the hypothesis that stable cyclic species could be generated, which would compete with conversion of the THT^+ -Redoxin to DHA-Redoxin, and explain the time course observed in Figure 1. In particular, we considered the possible formation of a cross-linked species (CL-Redoxin), distinct from THT^+ -Redoxin. This species, which represents a protein analogue of the busulfan-generated DNA intrastrand cross-link, would be indistinguishable from THT^+ -Redoxin upon being examined by mass spectrometry. Formation of CL-Redoxin would require nucleophilic substitution of the mesylate group in the initial Bu-Redoxin adduct by the thiol group of the second Cys residue, or nucleophilic attack at C2 of the sulfonium ion ring in THT^+ -Redoxin by the thiol group (Scheme 1).

Similarly, a second cyclic species (Cyclo-Redoxin) could be formed from DHA-Redoxin via a thiol-ene-based Michael addition reaction (Scheme 1). Because Michael addition can be catalyzed by phosphines,^{25,26} Cyclo-Redoxin formation could be favored by the presence of the reducing agent TCEP in the incubation mixture. Alternatively, Cyclo-Redoxin could result from nucleophilic attack of the thiol group at C2 of THT⁺-Redoxin with concomitant elimination of THT. Notably, DHA-Redoxin and Cyclo-Redoxin are isobaric and therefore cannot be distinguished by mass spectrometry.

In an effort to determine whether, and to what extent, the cyclic species were formed, Grx-1 samples previously incubated with busulfan for 24 h at 37 °C (at pH 7.4 and in the presence of 250 μ M TCEP) were rapidly buffer exchanged with 50 mM Tris-HCl and 250 μ M TCEP (pH 7.0) and then alkylated with 20 mM iodoacetamide (IAM) at RT for 4 h. Identical conditions had previously proven to be suitable for quantitatively alkylating each of the five Cys residues of Grx-1 (data not shown). While Tris-HCl was used both as a buffer and as a large source of amino groups to minimize spurious alkylation on lysines, a pH of 7.0 was used both to increase selectivity for thiols over amines and to minimize CL-Redoxin and Cyclo-Redoxin formation during the alkylation process. After alkylation, the samples were directly subjected to intact protein LC-MS analysis.

Alkylation provided a simple strategy for discriminating unambiguously between DHA-Grx-1 and Cyclo-Grx-1 and also between THT⁺-Grx-1 and CL-Grx-1. Grx-1 contains a total of five Cys residues, including the two found in the C-X-X-C motif. As a result, while DHA-Grx-1 and THT⁺-Grx-1 have four Cys residues available for alkylation, CL-Grx-1 and Cyclo-Grx-1 are expected to only have three. Therefore, by a simple analysis of the mass shift associated with the alkylation reaction, the nature of the species present in solution can be inferred, as long as complete alkylation is achieved. As shown in Figure 3a, alkylation caused a mass shift of 171 Da in two of the three species initially present (the third being the unmodified Grx-1 and hence displaying a mass shift of $5 \times 57 = 285$ Da), corresponding to S-carbamidomethylation (S-CAM) of three Cys residues. These results can be explained only by the initial presence of CL-Grx-1 and Cyclo-Grx-1 in the samples.

To confirm and validate this finding, we further studied the unexpected formation of Phospho-THT⁺-Grx-1 and Phospho-DHA-Grx-1 observed when incubations were performed in the presence of a large TCEP molar excess, as shown in Figure 2. For this, samples of 50 μ M Grx-1 previously incubated with busulfan in the presence of 5 mM TCEP (24 h at pH 7.4 and 37 °C) were alkylated with 20 mM iodoacetamide at pH 8.0 and RT for 1 h. In this case, the TCEP adducts, which unequivocally retain four Cys residues available for alkylation, also served as an internal probe of alkylation efficiency. Both these adducts displayed a mass shift of 228 Da (57×4 Da) upon IAM alkylation, thus confirming the structures shown in Scheme 1. The other species present showed a mass shift of 171 Da corresponding to S-carbamidomethylation of three Cys residues, in complete agreement with the previous results. Conceivably, the species at 11929 Da in Figure 3b represents an unresolved mixture of the fully alkylated Grx-1 ($[MH]^+ = 11930$ Da) and the fully alkylated THT⁺-Grx-1 ($[MH]^+ = 11927$ Da).

Although these results indicate that the main products of the incubation with busulfan (at 24 h) were CL-Grx-1 and Cyclo-Grx-1 rather than DHA-Grx-1 and THT⁺-Grx-1, it is possible that cyclization could have occurred, at least to some extent, during the alkylation process.

An alternative approach was also taken to selectively confirm the presence of DHA-Redoxin. Specifically, we attempted to trap any DHA-Grx-1 present at the end of the incubation with GSH. Busulfan-treated Grx-1 samples were rapidly buffer exchanged with PBS and 250 μ M DTT (or TCEP) (pH 7.4) to remove the unreacted busulfan, followed by addition of 5 mM GSH at 37 °C for 24 h. After treatment with 10 mM DTT at RT for 1 h to reduce redoxin/GSH disulfides that possibly formed, the samples were directly analyzed by LC-MS. Although protein precipitation was observed at 24 h, making quantification imprecise, the glutathione/DHA-Grx-1 adduct (GS-DHA-Grx-1) was detected (Figure 4), indicating that this species must be present. Qualitatively, significantly less GSH adduct was obtained versus the amount of Cyclo-Redoxin at the end of the 24 h incubation with busulfan.

It is also important to note that DTT adducts with DHA-Grx-1 were not detected under these experimental conditions, indicating relatively low reactivity of DTT toward this Michael acceptor or complete conversion of DHA-Grx-1 to GS-DHA-Grx-1.

Residue Identification for Grx-1 via MS/MS

To determine the specific amino acid residues that were adducted in the proposed species, LC-MS/MS analyses were performed on trypsin-digested samples. For these experiments, sample reduction (with DTT), alkylation (with IAM), and trypsin digestion were typically conducted at pH 8.0. However, for low-abundance species, these steps were conducted at pH 7.0 in an effort to minimize conversion of THT⁺-Redoxin to DHA-Redoxin as well as cyclization of the latter to Cyclo-Redoxin during the sample processing.

For Grx-1, the tryptic peptide 15-VVVF^{IK}PTCPYCR-27 was anticipated to contain all the chemical modifications proposed above. The expected monoisotopic masses were as follows: Cyclo-peptide [MH]⁺ = 1490.82 Da; CL-peptide [MH]⁺ = 1578.85 Da; DHA-peptide [MH]⁺ = 1547.84 Da; THT⁺-peptide [M]⁺ = 1635.87 Da; Phospho-DHA-peptide [M]⁺ = 1797.90 Da; Phospho-THT⁺-peptide [M]⁺ = 1885.94 Da; GS-DHA-peptide [MH]⁺ = 1854.92 Da.

In the LC-MS spectra of tryptic digests from Grx-1 samples incubated with busulfan in the presence of 250 μ M TCEP, precursor ions corresponding to Cyclo-peptide ([M + 3H]³⁺ = 497.61 Da), CL-peptide ([M + 3H]³⁺ = 526.96 Da), DHA-peptide ([M + 3H]³⁺ = 516.62 Da), and THT⁺-peptide ([M + 3H]⁴⁺ = 409.72 Da) were indeed detected. Figure 5 shows the fragmentation pattern of Cyclo-peptide and CL-peptide. The absence of y₂-y₄ ions further attests to the cyclic nature of these species. In the MS/MS spectra of THT⁺-peptide and DHA-peptide (Figure 6), the presence of y₂-y₄ ions as well as of y₅-y₁₂ ions identified the ninth residue as the site of modification, hence specifying Cys-23 of Grx-1 as the site of reaction with busulfan. Notably, the MS/MS spectrum of THT⁺-peptide presents y₅-y₁₀ ions characteristic of the DHA-peptide, thus implying a gas-phase decomposition of this species to form DHA-peptide. Moreover, in tryptic digests of samples incubated with busulfan in the

presence of 5 mM TCEP, precursor ions corresponding to Phospho-DHA-peptide ($[M + 3H]^{4+} = 450.23$ Da) and Phospho-THT⁺-peptide ($[M + 3H]^{4+} = 472.24$ Da) were also detected and the species identified by MS/MS (Figure 7). Because the phosphonium moiety was found to be in the ninth residue, these unique peptides provided confirmation of the initial site of reactivity of busulfan. Similarly, two precursor ions ($[M + 3H]^{3+} = 618.98$ Da and $[M + 4H]^{4+} = 464.49$ Da) corresponding to GS-DHA-peptide were found in trypsin digests of samples incubated with GSH. Also, in this instance, MS/MS data (Figure 8) unambiguously identified the peptide and the location (i.e., residue 9) of the modification.

Residue Identification for Thrx-1 via MS/MS

LC-MS/MS analyses were also conducted on Thrx-1 trypsin digests, in which the peptide 22-LVVVDFSATWCGPCK-36 was anticipated to bear the busulfan-induced chemical modifications. The expected monoisotopic masses were as follows: Cyclo-peptide $[MH]^+ = 1590.80$ Da; CL-peptide $[MH]^+ = 1678.83$ Da; DHA-peptide $[MH]^+ = 1647.82$ Da; THT⁺-peptide $[M]^+ = 1735.85$ Da.

Precursor ions corresponding to Cyclo-peptide ($[M + 2H]^{2+} = 795.90$ Da), CL-peptide ($[M + 2H]^{2+} = 839.92$ Da), DHA-peptide ($[M + 2H]^{2+} = 824.41$ Da), and THT⁺-peptide ($[M + 2H]^{3+} = 579.29$ Da) were found in LC-MS spectra of tryptic digests from samples incubated with busulfan in the presence of 250 μ M TCEP. The peptides were identified by MS/MS analysis (Figure S1), which indicated that the 11th peptide residue was the site of modification, hence identifying Cys-32 of Thrx-1 as the site of reaction with busulfan.

On the basis of the combined results for both Grx-1 and Thrx-1, LC-MS/MS results from trypsin digestions conducted at both pH 7.0 and 8.0, it appears that the most abundant species were the cyclic peptides CL-Redoxin and Cyclo-Redoxin, and that even at pH 7.0, only small amounts of THT⁺-peptide and DHA-peptide were present. Of course, some cyclization could have occurred during reduction, alkylation, and overnight trypsin digestion.

Also, the possibility of busulfan reacting at alternative cysteines was carefully investigated. Specifically, all of the available LC-MS/MS data sets were analyzed for possible adducts or chemical modification at the other Cys residues, but no modifications were found for either of the redoxins. This is indicative of the much greater nucleophilicity at pH 7.4 of Cys residues that participate in catalytic function compared to that of Cys residues that lie outside of active sites.

On the basis of the MS/MS analysis that yielded the specific sites of adduction for the TCEP adducts or busulfan adducts and the specific sites of DHA formation within the primary sequence of Grx-1, it is useful to map them onto the three-dimensional structure, as shown in Figure 9, where the residues that initially react with busulfan to form DHA are indicated, along with the other Cys that participates in subsequent formation of Cyclo-Redoxin or CL-Redoxin.

Studies with the C26S Mutant

Finally, to confirm that Cyclo-Redoxin and CL-Redoxin are formed in competition with the formation of TCEP adducts and to further confirm that the cyclic species result from nucleophilic attack by Cys-26 of Grx-1 on either Bu-Redoxin, THT⁺-Redoxin, or DHA-Redoxin, the C26S Grx-1 mutant was incubated with busulfan. This mutant has been characterized previously.²⁷ Consistent with the mechanism proposed in Scheme 1, no Cyclo-Grx-1 or CL-GRX-1 was detected under conditions that yielded both from wild-type Grx-1. As expected, DHA-C26S-Grx-1 was formed, and interestingly, a significant amount of TCEP adduct (in particular Phospho-DHA-C26S-Grx-1) was detected even in the presence of a low concentration of TCEP (250 μ M). The results with C26S-Grx-1 confirm the overall mechanism shown in Scheme 1.

Comparison of Reactivity with GSH

As noted above, it is established that low concentrations of EdAG can be detected in patients treated with busulfan.^{19,20} Therefore, the possible biological significance of our results depends on the ability of Cys residues in proteins to compete with GSH for reaction with busulfan. Because GSH is present at high concentrations in tissue (~1 mM), it might be expected to outcompete Cys residues in intact proteins. However, the thiolate form of GSH is the reactive form, and the Cys p*K*_a is ~9 in GSH, which results in 1.56% of the total pool of GSH existing as the reactive GS[−] at pH 7.4. The p*K*_a of Cys-23 of Grx-1 is ~4, which results in 99.9% of the active site Cys existing in the thiolate form. Thus, for equivalent concentrations of GSH and redoxin, the redoxin would be expected to react 64-fold faster than GSH. In fact, the nonenzymatic rate of reaction of GSH with busulfan has been reported to be 2.3 M^{−1} h^{−1}.²⁸ An approximate bimolecular rate constant for the reaction of busulfan with Grx-1 and Thrx-1 can be obtained from the early times points of Figure 1. Specifically, for a bimolecular reaction, the integrated rate equation is

$$\ln \frac{[B][A]_0}{[A][B]_0} = k([B]_0 - [A]_0)t$$

where [B] and [A] are the concentrations of GSH and busulfan at time *t*, respectively, and [B]₀ and [A]₀ are the initial concentrations. A plot of ln([B][A]₀/[A][B]₀) versus time yields a line with a slope of *k*([B]₀ − [A]₀), which yields a bimolecular rate constant of 111.3 M^{−1} h^{−1} (Figure S3). Note that the rate constant obtained from the complete data set in Figure 1 would underestimate the actual rate of reaction because busulfan undergoes hydrolysis on a time scale comparable to that of its reaction with the redoxin, and the latter sustained oxidation despite the presence of a small amount of reducing agent.²⁹ This complexity was not incorporated in our estimate. However, it is instructive that the recovered bimolecular rate constant for reaction of GRx-1 with busulfan is approximately 50-fold faster than the rate constant for GSH reacting with busulfan reported previously, which is very close to the difference predicted on the basis of the p*K*_as of GSH versus the redoxins. Thus, proteins at concentrations much lower than that of GSH could efficiently compete for reaction with busulfan, if they have reactive Cys residues with low p*K*_as. Such proteins would be even more likely to react during conditions of oxidative stress, in which GSH is depleted. Also,

we emphasize that we chose Grx-1 and Thrx-1 as models to demonstrate the previously unpredicted reactions that yield intramolecular cross-links and the ability of busulfan to react with intact proteins, in general. It is quite possible that other redoxins or other enzymes with acidic Cys residues could react faster than Grx-1 or Thrx-1.

CONCLUSIONS

Busulfan reacts with catalytic cysteines in two redox proteins, Grx-1 and Thrx-1, followed by a spectrum of reactions that collectively yield several novel protein species. The various reaction products are summarized in Scheme 1. The unambiguous assignment of the broad range of products resulting from multiple possible pathways emphasizes the rich landscape of chemistry available to proteins that interact with busulfan. The rearrangement of an initial busulfan adduct, followed by β -elimination to yield DHA, is especially noteworthy.

The reactions observed here are important for two specific reasons. Speculatively, the new chemistry demonstrated here could expand the set of reagents and strategies available for protein engineering or chemical modification, although additional studies will be needed to optimize it. While attempts to incorporate DHA into folded proteins have been widespread with modest success, they typically require high concentrations of nonaqueous solvent, high pH, or special bacterial strains with engineered tRNA pools or tRNA synthetases.³⁰ It is striking that a drug used clinically for decades can facilitate this protein modification under physiologic conditions. The resulting DHA provides an electrophilic center for chemical elaboration by biological thiols. Regardless, it is remarkable that a compound with this potential is actually used clinically in a large number of patients.

With regard to the second point of importance, the results are the first data that support the suggestion that Cys residues in proteins could be converted to DHA by busulfan.^{31,32} In addition, the data extend that suggestion by demonstrating that Cys residues that participate in catalytic function with low pK_a s are most susceptible. It should be emphasized that the redoxins represent a unique situation for the reaction with busulfan, because of the presence of two Cys residues in a single active site. This allows for the intramolecular cross-links observed in CL-Redoxin and Cyclo-Redoxin. However, it is reasonable to expect busulfan to react with other proteins with individual, isolated, Cys residues to generate DHA that does not subsequently react with Cys residues on the same protein. As a result, intermolecular cross-links with other proteins or irreversible glutathionylation would be more likely. Therefore, it is reasonable to consider the likelihood that busulfan reacts with proteins *in vivo* to ultimately result in irreversibly glutathionylated proteins, cross-linked proteins, or proteins with other covalent adducts in addition to reactions with redoxins.

It is important to stress that this possibility extends to many proteins other than Grx and Thrx. Many other proteins contain reactive Cys residues, either in C-X-X-C motifs or in other structural environments, and they could react with busulfan. Examples include other redoxins, acyl transferases, a wide range of cysteine proteases, and others. Therefore, we do not suggest that reactions with Grx and Thrx are the only interactions of busulfan with the proteome. Possibly, a complex combination of protein targets contributes to the pharmacology of busulfan, this “classic” drug. In effect, the reactivity of busulfan could be

spread across the proteome rather than targeted to Grx or Thrx, and this would likely include proteins with Cys residues that are activated for participation in catalysis. Additional studies will be required to explore these possibilities *in vivo*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ACN	acetonitrile
DHA	dehydroalanine
DMSO	dimethyl sulfoxide
DTT	DL-dithiothreitol
HEPES	4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid
EdAG	γ -glutamyl-dehydroalanyl-glycine
HED	hydroxyethyl disulfide
HSCT	hematopoietic stem cell transplant
IAM	iodoacetamide
Grx-1	glutaredoxin-1
GSH	glutathione
LC-MS	liquid chromatography-mass spectrometry
MW	molecular weight
MWCO	molecular weight cutoff
PBS	phosphate-buffered saline
RT	room temperature
TCEP	tris(2-carboxyethyl)phosphine
Thrx	thioredoxin
Tris-HCl	tris(hydroxymethyl)-aminomethane hydrochloride

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Figure 1.

Deconvoluted ESI-MS spectra of (a) Grx-1 or (b) Thrx-1 incubated with busulfan for 24 h at pH 7.4 and 37 °C in the presence of 250 μ M TCEP and after reduction with 10 mM DTT for 1 h at RT. The native redoxin, DHA-Redoxin, and putative THT⁺-Redoxin are clearly present. In panel b, the asterisk indicates Met1-cleaved Thrx-1 species. The insets show the time course of formation or depletion of the species present. The relative amount (percent of total protein) of each species was calculated from the fractional area of the most intense charge state (i.e., $[M + 11H]^{11+}$ or $[M + 10H]^{11+}$) in the MS spectra. The isobaric species DHA-Redoxin and Cyclo-Redoxin were integrated as if they were a single species. A similar approach was applied to THT⁺-Redoxin and CL-Redoxin.

Figure 2. Deconvoluted ESI-MS spectrum of Grx-1 incubated with busulfan for 24 h at pH 7.4 and 37 °C in the presence of 5 mM TCEP. Two new species with a mass shift of 250 Da were identified as TCEP adducts, $[M - 33 + 250]^+$ or $[M + 55 + 250]^+$.

Figure 3.

Products of iodoacetamide alkylation of Grx-1 samples previously incubated with busulfan (24 h at pH 7.4 and 37 °C) in the presence of (a) 250 μ M or (b) 5 mM TCEP. Grx-1/busulphan products formed in the presence of 250 μ M TCEP (a) had three cysteines available for S-carbamidomethylation, whereas the TCEP adducts formed in the presence of 5 mM TCEP (b) conserved four cysteines amenable for IAM alkylation. These results suggested a cyclic nature of the Grx-1/busulphan products.

Figure 4. Deconvoluted mass spectrum of a busulfan-incubated Grx-1 sample (24 h at pH 7.4 and 37 °C in the presence of 250 μ M TCEP) treated with GSH (24 h at pH 7.4 and 37 °C the presence of 250 μ M DTT). A mass shift of 307 Da indicates formation of a DHA-Grx-1/ glutathione adduct.

Figure 5. High-mass accuracy MS/MS spectra of the Grx-1 tryptic peptides (a) Cyclo-peptide and (b) CL-peptide obtained from fragmentation of precursor ions with masses of 745.91 Da (i.e., $[M + 2H]^{2+}$) and 789.93 Da (i.e., $[M + 2H]^{2+}$), respectively. Selected fragment ions are highlighted. Atoms colored green belong to the side chains of cysteine residues, whereas those colored blue are from busulfan. The symbol "CAM" in the peptide sequence indicates that the specific Cys residue was S-carbamidomethylated with iodoacetamide.

Figure 6. MS/MS spectra of the Grx-1 tryptic peptides (a) THT⁺-peptide and (b) DHA-peptide obtained from fragmentation of precursor ions with masses of 409.72 Da (i.e., $[M + 3H]^{4+}$) and 516.62 Da (i.e., $[M + 3H]^{3+}$), respectively. Atoms colored green belong to side chains of cysteine residues, whereas those colored blue are from busulfan. Asterisks denote fragments characteristic of the DHA-peptide formed by gas-phase decomposition of the THT⁺-peptide.

Figure 7.

MS/MS spectra of the Grx-1 tryptic peptides (a) Phospho-DHA-peptide and (b) Phospho-THT⁺-peptide obtained from fragmentation of precursor ions with masses of 450.23 Da (i.e., $[M + 3H]^{4+}$) and 472.24 Da (i.e., $[M + 3H]^{4+}$), respectively. Atoms colored green belong to side chains of cysteine residues, whereas those colored blue are from busulfan or TCEP.

Figure 8.

MS/MS spectrum of the Grx-1 tryptic peptide GS-DHA-peptide obtained from fragmentation of precursor ions with a mass of 464.49 Da (i.e., $[M + 4H]^{4+}$). In addition to ions resulting from fragmentation of the peptide backbone, a characteristic neutral loss of pyroglutamic acid (pGlu, monoisotopic mass of 129.04 Da) is observed for many fragments (colored red). Atoms colored green belong to side chains of cysteine residues, whereas those colored blue are from busulfan or GSH.

Figure 9. Electrostatic potential surface and cartoon representation of Grx-1 (adapted from Protein Data Bank entry 1JHB, model 1) and Thrx-1 (adapted from Protein Data Bank entry 1ERT). The close-ups show the structural details of the catalytic Cys residues (residues 23 and 26 and residues 32 and 35 for Grx-1 and Thrx-1, respectively). Images were generated using PyMol Molecular Graphics System, version 1.2r1 (Schrödinger, LLC). Arrows indicate the catalytic Cys residues that specifically react with busulfan. Other Cys residues do not react.

Scheme 1.

Proposed Reaction Mechanism for Products Formed from Reaction of Busulfan with Redoxin Thiols