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Thioredoxin Cross-Linking by Nitrogen Mustard in Lung Epithelial Cells: Formation of Multimeric Thioredoxin/Thioredoxin Reductase Complexes and Inhibition of Disulfide Reduction

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Abstract

The thioredoxin (Trx) system, which consists of Trx and thioredoxin reductase (TrxR), is a major cellular disulfide reduction system important in antioxidant defense. TrxR is a target of mechlorethamine (methylbis(2-chloroethyl)amine; HN2), a bifunctional alkylating agent that covalently binds to selenocysteine/cysteine residues in the redox centers of the enzyme, leading to inactivation and toxicity. Mammalian Trx contains two catalytic cysteines; herein, we determined if HN2 also targets Trx. HN2 caused a time- and concentration-dependent inhibition of purified Trx and Trx in A549 lung epithelial cells. Three Trx cross-linked protein complexes were identified in both cytosolic and nuclear fractions of HN2-treated cells. LC-MS/MS of these complexes identified both Trx and TrxR, indicating that HN2 cross-linked TrxR and Trx. This is supported by our findings of a significant decrease of Trx/TrxR complexes in cytosolic TrxR knockdown cells after HN2 treatment. Using purified recombinant enzymes, the formation of protein cross-links and enzyme inhibition were found to be redox status-dependent; reduced Trx was more sensitive to HN2 inactivation than the oxidized enzyme, and Trx/TrxR cross-links were only observed using reduced enzyme. These data suggest that HN2 directly targets catalytic cysteine residues in Trx resulting in enzyme inactivation and protein complex formation. LC-MS/MS confirmed that HN2 directly alkylated cysteine residues on Trx, including Cys32 and Cys35 in the redox center of the enzyme. Inhibition of the Trx system by HN2 can disrupt cellular thiol-disulfide balance, contributing to vesicant-induced lung toxicity.

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Notes

The authors declare no competing financial interest.

Supporting Information

Characterization of HN2-adducted Trx and TrxR tryptic peptides identified from a database search (summarized in Table 2 and 3) are shown in Figure S1-S8. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Graphical abstract

Introduction

The thioredoxin (Trx) system consists of Trx, thioredoxin reductase (TrxR) and NADPH. It is a major cellular thiol-reducing system that plays a crucial role in cellular antioxidant defense and growth control.^{1–3} Mammalian cells possess two thioredoxin systems; one localized in the cytoplasm (Trx1) and another in the mitochondria (Trx2). Mammalian Trxs, which contain a conserved Cys-Gly-Pro-Cys active site sequence, function as disulfide reductases for a variety of enzymes including ribonucleotide reductases, peroxiredoxins, and methionine sulfoxide reductases, many of which are important in the control of DNA synthesis and repair, antioxidant defense, signal transduction, and protein repair.^{1, 4} Trx acts as an oxidoreductase via thiol/disulfide exchange reactions between two catalytic cysteine residues (Cys32 and Cys35) and protein substrates. Oxidized Trx1 is reversibly reduced by TrxR and NADPH. Trx1 contains additional cysteines including Cys62, Cys69, and Cys73, which are absent in Trx2.¹ These structural cysteines are known to be important in redox regulation of Trx1 activity and nitric oxide signaling.^{5, 6} Cysteine residues in Trx1 and Trx2 are targets for chemical modification by electrophilic compounds and oxidation and nitrosylation reactions under conditions of oxidative and nitrosative stress.^{5, 7, 8} These processes can lead to alterations in the structure of Trx and its catalytic activity. Disruption of the Trx system can shut down Trx-regulated cellular processes presumably via their requirement for enzymes dependent on Trx.^{1, 2} This can compromise normal cellular metabolism and lead to the inhibition of proliferation and oxidative stress.^{2, 3, 7}

Sulfur mustard (2,2'-dichlorodiethyl sulfide) is a potent vesicant that has been used as a chemical warfare agent. The lung is a major target for sulfur mustard and pulmonary toxicity is a major cause of mortality and long-term complications.^{9, 10} Toxicity is associated with sulfur mustard-induced alkylation of nucleophilic sites in cellular macromolecules including DNA, RNA, and proteins.^{11, 12} Previously, we demonstrated that mechlorethamine (methylbis(2-chloroethyl)amine; HN2), a bifunctional alkylating agent structurally homologous to sulfur mustard and commonly used in cancer chemotherapy, selectively cross-linked TrxR forming TrxR dimers and oligomers in human lung epithelial A549 cells.¹³ Dimerization of TrxR was due to HN2 cross-linked cysteine 59 in one subunit of TrxR and selenocysteine 498 in the other subunit of the enzyme.¹³ In the present studies, the effects of HN2 on Trx were examined. In lung epithelial cells, HN2 was found to cross-link Trx and its redox partner TrxR, forming several high-molecular-weight Trx complexes, a consequence of HN2-induced alkylation of catalytic residues in Trx and TrxR. This resulted

in inactivation of the Trx system, a process that can disrupt Trx-regulated enzymes and contribute to HN2-induced cytotoxicity.

Materials and Methods

Chemicals and Enzymes

Human recombinant TrxR1 mutant enzyme (Sec498Cys) was from AbFrontier (Seoul, Korea). Dulbecco's modified Eagle's medium (DMEM; containing 4500 mg/L D-glucose, 110 mg/mL sodium pyruvate, and 584 mg/L L-glutamine; catalog no. 11995-065), fetal bovine serum, puromycin, and penicillin/streptomycin were from Life Technologies (Grand Island, NY). The SuperSignal West Pico Chemiluminescence Substrate kit was from Thermo Scientific (Rockford, IL). Purified rat cytosolic TrxR, recombinant human Trx1, HN2, insulin, NADPH, 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), phosphatase inhibitor cocktail (catalog no. P2850, which contains microcystinLR, cantharidin, and (–)-*p*-bromotetramisole), protease inhibitor cocktail (catalog no. P2714, which contains 4-(2-aminoethyl)-benzenesulfonyl fluoride, E-64, bestatin, leupeptin, aprotinin, and EDTA), and all other chemicals were from Sigma (St. Louis, MO).

Caution—HN2 is a highly toxic vesicant, and precautions were taken for its handling and preparation including double gloves, safety glasses, masks, and other protective equipment to prevent exposure. HN2 waste was disposed of following Rutgers University Environmental Health and Safety guidelines.

Cell Culture and Treatments

Human A549 lung carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μ g/mL streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C. Components in serum including albumin are known targets for covalent modification by HN2. Thus, solutions of HN2 were freshly prepared before use in serum-free DMEM. Cell viability was determined using alamarBlue¹³ (BioSource International, Camarillo, CA) or trypan blue dye exclusion (Life Technologies). For trypan blue dye exclusion assays, viable cells were analyzed on a Cellometer Vision CBA Analysis System (Nexcelom Bioscience, Lawrence, MA). Using either viability assay, treatment of cells with HN2 for 2 h did not induce toxicity at concentrations up to 500 μ M (not shown). The concentration of HN2 inhibiting cell viability by 50% was 25.3 μ M after 24 h treatment. For preparation of subcellular fractions, A549 cells (5×10^6 cells) were seeded into 15 cm culture dishes and treated with HN2 (1–500 μ M) or vehicle control. After 2 min to 24 h, cells were washed twice with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 2 mM KH₂PO₄, pH 7.4) and removed from the plates with a cell scraper in 5 mL of PBS. Cell pellets were collected after centrifugation (800g, 5 min).

Silencing of TrxR1 was accomplished by transfecting A549 cells with a negative control shRNA or SureSilencing shRNA plasmid for human TrxR1 (Qiagen, Valencia, CA) using Attractene Transfection Reagent (Qiagen), according to the manufacturer's instructions. Stably transfected cells were selected with 4 μ g/mL puromycin for 8 weeks.

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction

Total RNA was isolated from parental A549 cells and negative control shRNA-transfected cells or human TrxR1 shRNA-transfected cells using an RNeasy Mini Kit (Qiagen) according to the manufacturer's protocols. Following quantitation, 400 ng of total RNA was subjected to reverse transcription using RT² First Strand Kit (Qiagen) according to the manufacturer's protocol. qPCR was performed on an Applied Biosystem ViiA 7 Real-Time PCR system (Life Technologies) with RT² SYBR Green ROX qPCR Mastermix (Qiagen) according to the manufacturer's protocol. The primers for PCR reactions were for human Trx1, TrxR1, and β -actin (Qiagen). PCR mixtures contained 0.5 μ L of primers, 0.5 μ L of reverse transcription mixtures, and 5 μ L of SYBR Green Mastermix in a final volume of 10 μ L. PCR was run at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Samples were analyzed in triplicate, and the data normalized to β -actin levels using the Ct method.

Cell Fractionation, Immunoprecipitation, and Western Blotting

Nuclear fractions were prepared from A549 cells using NE-PER Reagents (Thermo Scientific) according to the manufacturer's protocols. For some experiments, cytosol was prepared from pelleted cells resuspended in cell lysis buffer (PBS containing 0.1% Triton X-100, pH 7.4, and protease inhibitor cocktail) followed by sonication as previously described.¹³ Homogenates were centrifuged at 9000g for 20 min at 4 °C to remove insoluble material and the resulting supernatants (S9 fractions), containing cytosolic proteins, collected. Protein concentrations were determined using the BCA protein assay (Thermo Scientific) with bovine serum albumin as the standard.

To identify Trx/HN2 cross-linked proteins, cytosol (500 μ g of S9 fractions in 0.5 mL PBS) was precleared with 20 μ L of 1 mg/mL mouse IgG1 antibody (Thermo Scientific) for 1 h on a tube rotator at 4 °C followed by the addition of 30 μ L of protein G plus agarose (Thermo Scientific). After an additional 1 h, cleared lysates, collected after centrifugation at 3000g for 5 min, were incubated with 20 μ L of anti-Trx1 monoclonal antibody (mAb, 0.5 mg/mL, BD Pharmingen, San Diego, CA) at 4 °C. Immune-complexes were precipitated 16 h later with 30 μ L of protein G slurry (binding at 4 °C for 4 h) and then collected by centrifugation (3000g for 5 min), followed by 4 washes with ice-cold PBS. Protein complexes were subjected to reducing and denaturing SDS-PAGE (10.5–14% Tris-HCl gel, Bio-Rad, Hercules, CA) followed by silver staining (Bio-Rad) or Western blotting. Bands containing Trx were analyzed by LC-MS/MS. Western blotting was performed as previously described.¹³

Trx and TrxR Activity Assays

An insulin reduction assay was used to assess Trx activity using the method of Luthman and Holmgren¹⁴ with minor modifications. Proteins were reduced by incubation with a reducing agent (DTT and NADPH for Trx and Trx/TrxR proteins, respectively) before the addition of HN2. Oxidized proteins in reaction mixes were not treated with DTT or NADPH. Recombinant human Trx1 (1 mg/mL) was incubated in the absence or presence of dithiothreitol (DTT, 10 mM) at 37 °C in reaction buffer (50 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, and 50 mM KCl) for 15 min and then purified using Chroma Spin

TE-10 columns (Clontech, Mountain View, CA) to remove DTT. Aliquots of purified Trx (final concentration, 1 μ M) with or without purified rat TrxR1 (50 nM) were incubated with NADPH (250 μ M) and increasing concentrations of HN2 (5 nM – 1000 μ M) or control in a final volume of 100 μ L of reaction buffer at room temperature. After 30 min, 100 μ L of a TrxR/insulin mixture (50 nM purified rat liver TrxR1, 250 μ M NADPH, and 107 μ M insulin in reaction buffer) was added and changes in absorbance at 340 nm recorded. Trx activity was calculated as the linear change in absorbance per min and expressed as a percentage of the enzyme activity in control samples. For some experiments, incubations with HN2 were run in the absence of NADPH, and additional NADPH was added to a final concentration of 250 μ M before the analysis of enzyme activity. TrxR enzyme activity in A549 cells was assayed using DTNB as a substrate as previously described.¹³

LC-MS/MS Analysis

For in-solution digestion, reduced human Trx1 (1 μ M) with or without purified rat TrxR1 was incubated with NADPH (250 μ M) and HN2 (100 μ M) at room temperature in a final volume of 100 μ L in 50 mM potassium phosphate, pH 7.0, 1 mM EDTA, and 50 mM KCl. After 1 h, the incubation mixture was desalted with Chroma Spin TE-10 columns to remove unreacted HN2. Aliquots of reaction mixtures were supplemented with urea (8M) and methylamine (20 mM) and then reduced with DTT (20 mM) at 60 °C. Thirty minutes later, reduced protein was alkylated with iodoacetamide (40 mM) for 1 h in the dark at room temperature. This was followed by digestion with trypsin (1:50 dilution, Roche, Indianapolis, IN) overnight at 37 °C. For in-gel digestion, aliquots of reaction mixtures or immunoprecipitates from A549 cell lysates were separated by SDS-PAGE using 7.5% or 10.5–14% Tris-HCl gels. After staining with Coomassie Blue or silver (Bio-Rad), bands containing Trx were cut from the gels. Reduction with DTT, alkylation with iodoacetamide, and digestion with trypsin were performed as previously described.¹³ Digested peptides were extracted from the gels with 100 μ L of formic acid/water/acetonitrile (5:35:60, v/v/v) and dried in a speed-vac concentrator. Peptides were reconstituted in 0.1% TFA and then analyzed by LC-MS/MS on a Dionex U3000 RSLC nanosystem (Dionex, Sunnyvale, CA) online with a Thermo LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The peptide mixtures were loaded onto an Acclaim PepMAP 100 nanotrap column (5 μ m particle, 100 Å pore size, 100 μ m $\times$ 2 cm, Dionex) and washed for 5 min with solvent A (0.1% formic acid in water) at a flow rate of 10 μ L/min. Separation was achieved with an Acclaim PepMAP RSLC nanocolumn (2 μ m particle, 100 Å pore size, 75 μ m $\times$ 15 cm, Dionex) with a 30-min gradient from 2 to 45% of solvent B (0.1% formic acid in acetonitrile) at a flow rate of 300 nL/min. The effluent from the HPLC column was subsequently analyzed by electrospray ionization mass spectrometry. MS spectra were acquired in an Orbitrap Analyzer with a mass resolution setting at 60,000. MS/MS spectra were acquired in LTQ using a data-dependent acquisition procedure with a cyclic series of a full scan followed by MS/MS scans of the most intense 20 ions with a repeat count of two and dynamic exclusion (DE) duration of 30 s.

The MS/MS data were searched using Thermo Proteome Discoverer (Thermo Scientific, version 1.3) via the SEQUEST algorithm against the UniProt rat and human protein database. SEQUEST searches were performed with tryptic specificity with a maximum of

two missed cleavages with parent mass error and MS/MS tolerance setting at 10 ppm and 0.8 Da, respectively. S-Carbamidomethylation at cysteine (+57.02 Da), HN2-induced alkylation at cysteine and lysine (+ 83.07 and + 101.08 Da for HN2 cross-link and monoadduct, respectively), and oxidation at methionine and tryptophan (+ 16.00 Da) were set as dynamic modifications to identify spectra of adducted peptides. Modifications on selenium-containing peptides were searched using dynamic peptide C-terminal modifications with UG (mass increase of 321.07 Da for one carbamidomethyl modification on C and one carbamidomethyl-modification on U) and HN2-modified UG (with mass increase of 290.10 Da for HN2 cross-linked on C and U, and 365.11 Da for one carbamidomethyl and one HN2 monoadduct modification on C or U; and 409.19 Da for two HN2 monoadduct modifications on C and U). HN2 intrapeptide cross-links were confirmed manually by the critical b and y ions in the MS/MS. For some experiments, MS/MS raw files were converted to mgf format using BioWorks (Thermo Scientific) and then analyzed using the Global Proteome Machine (GPM) open-source software (<http://www.thegpm.org>) against human and rat databases using parameter settings similar to those in the SEQUEST search.

Molecular Modeling and Data Analysis

Crystal structures of human Trx1-TrxR1 complex and human Trx1 were obtained from the Protein Data Bank (PDB IDs: 3QFA and 1ERT). The 3D models of human Trx-TrxR complex and site-directed mutagenesis on hTrxR1 (Ser497Cys, Cys498Sec) was generated using PyMOL software. Trx activity and TrxR activity were monitored for 10–30 min, and the initial velocities analyzed using SoftMax Pro 6.3 software (Molecular Devices). IC₅₀ values were determined by the nonlinear regression method of curve fitting using Prism 5 software (GraphPad Inc., San Diego, CA). Statistical differences were determined using Student's *t* test. A value of *p* < 0.05 was considered significant.

Results

HN2 cross-links thioredoxin and thioredoxin reductase in A549 lung epithelial cells

In initial studies, we examined the effects of HN2 on cross-linking of Trx in A549 cells. Western blotting of control cells showed Trx as a single band with an estimated molecular weight of 12 kDa. HN2 induced the formation of high molecular-weight Trx1 complexes in a time- and concentration-dependent manner (Figure 1A–E). These protein complexes, with estimated molecular weights of 70 kDa (Trx complex I), 130 kDa (Trx complex II), and 160 kDa (Trx complex III), were detected in both the cytosolic and nuclear fractions of the cells. HN2 cross-linking was observed 2 min post-treatment and persisted for at least 24 h. In contrast, HN2 did not cross-link related Trx proteins including Trx-like protein 1 and protein disulfide isomerase, which, like Trx, contain a CXXC (two cysteines separated by two amino acids) redox motif (Figure 1F,G). HN2 also did not cross-link various Trx substrate proteins, including peroxiredoxins and methionine sulfoxide reductases, and the Trx inhibitor protein Trx interacting protein (TXNIP) (data not shown).

To identify proteins in the Trx complexes formed in HN2-treated cells, they were immunoprecipitated from cell lysates and analyzed by LC-MS/MS. Figure 2 shows that all

high-molecular-weight Trx complexes were retained after immunoprecipitation of cell lysates from HN2-treated cells with Trx1 antibody. Proteins identified by LC-MS/MS are listed in Table 1; both Trx and TrxR were detected in these complexes. On the basis of estimated molecular weights of these complexes, Trx complex I likely resulted from HN2 cross-linked Trx monomers and TrxR monomers, and Trx complex II was formed by HN2 cross-linked Trx monomers and TrxR homodimers. Trx complex III likely resulted from HN2 cross-linked Trx oligomers and TrxR homodimers.

We next determined if modulating TrxR1 with shRNA in A549 cells altered the formation of high-molecular-weight Trx complexes following HN2 treatment. TrxR1 shRNA, but not negative control shRNA, successfully knocked down TrxR1 expression in A549 cells at both the mRNA and protein levels (Figure 3A). Protein expression of TrxR1 was maximally inhibited by more than 90% when compared to both shRNA negative control-transfected cells and wild type cells. After HN2 treatment, high-molecular-weight Trx complexes were detected in both shRNA negative control-transfected cells and wild type cells but not in TrxR1 knockdown cells (Figure 3B and not shown). These data provide support for our LC-MS/MS analysis of high-molecular-weight Trx complexes by immunoprecipitation and demonstrate that HN2 cross-linked Trx and TrxR forming high-molecular-weight Trx complexes in the A549 cell model.

HN2 inhibits the Trx system in a redox status-dependent manner

Mechanisms mediating the cross-linking of Trx and TrxR by HN2 were examined further using purified enzymes. Western blotting revealed that the cross-linking of Trx and TrxR by HN2 was dependent on the redox status of the proteins (Figure 4A). Thus, HN2 cross-linked Trx monomers forming Trx homodimers, which were detected in DTT-reduced but not in oxidized proteins. This implies that HN2 cross-linked cysteine residues in Trx. Similarly, HN2 cross-linked NADPH-reduced TrxR, but not oxidized protein. HN2 was found to cross-link the rat cytosolic TrxR wild type enzyme forming both TrxR dimers and TrxR oligomers, whereas HN2 cross-linked human TrxR Sec498Cys mutant enzyme (TrxRm) forming only TrxRm dimers. On the bases of the relative intensities of cross-linked and monomeric bands shown on the blot, HN2 was less effective in cross-linking the TrxR Sec498Cys mutant enzyme, when compared to that of the wild type enzyme. These results suggest that HN2 cross-linked cysteine and/or selenocysteine residues in the redox centers of TrxR, forming dimers or oligomers, and that selenocysteine is required to form HN2 cross-linked TrxR oligomers.

In the reaction mixture containing both Trx and TrxR, HN2 cross-linked protein complexes were also found to be redox status-dependent. HN2-induced Trx and TrxR cross-links were only detected in NADPH-containing reaction mixtures. Using the Trx antibody as a probe, two Trx/TrxR cross-links were identified in the reaction mixture containing the TrxR wild type enzyme, while only one Trx/TrxRm cross-link was detected in the reaction mixture containing the TrxR mutant enzyme. The migration of Trx/TrxR cross-linked proteins on the blot were in agreement with two Trx-complexed bands found in A549 cell lysates; these were bands representing cross-linked Trx monomers/TrxR monomers and Trx monomers/TrxR dimers. The migration of the cross-linked Trx/TrxRm complex on the blot was

consistent with cross-linked Trx monomers/TrxR monomers found in A549 cell lysates (Figure 4A). Using the TrxR antibody as a probe, fewer bands of cross-linked Trx/TrxR were detected when compared to those probed with the Trx antibody; only one Trx/TrxR cross-linked band and one relatively minor Trx/TrxRm cross-linked band were identified. These cross-linked bands had the same mobility as the cross-linked Trx monomers/TrxR monomers found in A549 cell lysates (Figure 4A). Moreover, pretreatment of the Trx system with cysteine/selenocysteine alkylating agents including, iodoacetamide or 2-chloroethyl ethyl sulfide, significantly blocked Trx/TrxR cross-linking by HN2 (Figure 4B). These results imply that HN2 acts on catalytic cysteine and/or selenocysteine residues on TrxR and cysteine residues on Trx forming Trx/TrxR complexed cross-links.

Enzyme inactivation by HN2 was also redox status-dependent; reduced enzymes were approximately 3- to 14-fold more sensitive to HN2 than oxidized enzymes for the inhibition of Trx (only Trx protein incubated with HN2) and the Trx system (both Trx and TrxR proteins incubated with HN2) (Figure 5). The IC₅₀ values for Trx inhibition by HN2 were $23 \pm 1.5 \mu\text{M}$ and $68 \pm 2.3 \mu\text{M}$ (mean $\pm$ SE, $n = 3$) for the reduced and oxidized forms, respectively. The IC₅₀ values for the Trx system (Trx plus TrxR) were $1.7 \pm 0.3 \mu\text{M}$ and $23 \pm 1.3 \mu\text{M}$ for the reduced and oxidized enzymes, respectively. Increased sensitivity to HN2 by reduced enzymes is likely due to direct targeting of catalytic residues on Trx and/or TrxR protein and by the formation of HN2 cross-links.

HN2 alkylates catalytic residues in Trx and TrxR

HN2 alkylation sites on Trx and TrxR proteins were characterized following LC-MS/MS analysis. HN2 contains two electrophilic chloroethyl groups, each of which can react with one nucleophilic site on protein or a water molecule forming HN2 cross-links or hydroxyl HN2 monoadducts. HN2 cross-links result from HN2 covalently modifying two amino acids on protein, leading to the formation of either intramolecular or intermolecular cross-links, with a mass increase of 83.07 Da per cross-linked modification. HN2 monoadducts result from modification on one amino acid on protein by hydroxyl HN2 with a mass increase of 101.08 Da per monoadduct modification.¹³ In addition, unmodified cysteine or selenocysteine residues on protein are alkylated by iodoacetamide (addition of carbamidomethyl groups; mass increase of 57.02 Da per modification) that is introduced during the process of protease digestion to protect free thiol and selenol groups on protein.

Table 2 and Table 3 list the HN2-modified peptides in the tryptic digest of Trx-containing bands (including Trx monomers and dimers, and Trx/TrxR cross-links) or direct solution digestion of HN2 reaction mixtures (containing only Trx or Trx/TrxR proteins). The tandem mass spectra of HN2-adducted peptides identified by a database search and their assignments are shown in Supporting Information, Figures S1-S8. HN2-linked covalent adducts were detected on peptides 9–36, 22–36, 49–72, and 73–80(81) from human Trx1 (Table 2) and peptides 53–67, 365–389, and 488–499 from rat TrxR1 (Table 3). Both monoalkylated and cross-linked HN2 modifications, which were covalently bound to cysteine, selenocysteine, lysine, threonine, glutamine, and tryptophan residues on the proteins, were detected (Tables 2 and 3). Modifications of cysteine residues by HN2 were detected on both Trx1 and TrxR1 proteins, whereas modifications of lysine, threonine,

glutamine, and tryptophan residues were only detected on Trx1, and modifications of selenocysteine were only detected on TrxR1. On human Trx1 protein, HN2 formed adducts on all five cysteine residues including two catalytic residues (Cys32 and Cys35), three structural residues (Cys62, Cys69, and Cys73), and three lysine residues (Lys23, Lys36, and Lys81). HN2 also alkylated Trp31, Gln63, and Thr76 on human Trx1. On rat TrxR1, HN2 preferentially modified cysteine and selenocysteine residues in the catalytic sites including Cys59, Cys64, Cys497, and Sec498. HN2 also adducted a structural cysteine residue (Cys383) in rat TrxR1.

We also performed a manual search for two peptides cross-linked by HN2 based on expected masses of the parent ions with all possible combinations of HN2-adducted peptides on Trx and TrxR. The cross-linked peptides were confirmed by critical fragment ions appearing after MS/MS. We found one interpeptide HN2 cross-link in the tryptic digest of an HN2-treated Trx1 reaction mixture (Table 2). We were unable to identify additional intermolecular cross-links in preparations from in-gel digestion of Trx/TrxR complexes or solution digestion of HN2 reaction mixtures containing both Trx1 and TrxR1. Figure 6 shows the tandem mass spectra of the identified interpeptide HN2 cross-link and its sequence assignment. The precursor ion was identified as a quintuply charged ion with a mass of 1169.87 Da ($[M+H]^+ 5845.35$) which matches the mass of peptides 9–36 (peptide α) and 49–72 (peptide β) from Trx1 with one HN2 and two carbamidomethyl modifications. MS/MS spectra revealed that several b ions corresponding to the fragments of peptide 9–36 ($b_{5\alpha}^{1+}$, $b_{16\alpha}^{3+}$, $b_{19\alpha}^{3+}$, and $b_{22\alpha}^{3+}$ ions) and peptide 49–72 ($b_{4\beta}^{1+}$ and $b_{5\beta}^{1+}$ ions) from human Trx1 were observed, indicating that these two tryptic peptides are present in the cross-linked complex. Fragment $y_{4\alpha}^{1+}$ was observed with a mass shift of 57.02 Da when compared to that of the unmodified y ion from peptide 9–35, suggesting that Cys35 on one Trx1 molecule was modified with iodoacetamide. A series of y ions ($y_{5\beta}^{1+}$ through $y_{9\beta}^{1+}$ ions) displayed a mass shift corresponding to the addition of one carbamidomethyl group on peptide 49–72, indicating that residue Cys69 on another Trx1 molecule was modified with iodoacetamide. Moreover, peaks assigned as $y_{8\alpha}+y_{13\beta}$, $y_{10\alpha}+y_{14\beta}$, and $y_{5\alpha}+y_{11\beta}$ ions displayed a mass shift corresponding to the addition of one HN2 and two carbamidomethyl groups, supporting the idea that these two peptides were cross-linked by HN2. Collectively, the assigned fragments from the MS/MS spectra demonstrated that HN2 covalently linked Cys62 in one Trx1 molecule and Cys32 on another Trx1 molecule, forming an interpeptide cross-link.

Discussion

In the present studies, we report on the molecular mechanism of Trx and TrxR cross-linking by a bifunctional alkylating agent, HN2. HN2 modified the Trx system forming several high-molecular-weight protein complexes in cytosolic and nuclear fractions of A549 cells. Similar complexes were identified using purified enzymes. Formation of these complexes is due to HN2-induced covalent modifications on catalytic residues in Trx and TrxR resulting in HN2 monoadducts, as well as HN2 cross-links including Trx/TrxR protein complexes, processes that lead to the inhibition of the Trx system. These result in alterations in thiol-dependent metabolic functions including DNA synthesis and repair, protein repair, defense

against oxidative stress, redox signaling, and induction of cell death, all processes that can contribute to HN2-induced toxicity.

Inactivation of the Trx system primarily resulted from HN2 alkylation of the redox motifs in Trx and TrxR and the formation of Trx/TrxR cross-links. This is supported by our findings using purified enzymes showing that (1) reduced Trx and TrxR were more sensitive to HN2 than the oxidized proteins; (2) Trx/TrxR protein complexes were only observed using an NADPH-reduced, but not oxidized, Trx system; (3) a TrxR Sec498Cys mutant protein was much less efficient in forming Trx/TrxR cross-links than the wild type enzyme; and (4) HN2 directly adducted to catalytic residues in Trx (including Cys32 and Cys35) and TrxR (including Cys59, Cys497, and Sec498). That HN2 predominantly bound to catalytic cysteine and/or selenocysteine residues on Trx and TrxR may be due to the fact that these residues are highly nucleophilic and are positioned in solvent-accessible domains in the proteins, factors that facilitate electrophilic HN2 binding.^{15–17} This is in agreement with previous studies showing that the Trx system was inhibited by several structural unrelated electrophiles including 1-methylpropyl 2-imidazolyl disulfide (PX-12), mercury chloride, arsenic trioxide, and 2-chloroethyl ethyl sulfide, via targeting redox-active cysteine and/or selenocysteine residues on the proteins.^{8, 18–20} It is not clear if adducts formed by these electrophiles can differentially affect Trx and TrxR.²¹ The preference of alkylation on cysteine and selenocysteine residues in the Trx system by HN2 is also consistent with earlier studies showing that different nitrogen mustards including mechlorethamine and tris(2-chlorethyl)amine, which contain soft electrophilic chloroethyl side chains, predominantly bind to soft nucleophilic sites on proteins including thiolate groups on cysteines and selenolate groups on selenocysteines.^{13, 22–25} This is supported by our findings that pretreatment of the Trx system with thiol alkylating agents including iodoacetamide and the half mustard, 2-chloroethyl ethyl sulfide, blocked HN2-induced formation of Trx/TrxR complexes. At the present time, we have no direct evidence on the mechanism mediating the formation of Trx and TrxR cross-links as we were unable to identify intermolecular protein cross-links. This may be due to limitations of our LC-MS/MS analysis as there may have been low recovery of the larger cross-linked peptides. These peptides may be difficult to extract from the gels and/or have low ionization efficiencies in LC-MS/MS which can lead to low recovery of the interpeptide cross-links. We also found that HN2 cross-linked several structural amino acids with neighboring cysteine residues in the same protein including Trp31, Lys36, Gln63, Lys72, and Thr76 in hTrx1 and Thr58 in rTrxR1, forming intrapeptide cross-links. Among these residues, Trp31 and Lys36 residues on hTrx1 and Thr58 residue on rTrxR1 formed cross-links with catalytic residues on hTrx1 (Cys32 and Cys35) and rTrxR1 (Cys59), which may also contribute to enzyme inactivation. The functional significance of HN2 adducted to Gln63 and Lys72 on hTrx1 is not clear. That HN2 can alkylate residues other than cysteine is consistent with previous studies demonstrating that mechlorethamine and tris(2-chlorethyl)amine readily form adducts with cysteine, followed by lysine and histidine using *in vitro* binding assays with model peptides containing nucleophilic amino acids.^{24, 26} Although no earlier reports described HN2 modifications on Trp, Thr, and Gln, these amino acids are known targets for covalent modifications by different classes of electrophiles.²⁷

Using purified enzymes, we found that Trx/TrxR cross-links were only detected in the NADPH-reduced Trx system, suggesting that catalytic residues on TrxR play a key role on the formation of Trx/TrxR complexes. Figure 7 shows molecular models of the human Trx1/TrxR1 complex and relative distances between redox-active residues on hTrxR1 and HN2-adducted cysteine residues on hTrx1 identified by LC-MS/MS analysis. The sulfur atom in the N-terminal Cys59/Cys64 redox motif of hTrxR1 is located at least 20 Å away from the sulfur atom on five cysteine residues on hTrx1 which is much greater than the size of an extended HN2 molecule (7.5 Å).²⁸ This suggests that the N-terminal redox motif of TrxR may not be involved in Trx/TrxR protein cross-linking (Figure 7B). In contrast, the C-terminal Cys497/Sec498 redox motif of TrxR is in the vicinity of several cysteine residues on Trx including redox-active Cys32 and Cys35, and the structural Cys73 residues. The distance of sulfur and/or selenium atoms on these residues between two proteins is around 1.4–9.3 Å (Figure 7C–D) which is close to or less than the size of an HN2 molecule; cross-linking of these residues on Trx and TrxR can lead to the formation of the Trx/TrxR protein complex. This idea is supported by our findings that a TrxR Sec498Cys mutant enzyme is much less efficient in cross-linking Trx than the wild type enzyme. It should be noted that the Sec498Cys enzyme is a human enzyme; human TrxR1 is known to be catalytically less efficient than rat TrxR1^{29, 30} and that this may account, at least in part, for the formation of fewer HN2-induced dimers and oligomers. Future studies will need to compare wild type and mutant proteins from the same species.

Of interest were our findings that HN2 appeared to selectively cross-link Trx and TrxR but not other Trx redox partners. Thus, HN2 did not cross-link Trx and various substrate proteins including peroxiredoxins and methionine sulfoxide reductases, or its inhibitor protein, TXNIP. We also found that HN2 did not cross-link TrxR and other substrate proteins such as Trx like protein 1 and protein disulfide isomerase, which contain CXXC redox motifs like Trx.^{31, 32} That Trx formed covalent complexes with TrxR are in agreement with a previous report showing that Trx cross-linked TrxR by structurally unrelated bifunctional electrophiles including HN2 in yeast and human cells; however, these data were based on Western blotting without additional molecular characterization.²⁵ Our observation that HN2 preferentially cross-linked Trx and TrxR, but not other redox partners, suggests that Trx and TrxR have strong affinities for the HN2 electrophile and are located in close proximity to one another in the cells. Trx is known to have a lower redox potential than protein disulfide isomerase and Trx like protein 1, likely resulting from different pKa's in their catalytic cysteine residues and distinct amino acids in the vicinity of the redox motifs in these proteins.³¹ This indicates that Trx more readily forms a dithiol motif in its redox center and that the thiolate in cysteine is more stable, which facilitates a nucleophilic attack by HN2.^{33–35} Among Trx interacting proteins, TrxR is unique in that it contains selenocysteine in its C-terminal redox center, while peroxiredoxins and TXNIP contain cysteine residues in their redox centers.³² Selenocysteine is known to have higher nucleophilicity than cysteine, due to the lower pKa of selenol (5.2) and larger atomic radius of selenium.³⁶ Both Trx and TrxR contain solvent-exposed and highly reactive catalytic residues, which facilitate HN2 binding and allow the formation of protein cross-links. Because the high reactivity of the catalytic residues in Trx and TrxR lead to extensive cross-linking by HN2, this likely limits cross-linking to other redox partners. However, we cannot rule out the possibility that Trx

and TrxR also cross-link with other redox partners, which are not evident in our assays, possibly due to low protein content in A549 cells and/or low reactivity of antibody probes used.

In both humans and in animal studies, vesicant exposure causes acute lung injury along with increased oxidative stress, inflammation, and fibrosis.^{9, 37, 38} Modulation of the Trx system is known to play a critical role in injury to the lung under conditions of stress. This is supported by animal studies showing that overexpression of Trx1 in transgenic mice or administration of recombinant human Trx1 to animals protects against stress-induced tissue injury and inflammation.^{39–43} Previous studies have also demonstrated that the HN2 analog, melphalan, activates mitogen-activated phosphorylated kinases (MAPK), nuclear factor κ B, and inflammatory responses in human lung epithelial cells.⁴⁴ This may partly be the result of melphalan-induced inactivation of the Trx system. Trx1 is an inhibitor of apoptosis signal-regulating kinase 1, a MAPK kinase kinase that can activate MAPK kinase and the c-Jun N-terminal kinase (JNK) and p38 MAPK pathways leading to cell death.^{1, 2} In addition, Trx1 is known to regulate the activities of several transcription factors that play important roles in inflammatory response and signaling pathways.^{42, 43} Our results showed that HN2 inhibited the Trx1 system in both the cytoplasm and nucleus of lung epithelial cells. This can reduce protection against oxidative stress in these cellular compartments and initiate stress-associated signaling leading to altered immune function, inflammatory responses, and cell growth control. Thus, inhibiting the Trx system can activate signaling pathways that contributes to HN2-induced lung injury and stress responses.^{37, 38} Previous studies have demonstrated that modification of TrxR on selenocysteine in its C-terminal redox center by electrophiles can induce selenium-compromised thioredoxin reductase-derived apoptotic proteins (SecTRAPs); SecTRAPs are thought to induce rapid cell death via apoptosis and/or necrosis.⁴⁵ The fact that HN2 can directly modify selenocysteine in the C-terminal of TrxR indicates that it also generates SecTRAPs, which may contribute to HN2-induced toxicity.

In summary, our studies demonstrate that Trx and TrxR are molecular targets for HN2, forming monoadducts, intramolecular and intermolecular cross-links on the same protein, and Trx/TrxR cross-linking complexes in lung A549 cells, as well as using purified enzymes. These processes also lead to inactivation of the Trx system resulting in the suppression of functions of many thiol-dependent systems including antioxidant pathways. This can contribute to HN2-induced cytotoxicity and tissue injury. Understanding the molecular basis of the action of HN2 on the Trx system may lead to the development of therapeutic agents that protect Trx and/or Trx-dependent pathways from vesicant-induced lung injury.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

HN2	mechlorethamine
Trx	thioredoxin
TrxR	thioredoxin reductase
Sec	selenocysteine
TXNIP	thioredoxin interacting protein

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Figure 1.

Effects of HN2 on Trx and Trx-like proteins in lung epithelial cells. A549 cells were incubated in serum-free medium without and with increasing concentrations of HN2. At the indicated times, subcellular fractions of the cells were prepared by differential centrifugation and analyzed for the expression of Trx and Trx-like proteins by Western blotting. β -Actin and TCF4 were used as protein loading controls for cytosolic and nuclear fractions, respectively. (A–C) Effects of HN2 on cytosolic Trx. (D, E) Effects of HN2 on nuclear Trx. The effects of HN2 on cytosolic Trx-like protein 1 (TXNL1; panel F) and protein disulfide isomerase (PDI, panel G) were also analyzed.

Figure 2.

Western blotting of Trx from control and HN2-treated lung epithelial cells. A549 cell lysates, immunoprecipitated with mouse anti-Trx1 monoclonal antibody (lanes 1–3), or total cell lysates (lanes 4–6) were analyzed for Trx expression using the rabbit anti-Trx1 polyclonal antibody. For immunoprecipitation studies, cell lysates (0.5 mg) from control and HN2-treated A549 cells were precleared with antimouse IgG1 antibody to remove nonspecific binding and then incubated with anti-Trx1 antibody. Proteins bound with the Trx1 antibody were subjected to pull-down with protein G/agarose and then analyzed by Western blotting. Molecular weight markers are shown.

Figure 3.

Effects of HN2 on the formation of Trx/TrxR cross-links in lung epithelial cells varying in TrxR1 content. (A) Knockdown of cytosolic TrxR1 expression. A549 cells were transfected with human TrxR1 or negative control shRNA and then selected using 4 μg/mL puromycin. Expression of TrxR1 was determined by real-time PCR, Western blotting (WB), and activity by the DTNB assay. (B) Effects of HN2 on Trx/TrxR cross-links in A549 cells varying in TrxR1 content. Cells were incubated in serum-free medium containing increasing concentrations of HN2 or vehicle-treated control. Two hours later, cytosolic fractions of the cells were prepared and analyzed for the expression of Trx and TrxR by Western blotting. βActin was used as protein loading controls.

Figure 4.

HN2 cross-links Trx/TrxR. (A) Effects of protein redox status on Trx/TrxR cross-linked by HN2. Recombinant human Trx1 (1 mg/mL) was incubated without (oxidized) or with (reduced) DTT (10 mM) at 37 °C for 15 min and then purified using Chroma Spin TE-10 columns to remove DTT. Purified Trx1 (1 μ M), purified rat cytosolic TrxR (0.15 μ M), and/or recombinant human TrxR1 Sec498Cys mutant (TrxRm; 0.15 μ M) were incubated with HN2 (100 μ M) or vehicle control in the presence or absence of NADPH (250 μ M) at room temperature for 30 min. Samples were then analyzed by Western blotting with anti-Trx1 or anti-TrxR1 antibody. A549 cell lysates were also included as controls for Western blotting. (B) Effects of cysteine alkylating agents on Trx/TrxR cross-linked by HN2. To analyze the effects of cysteine-binding agents on cross-linking of Trx/TrxR by HN2, recombinant human Trx1 (1 μ M) and purified rat cytosolic TrxR (0.15 μ M) were incubated at room temperature with NADPH (250 μ M) in the presence or absence of iodoacetamide (IAM, 100 μ M) or the half mustard 2-chloroethyl ethyl sulfide (CEES, 100 μ M) followed 30 min later by HN2 (100 μ M) or the vehicle control. After 1 h, samples were analyzed by Western blotting using anti-Trx1 or anti-TrxR1 antibody.

Figure 5.

Effects of redox status of Trx and TrxR on enzyme inhibition by HN2. For these studies, purified enzymes were used. (A) Inhibition of Trx by HN2. Recombinant human Trx1 was incubated at 37 °C without and with 10 mM DTT. After 10 min, Trx was purified using Chroma Spin TE-10 columns. Purified Trx (1 μM) was then incubated with increasing concentrations of HN2 or the vehicle control. After an additional 30 min, Trx activity was determined by the insulin reduction assay. Data are expressed as the mean $\pm$ SE ($n = 3$). (B) Inhibition of the Trx system by HN2. Purified rat liver cytosolic TrxR (10 nM) and recombinant human Trx1 (1 μM) were preincubated without and with NADPH (250 μM) at room temperature. Ten minutes later, HN2 was added to the enzyme reaction mixtures. After an additional 30 min, NADPH was added to a final concentration of 250 μM and the activity of the Trx system determined by the insulin reduction assay.

Figure 6.

MS/MS spectrum of HN2-modified tryptic ion at m/z 1169.87. The peptide was identified as a quintuply charged ion with one HN2 cross-link and two carbamidomethylated (cam) cysteines. The HN2 cross-link was between a peptide α (residues $Y_{49}SNVIFLEVDVDDCQDVASECEVK_{72}$) in one Trx molecule and a peptide β (residues $T_9AFQEALDAAGDKLVVDFSATWCGPCK_{36}$) in the other Trx molecule. Matched b and y fragments are marked.

Figure 7.

Molecular models of the human Trx-TrxR complex. Superimposition of the hTrx1 wild type structures and hTrx1-hTrxR1 complex (PDB IDs: 3QFA and 1ERT), and site-directed mutagenesis on hTrxR1 (Ser497Cys and Cys498Sec) were produced using PyMOL. (A) Ribbon representation of the overview of hTrxR1 bound with hTrx1. One hTrxR1 subunit is shown in yellow and the other in cyan. The hTrx1 molecules are shown in magenta. (B, C, D) Close-up of the hTrx1 and hTrxR1 interface. Redox motifs in Trx1 (Cys32 and Cys35) and TrxR1 (Cys59, Cys64, Cys497, and Sec498) and other relevant cysteine residues on Trx1 (Cys62, Cys69, and Cys73) are shown as sticks. Sulfur atoms are drawn in red, and selenium atoms are in green. The table shows the relative distances (Å) between the sulfur atom on cysteine residues of hTrx1 and sulfur or selenium atom on redox motifs of hTrxR1. The relative distance between the sulfur or selenium atom on the C-terminal redox center of hTrxR1 and the sulfur atom on Cys32, Cys35, and Cys73 on hTrx1 are labeled (panels C and D).

Table 1LC-MS/MS Analysis of Trx Immunoprecipitates from HN2 Treated A549 Cells^a

gel band	observed mass (kDa)	protein	accession number	calculated mass (kDa)	unique peptide ^b	total ^c	sequence coverage (%) ^d
Trx complex I	70	Thioredoxin reductase 1	ENSP000000421934	54.7	15	119	21
		Thioredoxin 1	ENSP000000363641	11.6	8	42	38
Trx complex II	130	Thioredoxin reductase 1	ENSP000000421934	54.7	19	112	31
		Thioredoxin 1	ENSP000000363641	11.6	8	32	47
Trx complex III	160	Thioredoxin reductase 1	ENSP000000421934	54.7	10	45	14
		Thioredoxin 1	ENSP000000363641	11.6	5	12	31

^aTrx-containing complexes were immunoprecipitated from lysates of cells treated with 200 μ M or 500 μ M HN2.^bThe number of unique peptide sequences associated with this protein assignment.^cThe total number of tandem mass spectra that can be assigned to this protein.^dAmino acid sequence coverage of this protein.

Table 2

HN2-Modified Peptides from Human Trx1^a

peptide sequence (residue position)	m/z	charge	t _R (min)	modifications
TAFQEALDAAGDKLVVVDFAATWCGPCK (9–36)	622.52	5	40.39	W ₃₁ -C ₃₂ HN2 cross-link, C ₃₅ -K ₃₆ HN2 cross-link
	757.13	4	35.00	C ₃₂ -C ₃₅ HN2 cross-link
	775.89	4	38.19	C ₃₂ HN2 monoadduct, C ₃₅ carbamidomethyl or C ₃₂ carbamidomethyl, C ₃₅ HN2 monoadduct
	786.90	4	37.87	C ₃₂ HN2 monoadduct, C ₃₅ HN2 monoadduct
	1034.18	3	38.19	C ₃₂ HN2 monoadduct, C ₃₅ carbamidomethyl or C ₃₂ carbamidomethyl, C ₃₅ HN2 monoadduct
LVVVDFAATWCGPCK (22–36)	854.44	2	38.94	C ₃₂ -C ₃₅ HN2 cross-link
	904.98	2	32.94	C ₃₂ HN2 monoadduct, C ₃₅ -K ₃₆ HN2 cross-link
YSNVIFLEVDVDDCQDVASECEVK (49–72)	722.10	4	34.11	C ₆₂ -Q ₆₃ HN2 cross-link, C ₆₉ -K ₇₂ HN2 cross-link
	934.77	3	36.09	C ₆₂ -C ₆₉ HN2 cross-link
	959.78	3	33.40	C ₆₂ HN2 monoadduct, C ₆₉ carbamidomethyl or C ₆₂ carbamidomethyl, C ₆₉ HN2 monoadduct
	974.47	3	34.94	C ₆₂ HN2 monoadduct, C ₆₉ HN2 monoadduct
	1401.65	2	33.40	C ₆₂ -C ₆₉ HN2 cross-link
CMPTEQFFK (73–81)	416.54	3	31.70	C ₇₃ -T ₇₆ HN2 cross-link, M ₇₄ oxidation
	625.31	2	32.87	C ₇₃ HN2 monoadduct
	633.31	2	28.66	C ₇₃ HN2 monoadduct, M ₇₄ oxidation
CMPTEQFFKK (73–82)	459.91	3	30.17	C ₇₃ HN2 monoadduct
TAFQEALDAAGDKLVVVDFAATWCGPCK (9–36)	1169.87	5	32.47	C ₃₅ carbamidomethyl, C ₃₂ -C ₆₂ HN2 cross-link,
				C ₆₉ carbamidomethyl

^a Adducts were identified from tryptic digest of SDS-PAGE protein bands representing Trx monomers, Trx dimers, Trx monomers+TrxR monomers, Trx monomers+TrxR dimers or solution digestion of HN2 reaction mixtures containing human Trx1 with or without rat TrxR1.

Table 3

HN2-Modified Peptides from Rat TrxR1^a

peptide sequence (residue position)	m/z	charge	t _R (min)	modifications
WGLGGTCVNVGCIPIK (53–67)	822.40	2	37.12	T ₅₈ -C ₅₉ HN2 cross-link, C ₆₄ carbamidomethyl
	839.39	2	33.73	W ₅₃ oxidation, C ₅₉ HN2 monoadduct, C ₆₄ carbamidomethyl
CDYDNVPTTVFTIPLEYGCCGLSEEK (365–389)	1000.09	3	39.47	C ₃₆₅ carbamidomethyl; C ₃₈₂ carbamidomethyl; C ₃₈₃ HN2 monoadduct
SGGDILQSGCUG (488–499)	614.23	2	27.04	C ₄₉₇ -U ₄₉₈ HN2 cross-link
	651.73	2	24.68	C ₄₉₇ carbamidomethyl, U ₄₉₈ HN2 monoadduct

^aAdducts were identified from tryptic digest of SDS-PAGE protein bands representing Trx monomers+TrxR monomers, Trx monomers+TrxR dimers or solution digestion of HN2 reaction mixtures containing human Trx1 and rat TrxR1.