Published in final edited form as:

ACS Chem Biol. 2017 August 18; 12(8): 2201–2208. doi:10.1021/acscchembio.7b00444.

Discovery of Heteroaromatic Sulfones As a New Class of Biologically Compatible Thiol-Selective Reagents

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Abstract

The selective reaction of chemical reagents with reduced protein thiols is critical to biological research. This reaction is utilized to prevent cross-linking of cysteine-containing peptides in common proteomics workflows and is applied widely in discovery and targeted redox investigations of the mechanisms underlying physiological and pathological processes. However, known and commonly used thiol blocking reagents like iodoacetamide, N-ethylmaleimide, and others were found to cross-react with oxidized protein sulfenic acids (–SOH) introducing significant errors in studies employing these reagents. We have investigated and are reporting here a new heteroaromatic alkylsulfone, 4-(5-methanesulfonyl-[1,2,3,4]tetrazol-1-yl)-phenol (MSTP), as a selective and highly reactive –SH blocking reagent compatible with biological applications.

Graphical abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscchembio.7b00444.

Supporting Figure S1 and additional information for compounds shown in Figure 2 (PDF)

Notes

The authors declare no competing financial interest.

Reversible oxidation at protein cysteine residues plays important regulatory roles in protein folding, function, and intracellular trafficking similar to phosphorylation and other reversible post-translational modifications. As demonstrated by an increasing number of studies of proteins undergoing multiple post-translational modifications, it is often the balance between the reduced and oxidized states of a protein that ultimately informs on protein activity, trafficking, or inhibitor-binding properties.¹⁻⁴

The high interest in studying oxidative protein modifications has brought into focus the need for chemical and analytical tools to enable selective monitoring of the vast variety of oxidative modifications occurring at protein cysteine residues. We have learned over past decades that (a) susceptibility of cysteines to oxidation is controlled by factors beyond the commonly recognized pK_a constant,⁵⁻⁸ (b) reduced cysteine thiols have overlapping reactivity profiles with some of the oxidized thiol species,⁹ (c) multiple oxidative modifications may occur at the same cysteine site either by direct action of reactive oxidants or by interchangeable substitutions of oxidative modifications (e.g., cycling of sulfenic acid –SOH and sulfonylamide –SN species),¹⁰ and (d) thiol–disulfide exchange and other oxidative reactions at cysteine sites during sample workup procedures occur with rapid kinetics raising the need for effective thiol blocking or quenching techniques.¹¹ Cumulatively, these chemical and kinetic properties make the selective monitoring of oxidative modifications at cysteine sites a highly challenging task.

More recent discoveries have overcome many of the roadblocks in the field but have also revealed unexpected issues with some of the most commonly used methods like the biotin-switch assays.^{9,10} Our group showed that commonly used thiol-blocking reagents such as iodoacetamide (IAM), N-ethylmaleimide (NEM), and methylmethanethiosulfonate (MMTS) are not selective for –SH and display cross-reactivity with sulfenic –SOH species producing –S(O)R species (Figure 1a).¹² While the chemical nature of the adduct (sulfoxide (–S(=O)R) or a thioperoxide (–S–O–R)) has not been elucidated, its reduction to the –SH state by mild reducing agents like DTT suggests a thioperoxide adduct.¹² The reported findings rendered these thiol blockers unsuitable for workflows seeking quantitative analysis of reversibly oxidized protein species such as protein disulfides, protein sulfenylation (–SOH), and protein S-nitrosylation (–SNO) due to the conversion of –S(O)R adducts to –SH with reductants commonly used in experimental workflows for switch assays.¹²

In addition to these applications, thiol-reacting compounds are commonly utilized in proteomics workflows to either prevent cross-linking of cysteine-containing peptides during analysis by mass spectrometry (e.g., IAM) or act as reactive groups in cross-linking reagents (e.g., iodoacetyl or maleimide-based reagents) for the elucidation of protein structure and protein–protein interactions. In certain cases, cross-linking reagents are added directly to

cells in an effort to fix pathophysiological protein complexes and identify interacting proteins by mass spectrometry. The cell membrane permeable NEM compound has also been added to cells in redox studies as a means to block protein and small molecule thiols and prevent thiol–disulfide exchanges or oxidation during lysis.^{14–16} Thus, cell membrane permeability is an important property of thiol-reacting compounds.

In this context, the major goal of the work presented here was to identify new thiol blocking reagents selective toward reduced –SH species and to characterize their kinetics and compatibility with cell culture studies in comparison with current nonselective thiol blockers.

Methylsulfonyl benzothiazole (MSBT) has been introduced recently as a new blocking reagent for –SH in procedures aimed at the identification of protein persulfides (–S_(n)–SH; Figure 1b).^{17,18} Combined with its lack of reactivity toward other nucleophilic amino acids and good water solubility, we envisioned that MSBTA (the carboxylic acid form of MSBT) could be a promising thiol-blocking agent. Upon successful confirmation of its selectivity toward –SH, a screening of an additional four compounds identified MSTP (4-(5-methanesulfonyl-[1,2,3,4]tetrazol-1-yl)-phenol) as the most reactive –SH blocker and potential lead reagent based on its kinetic properties and stability in aqueous solutions. Further side-by-side comparison analysis of lead MSTP and NEM reagents in a variety of experimental setups was performed to confirm the selectivity of MSTP toward –SH, determine its relative efficiency for –SH blocking, and investigate cell membrane permeability. The results demonstrate MSTP as a highly reactive and selective –SH blocker compatible with the analysis of biological samples. MSTP, like NEM, is also cell membrane permeable, enabling quenching of intracellular thiols with short incubation times.

RESULTS

Model Protein Constructs and Chemical Models for Testing the Selectivity of Thiol-Reacting Compounds

To determine the selectivity of thiol-reacting compounds toward –SH in the presence of sulfenic acids (–SOH) and other oxidized species, we employed two protein models containing single reactive thiols. The *S. typhimurium* peroxiredoxin AhpC is known to form a stable sulfenic acid at Cys46 upon oxidation when the resolving Cys165 is mutated to alanine or serine.¹⁹ The C165S and C165A AhpC mutants can also be used to generate a variety of other oxidative modifications at the reactive Cys46 site such as –SNO, disulfides, and others as we reported before and applied here.¹² Similarly, the *E. coli* free methionine-(R)-sulfoxide reductase (fRMsR) harboring C84S and C94S mutations can be utilized to generate –SOH and other oxidative modifications at the reactive cysteine site Cys118 and was used here as an additional –SOH and –SNO protein model.²⁰ Selectivity studies also included Fries' acid, a small molecule sulfenic acid.²¹

MSBTA-A Selective Thiol-Reacting Compound

As seen in Figure 2a, treatment of the reduced enzyme (C165S AhpC-SH; 50 μ M; 20601 a.m.u.) with MSBTA (5 mM) resulted in complete labeling of the protein thiol within 30

min, forming an adduct corresponding to 20 734 a.m.u. with a pseudo-first order rate constant k_{obs} of 0.11 min^{-1} (k $0.3 \text{ M}^{-1} \text{ s}^{-1}$). This reaction rate is comparable with the rate of the IAM reaction with thiols (k $0.15 \text{ M}^{-1} \text{ s}^{-1}$).¹² Exposure of C165A AhpC-SH to H_2O_2 provided the mixed oxidized forms of the enzyme ($-\text{SN}$, $-\text{SOH}$, $-\text{SO}_{2/3}\text{H}$), consistent with our previous findings.^{19,22–24} Following treatment with MSBTA, no reactivity was observed with the $-\text{SOH}$ and other oxidized forms of the enzyme ($-\text{SN}$, $-\text{SO}_{2/3}\text{H}$), pointing to the highly selective nature of MSBTA (Figure 2b). Some hyperoxidation of protein $-\text{SOH}$ to $-\text{SO}_{2/3}\text{H}$ is observed over the time course of the reaction that was independent of MSBTA or dimethyl sulfoxide (DMSO) used as a solvent in preparation of MSBTA stocks. The hyperoxidation was slower when the reaction was performed on ice, but still without evidence of adduct formation (data not shown).

Comparison of $-\text{SH}$ Blocking by MSBTA with Other Heteroaromatic Sulfonyl Amides and Alkylsulfones

Knowing that other $-\text{SH}$ blockers like NEM have a faster reaction rate with protein $-\text{SH}$ than IAM,¹³ we wanted to determine if there are heteroaromatic sulfonyl amides or alkylsulfones that would similarly display increased reaction rates with protein $-\text{SH}$ relative to MSBTA. For this purpose, we employed the compounds shown in Figure 2c, which are all expected to react with anionic thiols ($-\text{S}^-$) through nucleophilic aromatic substitution as described in previous publications.^{18,25} Pretreatment of cell extracts with these compounds for 30 min, followed by 30 min of incubation with biotin-tagged iodoacetamide (IAM-biotin), demonstrated that, similar to MSBTA, compounds **1** and **4** are efficient $-\text{SH}$ blocking agents, with compound **4** (MSTP, 4-(5-methanesulfonyl-[1,2,3,4]tetrazol-1-yl)-phenol) being superior to all other compounds and providing comparable blocking of thiols as NEM (Figure 2c). In these experiments, residual labeling observed with MSTP could be due to incomplete blocking or to the reaction of IAM-biotin and NEM with protein $-\text{SOH}$ species as we reported earlier.¹² C165S AhpC was spiked in as control protein. Complete blocking of the free thiol in this protein is observed with compounds **1** and **4** as well as NEM and IAM as revealed by the anti-biotin Western blot and the lack of a shift to higher molecular weight (biotin-tagged AhpC) when the blot was exposed to reversible protein stain. We further characterized the reaction of MSTP with protein $-\text{SH}$ using reduced C165S AhpC-SH (Figure 2d). Complete labeling with MSTP (20 762 a.m.u.) was achieved within 2 min (k_{obs} of 5.1 min^{-1} , $k = 16.6 \text{ M}^{-1} \text{ s}^{-1}$), a ~50 fold higher rate constant compared with MSBTA.

Analysis of MSTP Selectivity toward Reduced Thiols and Chemical Compatibility with $-\text{SOH}$ Labeling Probes

To further confirm the selectivity and relative reactivity of $-\text{SH}$ blockers, we performed a series of side-by-side comparison studies focusing on the lead MSTP compound and NEM and using two protein systems (C165A AhpC and C84S/C94S fRMs). As introduced above, these genetically engineered proteins carry single reactive thiols, which can undergo oxidation to a variety of species ($-\text{SOH}$, $-\text{SO}_{2/3}\text{H}$, $-\text{SNO}$, intermolecular disulfides with cysteine, $-\text{SSH}$, etc.). Full time course analysis of C165A AhpC in reduced ($-\text{SH}$) and oxidized states was performed first, and the ESI-TOF MS (Electrospray Ionization Time-of-Flight Mass Spectrometry) profiles at the 30 min time point are shown in Figure 3a–c. As

expected based on previous published data¹² and data in Figure 2, all four –SH blockers produced adducts within minutes and resulted in complete blocking of cysteine –SH in C165A AhpC within 30 min (Figure 3a). Also, consistent with data in Figure 2, only NEM reacted with C165A AhpC-SOH, and there was no evidence of product formation with MSTP (Figure 3b). Neither NEM nor MSTP reacted with C165A AhpC-SNO or –SSCys species (Figure 3c). Short time point data for the reactions with –SH and –SOH species are shown in Supporting Figure S1a,b. The results of this analysis were highly consistent with the fRMSr data included in Figure 3d–f and Supporting Figure S1d,e. The short time point ESI-TOF data for the –SNO reaction with NEM and MSTP is not included for either protein, as there was no adduct formation at 30 min.

To ensure that the observed selectivity and lack of reaction with protein –SOH species were not due to perhaps unique protein features blocking the MSTP access to the –SOH site, we analyzed next the reaction of these compounds with –SOH in a protein free environment using Fries' acid. The reactions were monitored by UV–vis spectrophotometry at 453 nm, and the results at 30 mM NEM and MSTP are shown in Figure 3g. BCN, the –SOH reacting probe, was used as a control.²² Similar to the protein data, there was no measurable reaction of Fries' acid with MSTP, while NEM did indeed react with this –SOH model compound with a rate constant of $0.84 \text{ M}^{-1} \text{ s}^{-1}$. The BCN kinetics ($k = 12 \text{ M}^{-1} \text{ s}^{-1}$) are consistent with our previously published value ($12 \text{ M}^{-1} \text{ s}^{-122}$).

Concentration Dependence Studies of –SH Blocking by Selective MSTP and Nonselective NEM Using Cell Extracts

To further evaluate the efficacy of –SH blocking by MSTP compared with NEM, we have used reduced lysates and incubated with increasing concentrations of –SH blocker for 30 min followed by the addition of biotin-IAM for an additional 30 min. The results are shown in Figure 4a. The IAM-biotin labeling profiles demonstrate highly effective blocking of the IAM-biotin signal by NEM, followed by MSTP. Taking into consideration the additional reactivity of NEM, and IAM-biotin with –SOH species, the conclusion from these studies is that MSTP is at least as efficient as NEM in reacting with reduced –SH species when used at a 10 mM concentration.

Comparison Analysis of Cell Membrane Permeability of Thiol-Reacting Compounds

As described in the introduction, the addition of –SH blocking reagents to intact cells is often considered as a means to distinguish between true biological protein redox states and modifications that may occur postlysis. Published examples include studies of thiol–disulfide relays in signaling¹⁶ and evaluation of PrxII redox state in blood erythrocytes.¹⁴ In these cases, the cells were treated for short periods of time (typically 5 min) with >50 mM NEM prior to lysis. We have compared the cell membrane permeability of MSTP with that of NEM by incubating the cells for 5 min with these compounds followed by lysis in the presence of IAM-biotin. Although both reaction kinetics, selectivity and cell membrane permeability, contribute to the observed profile, it can be concluded that MSTP like NEM is cell membrane permeable as there is a decrease in IAM-biotin labeling in cells exposed to MSTP relative to the control without –SH blocker (Figure 4b). We have also monitored the redox state of peroxiredoxins to further evaluate the efficacy of thiol blocking in cells.

Monitoring of PrxI-IV hyperoxidation in the same lysates analyzed above demonstrates that MSTP is as effective as NEM in preventing the oxidation of Prx-SH to $-SO_2/3H$ (Figure 4c).

DISCUSSION

Alteration of redox homeostasis underlies many physiological and pathological conditions impacting human health. The predominant small molecule effectors of redox regulation include reactive oxygen and nitrogen species (ROS/RNS), as well as reactive sulfur species (RSS). While controlled and localized production of these reactive oxidants (e.g., H_2O_2 , NO, H_2S) by dedicated systems is critical to normal physiological processes, acute or chronic accumulation of these species contributes to disease development and modulates responses to therapies. Decades of studies have linked oxidative stress to metabolic and inflammatory diseases, cancer, aging, and age-related pathologies. However, the studies of mechanisms linking redox imbalance to disease initiation, progression, and treatment remain poorly understood. One of the reasons has been the limited availability of reliable tools to study redox-regulated processes, which are often reduced to nonselective monitoring of reactive oxidants (e.g., 2',7'-dichlorofluorescein diacetate (DCF) for general ROS assessment)²⁶ and their products of reaction with proteins (e.g., 2,4-dinitrophenylhydrazine (DNPH), for general carbonylation).²⁷ However, this has dramatically changed in recent years, and we are witnessing an exponential increase in selective and highly reactive molecular probes to enable precise analysis of reactive oxygen species and protein oxidation in biological systems from all genera (e.g., refs ^{28–31}).

In particular, redox workflows are applied widely today in discovery and targeted studies of biological oxidative mechanisms.^{32–36} In general, these target specific oxidized protein species and involve either direct labeling of oxidized species with selective chemical probes or tag-switch methods, where the targeted oxidized species of interest is labeled with biotin or other enrichment or detection tags. Naturally, a critical common step to all approaches targeting cysteine oxidative modification in proteins is the blocking of reduced thiol species ($-SH$) with the goal of preventing undesired reactions at the time of lysis (e.g., postlysis oxidation, stabilizing protein oxidized states by preventing thiol–disulfide exchange reactions or sulfenic acid condensation reactions with free or protein bound thiols) or during sample processing procedures (e.g., reduction of proteolytic digests and blocking of resulting cysteine-containing peptides with IAM in mass spectrometry analysis). Intriguingly, known and commonly used thiol blocking reagents like IAM, NEM, and MMTS were found to also react with sulfonylated proteins ($-SOH$), negatively impacting workflows aimed at identification and quantitation of these species but also protein nitrosylation ($-SNO$) and global detection of reversibly oxidized protein species.¹²

Thus, the main scope of the work presented here was to identify new selective and highly reactive $-SH$ blocking reagents to alleviate the issue of cross-reactivity with $-SOH$ reported for the widely used reagents IAM and NEM compounds.

Heteroaromatic sulfonyl amides and alkylsulfones have been developed to use as blocking reagents in tag-switch assays aimed at identification of protein persulfides and polysulfides.¹⁸ However, their selectivity for $-SH$ has not been tested. In an effort to

determine if this class of reagents could provide viable substitute options for IAM or NEM workflows, we have performed the studies described here using first MSBTA as the proof of concept reagent. Indeed, the results demonstrate that MSBTA is a selective compound reacting with protein thiols at rates comparable to those of IAM. Further screening of a series of sulfonyl reagents shown in Figure 2c has identified a lead compound (compound 4/MSTP), which was 50-fold more reactive than MSBTA and 160-fold more reactive than IAM toward protein –SH, displaying kinetics comparable with NEM toward protein –SH. The selectivity toward –SH was further investigated by focusing on top reacting compounds (MSTP and NEM) and using two protein models carrying single reactive cysteine sites that can be used to generate single or mixed oxidized species (C165S/A AhpC and C84S/C94S fRMs_r) and a small molecule –SOH model compound, Fries' acid (Figure 3 and Figure S1). The results confirmed the cross-reactivity of NEM with –SH and –SOH species reported before¹² and the selectivity of MSTP toward protein –SH.

The side-by-side comparison analysis of the effects of MSTP and NEM on protein –SH blocking using cell extracts supported the advantage of selectively trapping reduced thiols using MSTP over NEM when these compounds were added either to lysates or intact cells (Figure 4). These findings open new possibilities for the design of workflows in redox proteomics studies and are expected to improve the analysis of protein sulfenylation as MSTP would not compete with –SOH targeted probes. Similarly, side-by-side comparison of NEM versus MSTP in creative workflows could provide information on the prevalence of –SOH species and better distinguish between biologically relevant oxidative events and postlysis artifacts.

To summarize, we demonstrate here that certain heteroaromatic alkylsulfones like MSTP are selective and highly reactive –SH blocking reagents that are compatible with a variety of experimental setups in biological research. We anticipate the –SH blocking compound MSTP identified here to find broad applicability in studies relying on selective and quantitative blocking of reduced thiols.

METHODS

Materials

Dithiothreitol (DTT), a bicinchoninic acid (BCA) assay kit, immobilized tris-2-carboxyethylphosphine (TCEP) resin, EZ-Link Iodoacetyl-PEG₂-Biotin (IAM-biotin), mass spectrometry grade acetonitrile (ACN), and a Pierce Reversible Protein Stain Kit for Nitrocellulose Membranes (Cat. # 24580) were purchased from ThermoFisher Scientific. Hydrogen peroxide and 9-hydroxymethylbicyclo[6.1.0]nonyne (BCN; Cat. # 742678) were obtained from Sigma-Aldrich. Fries' acid was synthesized in our group as previously described.^{22,23,37} Antibiotin HRP-linked antibody (Cat. # 7075S) and antirabbit IgG HRP-linked secondary antibody (Cat. # 7074S) were purchased from Cell Signaling Technology. Anti-Prx-SO₂/3 (Cat. # 16830) rabbit antibody was purchased from Abcam. Protease and phosphatase inhibitor tablets were acquired from Roche (Cat. # 04693159001 and 04906837001, respectively). Enhanced chemiluminescence (ECL) reagents were obtained from Advantia (WesternBright Quantum HRP substrate, Cat. # K-12042) and GE Healthcare Life Sciences (Amersham ECL Prime Western Blotting Detection Reagent, Cat. #

RPN2232). Methylsulfonyl benzothiazole (MSBTA) and other heteroaromatic sulfonyl amides and alkylsulfones were synthesized as previously described.^{18,25} For a detailed discussion of the syntheses, see the Supporting Information.

UV–Vis Kinetics of Fries' Acid Reaction with NEM or MSTP

Stock solutions of Fries' acid (3 mM) and thiol blocking reagents (300 mM) were prepared in acetonitrile (MeCN). The Fries' acid stock (0.1 mL) was added to MeCN (0.3 mL) and ammonium bicarbonate buffer (0.5 mL, 50 mM, pH 7.5) in a 1 mL UV–vis cuvette; an aliquot of the stock solution of reagent (0.1 mL) was added, followed by a quick shake of the cuvette. The cuvette was immediately loaded into a Cary 50 UV–vis spectrophotometer and measurements began. UV–vis data were recorded at 453 nm with 6 s intervals. The BCN data were collected similarly using a 3.6 mM final BCN concentration. SigmaPlot 11.0 (Systat Software, Inc.) was utilized to plot data using exponential decay with the equation: $f = y_0 + a(e^{-bx})$.

Reaction of –SH Blocking Reagents with Reduced AhpC and fRMsr Proteins

The C165S or C165A mutants of *Salmonella typhimurium* AhpC were overexpressed and purified from *E. coli* as previously described.¹⁹ C165S/A AhpC was reduced with DTT (10 mM) for 30 min at RT, and DTT was then removed by passing the protein solution through a Bio-Gel P6 spin column equilibrated with ammonium bicarbonate (50 mM, pH 7.5). Protein concentration was determined based on the absorbance at 280 nm ($\epsilon_{280} = 24\,300\text{ M}^{-1}\text{cm}^{-1}$).¹⁹ Stock solutions (0.5 M) of the –SH blocking reagents MSTP and NEM were prepared in DMSO. The C165S/A AhpC samples (50 μM) were incubated with 5 mM of –SH blockers at RT. Aliquots were taken at various time intervals, passed through a Bio-Gel P6 spin column equilibrated with 0.1% (v/v) formic acid, and analyzed by Electrospray Ionization Time-of-Flight Mass Spectrometry (ESI-TOF MS) as described below. The C84S/C94S fRMsr was purified as previously described.²⁰ The exact same procedure was followed as with the C165A AhpC to monitor the reaction of –SH blockers with the reduced protein.

Generation of AhpC and fRMsr Sulfenic Acid (–SOH) and Reaction with –SH Blockers

C165S/A AhpC was reduced and transferred into ammonium bicarbonate buffer as described above. The sulfenic acid species of C165S/A AhpC (C165S AhpC-SOH) was generated by treatment with 1–1.2 equiv of H_2O_2 for 40 s at RT in 50 mM ammonium bicarbonate buffer (pH 7.5). Excess H_2O_2 was removed by passage through a Bio-Gel P6 spin column equilibrated with 50 mM ammonium bicarbonate. Formation of C165S/A AhpC-SOH was confirmed by ESI-TOF MS. fRMsr sulfenic acid was generated by incubating the reduced enzyme with 100-fold equivalents of methionine sulfoxide (Met(O)) for 2 min, exchanged to 50 mM ammonium bicarbonate buffer using a Bio-Gel P6 spin column, and analyzed by ESI-TOF MS. The oxidized C165S/A AhpC or C84S/C94S fRMsr samples (50 μM) containing –SOH and other oxidized species (–SN, – $\text{SO}_2/3\text{H}$) were incubated with 5 mM of –SH blockers at RT. Aliquots were taken at various time intervals, passed through a Bio-Gel P6 spin column equilibrated with 50 mM ammonium bicarbonate, and analyzed by ESI-TOF MS as described below.

Generation of Nitrosylated AhpC and fRMsr (–SNO) and Reaction with –SH Blockers

C165S/A AhpC and C84S/C94S fRMsr were reduced by 10 mM DTT and transferred into 50 mM HEPES buffer supplemented with 1 mM DTPA (pH 7.1). S-nitrosocysteine (L-Cys-SNO) was first generated as previously described by mixing L-cysteine (0.34 mmol) with sodium nitrite under acidic conditions and neutralizing with NaOH.³⁸ Reduced AhpC or fRMsr was incubated with freshly prepared S-nitrosocysteine (1 mM) at RT for 60 min in the dark. Removal of S-nitrosocysteine was performed using Bio-Gel P6 spin columns equilibrated in 50 mM HEPES containing 1 mM DTPA buffer (pH 7.1). The formation of C165S/A AhpC-SNO or C84S/C94S fRMsr-SNO was monitored by ESI-TOF MS, and then the protein samples (50 μ M) were incubated with 5 mM MSTP or NEM at RT. Aliquots were taken at various time intervals, passed through a Bio-Gel P6 spin column equilibrated with 50 mM ammonium bicarbonate and analyzed by ESI-TOF MS as described below.

Intact Protein Analysis by Mass Spectrometry

ESI-TOF MS analyses were performed on an Agilent 6120 MSD-TOF system operating in positive ion mode with the following settings: capillary voltage of 3.5 kV, nebulizer gas pressure of 30 psig, drying gas flow of 5 L min⁻¹, fragmentor voltage of 175 V, skimmer voltage of 65 V, and gas temperature of 325 °C. Samples were introduced *via* direct infusion at a flow rate of 20 μ L min⁻¹ using a syringe pump. Mass spectra were averaged and deconvoluted using the Agilent Mass-Hunter Workstation software v B.02.00.

Cell Culture

All studies using intact cells or cell extracts were performed with human lung carcinoma A549 cells obtained from the American Type Culture Collection. Cells were cultured at 37 °C under 5% CO₂, in Ham's F-12K (Kaighn's) Medium supplemented with 10% (v/v) FBS, 100 U mL⁻¹ penicillin, and 100 μ g mL⁻¹ streptomycin.

Blocking of Protein –SH in Cell Extracts and Intact Cells

A549 cells were lysed on ice for 30 min in modified RIPA (mRIPA) buffer (50 mM Tris-HCl at pH 7.4, 1% (v/v) NP-40, 0.25% (w/v) sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM NaF) supplemented with catalase (200 U mL⁻¹), protease, and phosphatase inhibitor tablets. The lysate was cleared by centrifugation at 13 000 rpm for 10 min at 4 °C. Immobilized TCEP disulfide reducing beads were washed with mRIPA buffer and then mixed with the cleared lysate. The mixture was incubated at RT for 1 h. The supernatant containing the reduced proteins were collected, and C165S AhpC was added (100 μ g mg⁻¹ lysate). The mixture was treated first with –SH blockers (5 mM and 0.5–10 mM for experiments in Figures 2c and 4, respectively) at RT for 30 min followed by treatment with 5 mM IAM-biotin for an additional 30 min to trap the remaining unblocked protein thiols. Western blot analysis was performed using an anti-biotin antibody to visualize the relative content of IAM-biotin labeled proteins with and without pretreatment with nonbiotin tagged –SH blockers.

For experiments in intact cells, A549 cells were treated with 10 mM –SH blockers in PBS at RT for 5 min and lysed in mRIPA buffer supplemented with 5 mM IAM-biotin, protease and phosphatase inhibitor tablets at RT for 30 min. The lysate was cleared by centrifugation at

13 000 rpm for 10 min at 4 °C. Protein concentration was measured by using the BCA assay. Equal amounts of samples were separated by 12% SDS-PAGE gels, transferred to nitrocellulose membranes, and analyzed by Western blot using anti-PRX-SO₂/3 antibodies. The chemiluminescence signal was generated using ECL reagents. The blots were stained with Reversible Protein Stain as controls for equal protein loading.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Research reported in this publication was supported by the National Cancer Institute, National Institute of Environmental Health Sciences, and National Heart, Lung, and Blood Institute of the National Institutes of Health under award numbers R33 CA177461 (C.M.F., L.B.P., S.B.K.), R21 ES025645 (C.M.F., S.B.K., L.B.P.), and R01 HL116571 (M.X.). We would also like to acknowledge the Kimbrell family for the support of high-end mass spectrometry instrumentation in C.M.F.'s laboratory, the Comprehensive Cancer Center of Wake Forest University NCI CCSG P30CA012197 grant for support of shared resource facilities, and the Wake Forest Center for Redox Biology and Medicine.

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a) Cross-reaction of classical -SH blockers with protein sulfenic acids (-SOH) **b) Heteroaromatic alkylsulfones as selective -SH blockers** **Figure 1.**

Reaction of current and prospective thiol-reacting compounds with protein thiols (-SH) and sulfenic acids (-SOH). (a) Classical -SH blocking reagents iodoacetamide (IAM) and N-ethylmaleimide (NEM) react with both thiols and sulfenic acids. The resulting -S(O)R adducts can be converted to -SH by reductants such as DTT. The second order rate constants for IAM reaction with proteins -SH and -SOH species are based on the AhpC model system reported earlier.¹² The corresponding rate constants for NEM are based on ref¹³ for -SH and ref¹² for its reaction with -SOH. (b) Reaction of MSBTA (R: -CH₂-COOH) with protein thiols ($k = 0.3 \text{ M}^{-1} \text{ s}^{-1}$ at pH 7.5, determined here using AhpC-SH).

Figure 2.

Reaction of MSBTA and other heteroaromatic sulfonyl amides and alkylsulfones with protein thiols (–SH). (a) Upper panel: Selected region of the ESI-TOF mass spectra showing C165S AhpC-SH (black trace) and the adduct formed following 30 min of incubation with MSBTA at RT (blue trace). Lower panel: Time course of adduct formation upon treatment of C165S AhpC-SH (50 μM) with MSBTA (5 mM) in ammonium bicarbonate buffer at RT ($k = 0.36 \text{ M}^{-1} \text{ s}^{-1}$). (b) Selected region of the ESI-TOF mass spectra showing C165S AhpC-SOH immediately after generation (top), and after 30 min of incubation at RT without or with MSBTA or DMSO control. (c) Potential compounds with anticipated selectivity toward –SH species and Western blot analysis of cell extracts treated with 5 mM compounds **1–5** for 30 min at RT followed by 30 min of incubation with biotin-tagged iodoacetamide (IAM-biotin, 5 mM). C165S AhpC was spiked in as a control. (d) Selected region of ESI-TOF mass spectra showing C165S AhpC-SH (black) and the adduct formed following 2 min of incubation with compound **4** (MSTP; red). Time course of adduct formation upon treatment

of C165S AhpC-SH (50 μM) with MSTP (5 mM) in ammonium bicarbonate buffer at RT is shown on the right panel ($k = 16.6 \text{ M}^{-1} \text{ s}^{-1}$).

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Figure 3.

Comparison of the reactivity of MSTP and NEM toward different sulfur species. (a) Selected regions of ESI-TOF mass spectra showing C165A AhpC-SH (black) and the adducts formed with NEM (green) and MSTP (magenta) following 30 min incubation at RT. (b) Selected regions of ESI-TOF mass spectra showing C165A AhpC-SOH (black) and the adducts formed with NEM (green) but not with MSTP (magenta) following 30 min incubation at RT. The % of each species was calculated based on the peak area of - SN, -SOH, -SO₂H, and -S(O)-NEM species. (c) Selected region of ESI-TOF mass spectra showing the lack of adduct formation upon 30 min incubation of C165S AhpC-SNO (black) with NEM (green) or MSTP (magenta). (d-f) The corresponding ESI-TOF MS profiles of C84S/C94S fRMs-SH, -SOH, and -SNO reactions with NEM and MSTP. (g) Fries' acid reaction kinetics with 30 mM -SH blockers as monitored by UV-vis spectrophotometry at 453 nm showing that only

NEM reacts with –SOH but not MSTP. BCN reaction with Fries' acid was used as a control for the assay.

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Figure 4.

Evaluation of thiol-blocking efficacy by selective MSTP and nonselective NEM. (a) A549 cell lysate was reduced and then incubated with increasing concentrations of MSTP and NEM (0.5, 1, 5, and 10 mM) followed by addition of IAM-biotin to quantify remaining unreacted –SH and –SOH species. Shown are the antibiotin Western blot for IAM-biotin incorporation and the reversible protein stain for loading control and evaluation of –SH blocking in the control C165S AhpC-SH protein. Comparable blocking of AhpC-SH is noted at 5 and 10 mM MSTP or NEM. (b and c) Same as a except MSTP and NEM were added to intact cells for 5 min prior to lysis in the presence of IAM-biotin; b shows the antibiotin Western blot and c the anti-Prx-SO₂/3 blot as further evidence supporting effective blocking of protein –SH by MSTP.