

Proteome-wide survey reveals norbornene is complementary to dimedone and related nucleophilic probes for cysteine sulfenic acid

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ABSTRACT: Detection of cysteine sulfenic acid in live cells is critical in advancing our understanding of cysteine redox chemistry and its biological function. Accordingly, there is a need to develop sulfenic acid-specific chemical probes with distinct reaction mechanisms to facilitate proteome-wide detection of this important posttranslational modification. Herein, we report the first whole-cell proteomics analysis using a norbornene probe to detect cysteine sulfenic acid in live HeLa cells. Comparison of the enriched proteins to those identified using dimedone and other C-nucleophilic probes revealed a complementary reactivity profile. Remarkably, 148 new members of the sulfenome were identified. These discoveries highlight how subtle differences in chemical reactivity of both the probes and cysteine residues influence detection. Overall, this study expands our understanding of protein oxidation at cysteine and reveals new proteins to consider for future studies of cysteine oxidation, redox regulation and signaling, and the biochemistry of oxidative stress.

The study of oxidative stress is a broadly important area of research due to its involvement in many diseases such as heart disease,¹ diabetes,² neurodegenerative diseases,³ and cancer,⁴ as well as the ageing process.⁵ Cysteine residues are particularly susceptible to oxidation during the generation of reactive oxygen species (ROS). This oxidation can serve a scavenging role—destroying ROS—but cysteine oxidation can also be an integral step in redox signaling and regulation.^{6–8} One product of this oxidation, cysteine sulfenic acid, is thought to be a biomarker of oxidative stress and in some cases a precursor to further modifications such as disulfide formation, oxidation to sulfenic and sulfonic acids, disproportionation, or other modifications.^{9,10} In some cases, the sulfenic acid itself is directly involved in protein function, catalysis, and signaling. For example, protein tyrosine phosphatases (PTP) are inactivated by

oxidation of the cysteine to its sulfenic acid^{11–14} and the monoacylglycerol lipase is inhibited.¹⁵ Epidermal growth factor receptor (EGFR) signaling is enhanced by sulfenic acid formation^{16,17} whereas the Src kinase is regulated by cysteine oxidation through structural alterations that contribute to activation of Src activity.¹⁸ These specific examples highlight the various ways in which sulfenic acid formation can affect protein function.^{19,20} To identify and understand such protein functions, the sulfenic acid needs to be readily detected and quantified. However, sulfenic acids are often highly reactive and short-lived, making such analyses challenging. New probes that meet this need will help map the cellular roles of cysteine sulfenic acid and how oxidative stress contributes to disease.^{7–9}

A diverse range of chemical probes for detecting cysteine sulfenic acids have been reported, with many advancing our understanding of the role of cysteine oxidation during oxidative stress.²¹⁻³¹ However, a comprehensive assessment of cysteine oxidation and its cellular role remains elusive. To fill this knowledge gap, there is a need for new chemical probes with complementary selectivity and modes of reaction.

this work

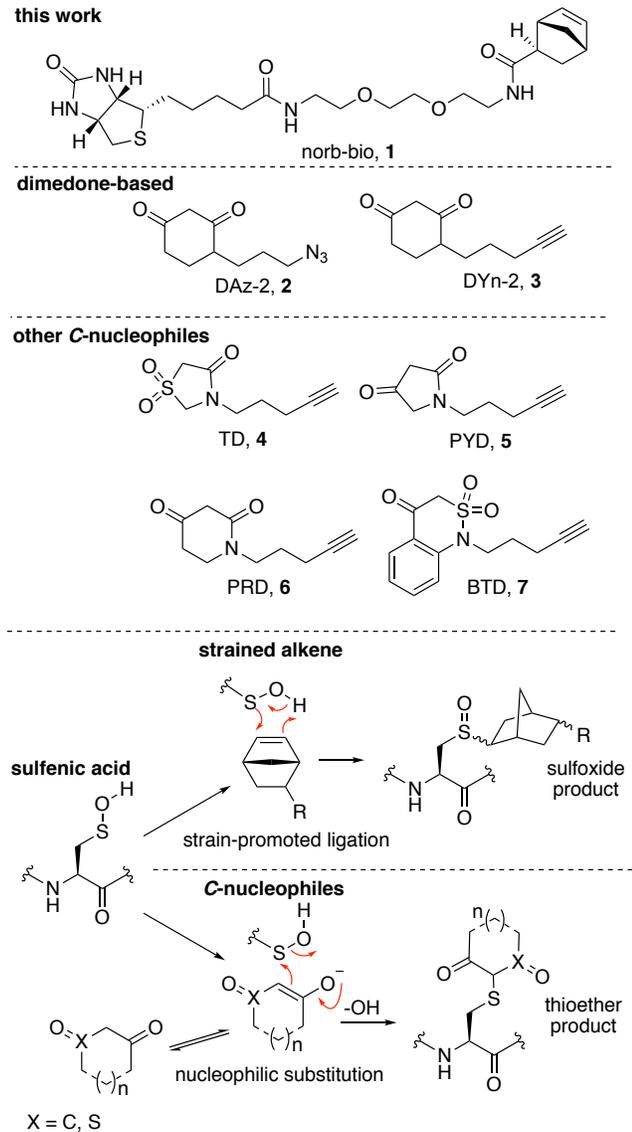


Figure 1. Probes used to detect cysteine sulfenic acid in cell lysates and live cell studies. Probe **1** was used in the current study and reacts with sulfenic acids through a strain-promoted group transfer reaction.³² Dimedone-based probes **2**²¹ and **3**²² have been used extensively in previous studies.^{17,33-36} Other C-nucleophilic probes such as **4-7** as well as **3** were compared in a cell lysate study.²³ Probe **7** has also independently been analyzed for a proteome-wide survey.²⁴

We recently documented the use of norbornene probes as cysteine sulfenic acid traps on small molecules, proteins, and live cells.^{32,37} In both studies, norbornene probes were shown to react rapidly and selectively with sulfenic acids. Notably, a norbornene derivative tagged with biotin (norb-bio, **1**) was used to probe cysteine sulfenic acid formation in living cells

during H₂O₂-induced oxidative stress. These studies validated the probe as a viable tool for the detection of cysteine sulfenic acid on proteins in a cellular context.³² Here, we report the next advance in the use of our norbornene probe in the study of cysteine oxidation: a proteome-wide survey of cysteine sulfenic acid formation in HeLa cells under oxidative stress. In doing so, the set of protein hits using the norbornene probe were identified, for the first time, by comprehensive proteomic analysis. The identified protein hits were also compared to published datasets using various dimedone-based^{21,22} or related carbon-centered nucleophilic^{23,24} probes (Fig. 1). This analysis revealed which proteins were detected by more than one probe and also entirely new proteins previously not identified as part of the sulfenome.

Results and Discussion

Guided by the results obtained in our previous study,³² we chose the conditions which would provide the best opportunity to label, enrich, and identify proteins containing cysteine sulfenic acid. Our approach was to compare any protein hits identified in a negative control sample of HeLa cells not treated with hydrogen peroxide to a sample of HeLa cells treated with the hydrogen peroxide oxidant. Comparison of these two sets of protein hits would allow quantification of enrichment due to oxidation (e.g. sulfenic acid formation). This strategy would also help filter out any false-positives enriched in the negative control, or those not significantly enriched in either (e.g. endogenously biotinylated proteins). In the event, HeLa cells were incubated with norb-bio (3 mM, **1**) in DMSO 1 %v/v for 2 h at 37 °C in 5 % CO₂ humidified atmosphere. After the initial 2 h of incubation with probe **1**, H₂O₂ (2 mM) was added to the set of cells designated for oxidation. This high concentration of oxidant was used based on our previous studies to ensure significant protein oxidation. Both the control samples and the oxidized samples were prepared in biological triplicates. All samples were then left to incubate for a further 2 h at 37 °C in a 5 % CO₂ atmosphere. After this time, the media was removed, and the cells were trypsinized, collected, washed, and lysed. Samples were then purified using a size exclusion spin column to remove any unbound probe (Fig. 2).

Before affinity purification, a small portion of each sample was analyzed by SDS-PAGE and western blot to ensure adequate enrichment of oxidized proteins (e.g. those that contain cysteine sulfenic acid). Only the oxidized samples displayed a streptavidin signal, indicating presence of the norb-bio probe **1** (Fig. 3A-B). All samples were subject to affinity purification using streptavidin agarose beads followed by on-bead reduction with dithiothreitol (DTT) and alkylation with *N*-ethylmaleimide (NEM) to block free thiols. This blocking was designed to prevent disulfide bond formation or other reactions of cysteine thiols during the remainder of the sample preparation. Samples were then eluted from the beads in SDS-PAGE sample buffer containing free biotin (Fig. 2). After this affinity purification and elution of bound proteins from the streptavidin agarose beads, a small portion of the sample was analyzed by SDS-PAGE. Silver staining was used to assess the total protein extracted during the affinity purification and release step. Once again, significant enrichment in protein loading was only observed for the oxidized samples compared to the control (Fig. 3C). This result is consistent with our previous reports that indicate, at 37 °C or lower, a significant reaction between cysteine and norbornene derivatives only occurs

when an oxidant converts the cysteine thiol to a sulfenic acid.^{32,37}

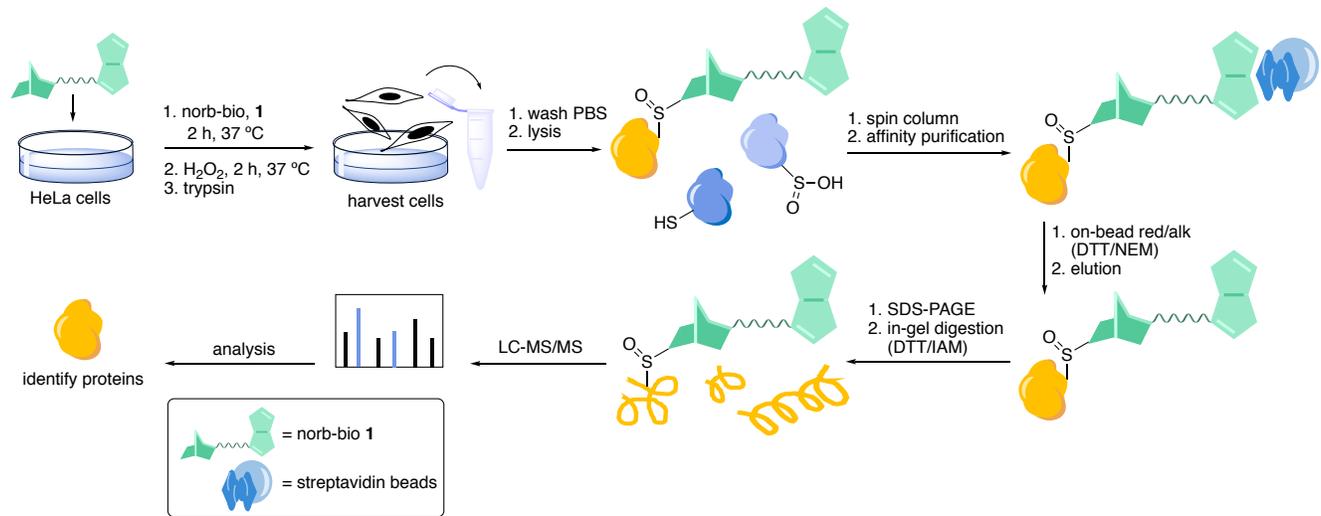


Figure 2. Summary of protocol for using norb-bio 1 to trap, enrich, and identify proteins containing cysteine sulfenic acid. HeLa cells were used in this study and oxidative stress was simulated by treating the cells with 2 mM H_2O_2 .

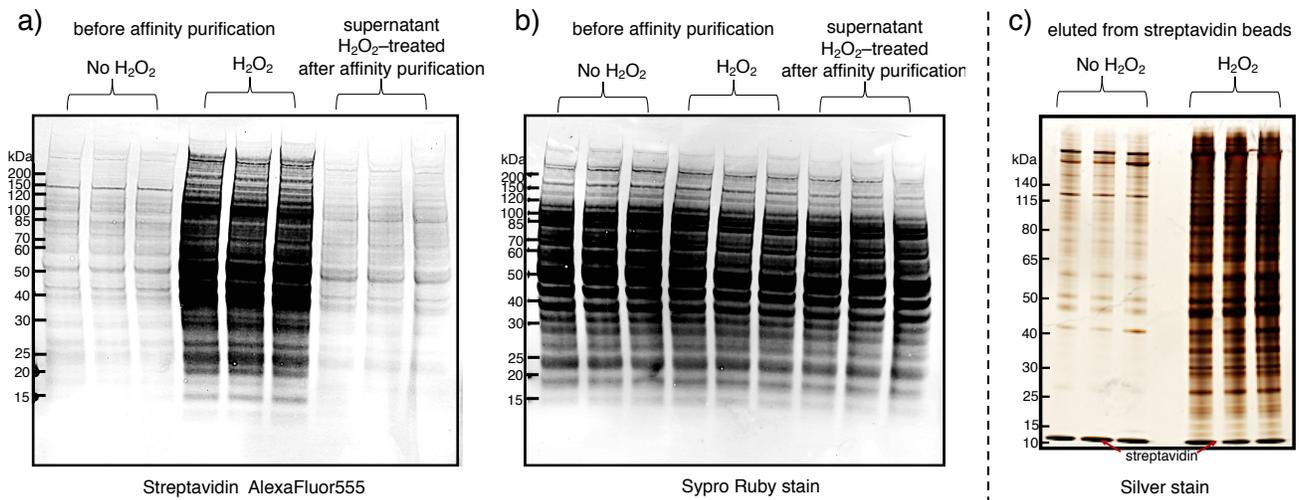


Figure 3. (a) Western blot analyzed with streptavidin AlexaFluor555 showing the enrichment of proteins labeled with norb-bio in live HeLa cells after treatment with and without H_2O_2 . Streptavidin signals are only observed for the H_2O_2 -treated samples (middle three lanes). After affinity purification with streptavidin agarose beads, the supernatant (non-bound proteins) was analyzed for the H_2O_2 -treated samples and no streptavidin labeling was observed, indicating efficient extraction of probe-bound proteins (right three lanes). (b) Western blot treated with Sypro Ruby total protein stain to show total protein loading across all experiments. Each lane was loaded with approximately 20 μ g total protein. (c) SDS-PAGE analysis of total proteins eluted from streptavidin agarose beads after affinity purification of samples treated with and without H_2O_2 . Silver staining was used to visualize total proteins. Significant enrichment is observed for H_2O_2 -treated samples.

Eluted proteins were then prepared for proteomics analysis by desalting (SDS-PAGE), reduction (DTT) and alkylation with iodoacetamide (IAM), trypsin digestion, and peptide extraction. At this stage of analysis, free cysteines were blocked with IAM to distinguish these free cysteines from those blocked in the on-bead reduction/alkylation step with NEM. Therefore, any cysteine containing an NEM modification could not be a sulfenic acid site. Initial LC-MS/MS analysis

revealed a broad spectrum of proteins, with 1056 hits identified across both sample sets based on one unique peptide match. After applying a stringent set of selection criteria (proteins identified with at least two unique peptides, a peptide FDR < 0.01, a protein FDR < 0.01, and a search engine score > 20 for Mascot and > 100 for MS Amanda), 635 proteins were quantified from both sample sets. This data set was further filtered by applying selection criteria for those proteins

that were significantly enriched in the oxidized sample compared to the control (fold change > 2 and ANOVA score < 0.01) to give a narrower set of 482 potential protein hits. These 482 protein hits were further filtered to eliminate any proteins which do not contain a cysteine residue. This provided 473 proteins that may contain the sulfenic acid modification, and therefore constitute a class of proteins that are modified during oxidative stress. It was interesting to note that 9 of the

significantly enriched proteins did not contain cysteine residues. Of these 9 proteins, most were histone or protein subunits, so their enrichment might be due to their strong interaction with a separate protein that contains the cysteine sulfenic acid modification. In this way, the assay provides a way to identify proteins that interact with the sulfenome, without necessarily containing a cysteine residue. The list of 9 proteins is included in the supporting information (Spreadsheet S1).

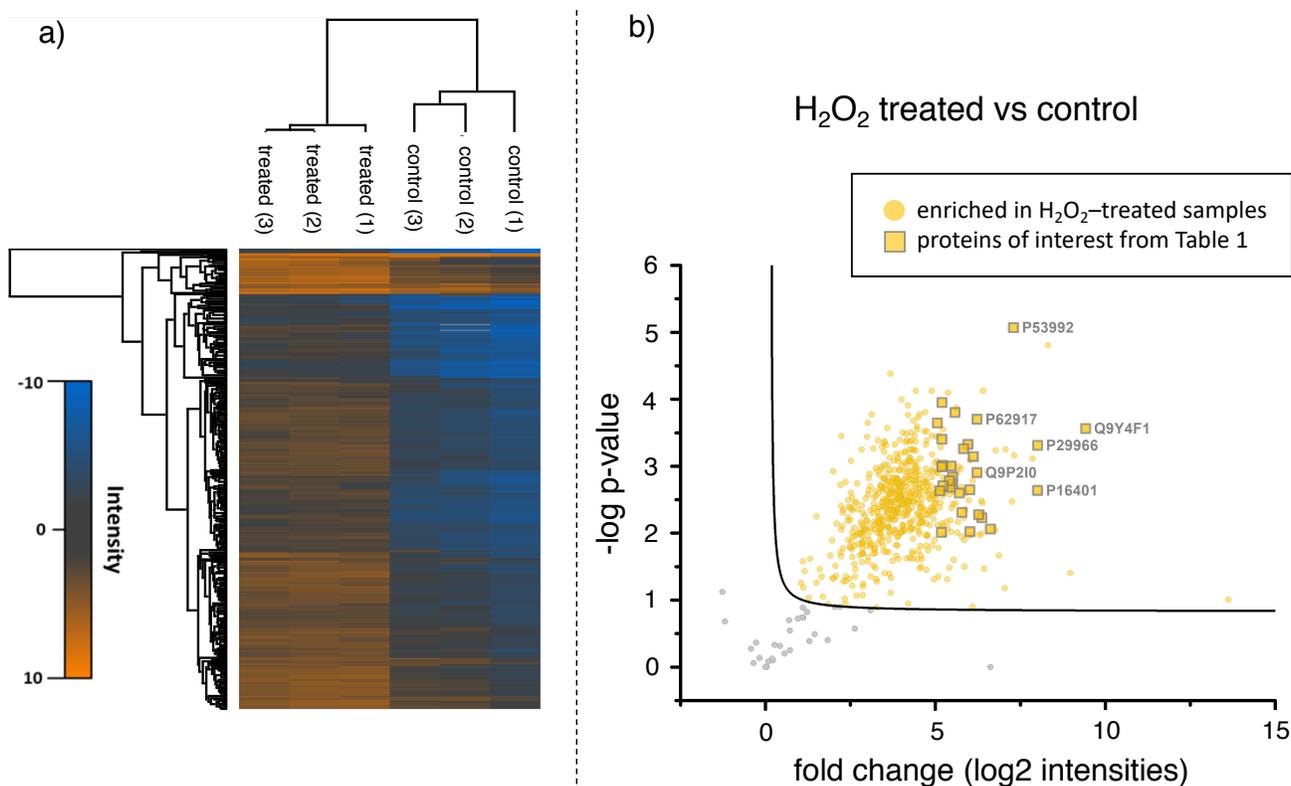


Figure 4. (a) Heatmap showing the similarity between samples in both the negative control (no treatment with H₂O₂) and oxidized samples (treated with H₂O₂). The difference between samples is represented by the length of the connecting line, with a greater difference resulting in longer connecting lines. The heatmap was generated with Perseus 1.6.0.7 on log₂ transformed protein intensities and clustered hierarchically with Euclidean distance. (b) Volcano plot depicting the protein significance (y-axis) compared to the fold change (x-axis). Proteins towards the top right of the plot display large magnitude fold changes that are also statistically significant. The black line depicts the significance border which was corrected for multiple testing in Perseus with S0 value = 0.1 and FDR = 0.01, performed as a two-sided Student's T-test. The 30 proteins in Table 1 are highlighted to show their significant enrichment, with labels for a select few.

It should be noted that these (473) hits require additional validation to verify the site of the cysteine sulfenic acid formation and also to establish a cellular role of this protein oxidation. With that said, it should be reiterated that these 473 proteins all have cysteine residues and only react efficiently with norbornene in the presence of hydrogen peroxide. This is consistent with sulfenic acid formation and trapping with the norbornene probe. Therefore, this set of proteins should be of interest to the community of researchers investigating the sulfenome and broader aspects of protein oxidation and redox regulation. To facilitate these efforts, a full list of the proteins is provided in supporting information (Spreadsheet S1). This includes protein description, confidence score, Anova (p), max fold change, and amount detected (fmol) for each replicate. A graphical representation of

the comparison between the control (not treated with H₂O₂) and oxidized (treated with H₂O₂) sample sets and statistical analysis of the identified proteins is shown in Figure 4.

These 482 enriched protein hits are linked to a diverse range of biological pathways and cellular locations (Fig. 5). Regarding the latter, a large number of proteins in the cytosol or nucleus were detected. With respect to biological pathways, the majority of protein hits were associated with generalized cellular metabolism or gene expression. This includes well documented proteins such as alpha-enolase, GAPDH, lactate dehydrogenase (A chain), and fructose-bisphosphate aldolase A, all of which are involved in glycolysis and have been shown to be susceptible to cysteine oxidation, some with implications in maladies such as Alzheimer's disease.³⁸⁻⁴³

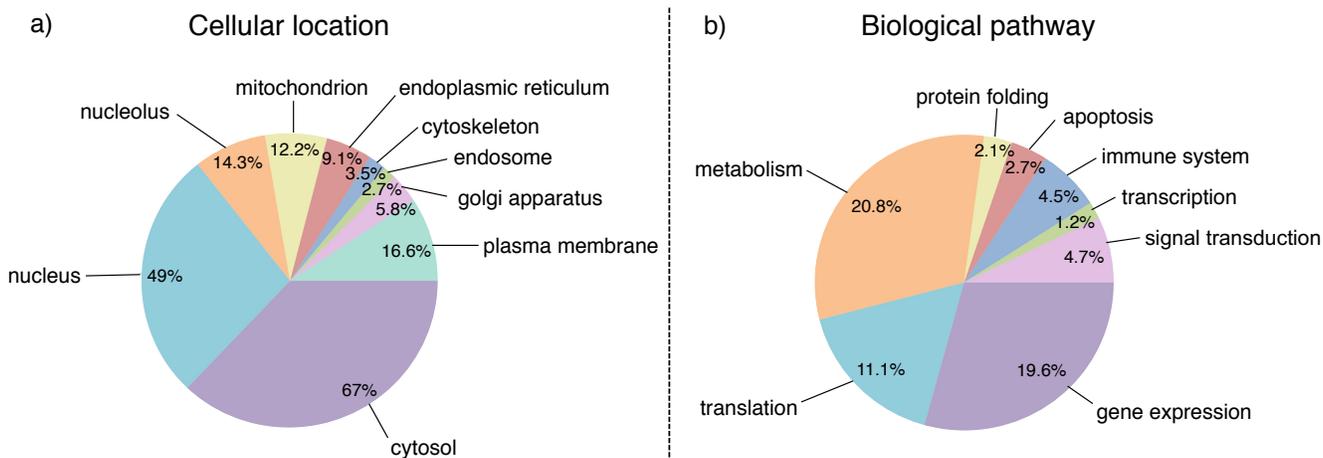


Figure 5. (a) 482 enriched proteins identified using norb-bio probe **1**, categorized into generalized cellular locations. (b) 482 enriched proteins identified using norb-bio probe **1**, categorized into generalized biological pathways. In both cases, some proteins may have multiple cellular locations or participate in multiple cellular pathways, which is reflected in the number of proteins assigned to each category. As such, total protein components or pathways percentages reflect the percentage of proteins in the dataset which exhibit the particular feature. The 9 enriched proteins without cysteine residues were included in this analysis since they may play a role by association with other proteins that contain oxidizable cysteine residues.

The data set of 473 enriched proteins with cysteine residues was then compared to protein hits identified in a previous cysteine sulfenic acid survey on HeLa cells using dimedone-based probe **2**.²¹ Of the 193 reported proteins, and the 59 able to be cross-referenced in the UniProt (Swiss-Prot) database, only 26 proteins were common with this current study. This difference in profile could relate to the distinct mechanisms of the two probes (Fig. 1) or steric differences. This observation is not unexpected as previous studies by Carroll and co-workers have demonstrated how subtle differences in probe design can lead to interactions with different proteins.^{23,44} In fact, our previous studies on cell lysates using a similar dimedone-based probe appeared to display a different reactivity profile to our norb-bio probe by western blot, which is consistent with the outcome observed in this proteomics study.³²

We next compared the hits in our sulfenome survey with those reported for other probes. These studies all used different chemical probes such as **3** (human RKO cell line, 778 protein hits),²² **7** (human RKO cell line, 1202 protein hits),²⁴ and a comparison of probes **3-7** (human RKO cell lysates, 761 protein hits combined),²³ some of which have reported greater reactivities than typical dimedone-based probes. These comparisons should be taken with due care as the conditions and methods differ slightly across each study, which could account for the differences in protein identification. These differences can include the cell line used in the analysis, the concentration and method of adding the oxidant, and whether the analysis was performed on cell lysates or live cells. With these qualifications, we still thought it would be valuable to investigate if any of the 473 protein hits we had identified with our norb-bio probe had been previously reported to contain cysteine sulfenic acids, and also to determine if there are any new proteins identified in the sulfenome.

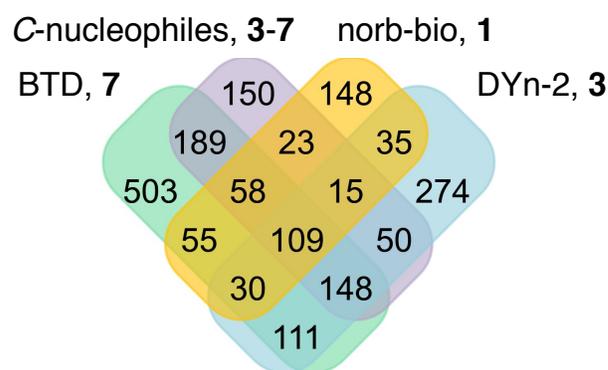


Figure 6. Venn diagram comparing different datasets of identified protein sulfenic acids. Orange represents the 473 proteins identified in this data set using norb-bio **1**. Blue represents hits found using **3** (772/778 proteins matched).²² Purple represents a combined dataset of proteins found using probes **3-7** (742/761 proteins matched).²³ Green represents hits found using probe **7**.²⁴ Note: not all proteins were able to be cross-referenced in the database for comparison and this is indicated by proteins matched out of total proteins reported in the original dataset. The 9 enriched proteins without cysteine residues were excluded from this comparison since they do not contain sulfenic acids.

We found that of the 473 enriched proteins we identified as plausible hits, 148 (31 %) appear to be previously unknown sulfenic acid sites. It was also reassuring that 325 of the 473 hits (69 %) had been previously identified in other studies, indicating our probe is reacting with a similar subset of proteins as other probes. A graphical representation of the number of common proteins between the datasets is shown in Figure 6. The full list of proteins as analyzed from each study is included in the supporting information (Spreadsheet S3).

From these 148 previously unknown hits, we analyzed 28 proteins which appeared to have the greatest fold change (>30) in enrichment and therefore proteins that react significantly with norb-bio (1) in the presence of hydrogen peroxide. These selected proteins are compiled in Table 1. A full list of the 148 proteins is also included in the supporting information (Spreadsheet S2), along with a list of the most enriched proteins (66 with a fold change >30), and the proteins listed in Table 1. We provide these protein hits here as potential leads for future investigation of cysteine sulfenic acid in redox regu-

lation and other cellular functions. The last two entries of Table 1 represent two of the 9 proteins which do not contain cysteines but were significantly enriched in the analysis. These proteins may be of interest for their interactions with proteins that do contain oxidizable cysteine residues. All 30 protein hits in Table 1 are highlighted in Figure 4B to emphasize their statistical significance and magnitude of enrichment in the comparison between cells treated with H₂O₂ and those not subjected to oxidative stress.

Table 1 List of 30 selected proteins, their cellular location, and biological process (if known) from UniProt database. Proteins were selected from the 148 previously unknown hits and chosen based on largest fold change (>30). The last two entries represent proteins that do not contain cysteine residues but were significantly enriched in the analysis when treated with hydrogen peroxide. Additional details of the 30 selected proteins is included in supplementary information (Spreadsheet S2).

UniProt ID	Protein name	Cellular location [#]	Biological process
A2RRP1	Neuroblastoma-amplified sequence	ER	protein transport
P30530	Tyrosine-protein kinase receptor UFO	PM	differentiation, immunity
Q12802	A-kinase anchor protein 13	C, N	
Q9C0D5	Protein TANC1	PM	
P49756	RNA-binding protein 25	N, C	mRNA processing, mRNA splicing
Q8NI27	THO complex subunit 2	N	mRNA processing, mRNA splicing, mRNA transport, transport
Q9H3S7	Tyrosine-protein phosphatase non-receptor type 23	E, N, CS	cilium biogenesis/degradation, protein transport, transport
P49589	Cysteine--tRNA ligase, cytoplasmic	C	protein biosynthesis
P29966	Myristoylated alanine-rich C-kinase substrate	CS, PM	
P33527	Multidrug resistance-associated protein 1	M	transport
Q9ULH0	Kinase D-interacting substrate of 220 kDa	E, PM	neurogenesis
Q9Y4F1	FERM, ARHGAP and pleckstrin domain-containing protein 1	C, PM	
P11274	Breakpoint cluster region protein	PM	
Q9Y3P9	Rab GTPase-activating protein 1 activating protein GAPCenA)	C, CS	cell cycle
Q6KC79	Nipped-B-like protein	N	cell cycle, transcription, transcription regulation
Q15276	Rab GTPase-binding effector protein 1	E, C	apoptosis, endocytosis, protein transport, transport
Q8TF05	Serine/threonine-protein phosphatase 4 regulatory subunit 1		
Q96SU4	Oxysterol-binding protein-related protein 9	E, G	lipid transport, transport
Q9P210	Cleavage and polyadenylation specificity factor subunit 2	N	mRNA processing
P46781	40S ribosomal protein S9	C	
P46821	Microtubule-associated protein 1B	CS, C	

P46379	Large proline-rich protein BAG6	C, N, S	apoptosis, differentiation, immunity, transport
P62917	60S ribosomal protein L8	C	
Q5SRE5	Nucleoporin NUP188 homolog	N	mRNA transport, protein transport, translocation, transport
P53992	Protein transport protein Sec24C	ER, C	ER-golgi transport, protein transport, transport
Q27J81	Inverted formin-2	C	actin binding
Q6YHU6	Thyroid adenoma-associated protein	C, ER	
Q99570	Phosphoinositide 3-kinase regulatory subunit 4	E, M	kinase, serine/threonine-protein kinase, transferase
P16401*	Histone H1.5	N	DNA-binding
P16402*	Histone H1.3	N	DNA-binding

#Cellular locations: C = cytoplasm, PM = plasma membrane, N = nucleus, ER = endoplasmic reticulum, CS = cytoskeleton, G = Golgi apparatus, E = endosome, S = secreted

* These proteins do not contain a cysteine residue but are significantly enriched in the analysis.

Additionally, 109 proteins were identified as common across all studies compared in this analysis (Fig. 6). A full list of the 109 proteins is also included in supplementary information (Spreadsheet S2). Because these proteins are consistently detected across multiple studies with different probes and labeling conditions, these proteins clearly contain oxidizable cysteine residues that can be readily converted into cysteine sulfenic acid.

For future studies, specific proteins of interest could be tested to determine what function, if any, the sulfenic acid residue plays in redox regulation or signaling. Further studies could also use this survey as a starting point in physiological studies relating to oxidative stress. Additionally, a total of 1056 proteins were identified in this study before applying stringent selection criteria, so there is a possibility of an even broader population of proteins in the sulfenome. To assess these proteins, we are developing modified norbornene probes to facilitate more sensitive and general detection of proteins containing cysteine sulfenic acid.

Conclusion

This proteomics study has provided new insight into members of the sulfenome. A total of 148 out of 473 proteins were detected that had not been previously identified in other studies. This result reveals that the norbornene probes for cysteine sulfenic acid are complementary to dimedone and related nucleophilic probes.⁴⁵ The full list of protein hits identified using the norbornene probe provides motivation for further analysis into the role of cysteine oxidation in cellular function and disease. Our own laboratories are pursuing these hits further with an aim to discover new roles for cysteine oxidation.

Methods

General considerations

All purchased chemicals were used as received without further purification. All fluorescence and UV-Vis spectroscopy in a 96-well plate format was performed on a SpectraMax i3x plate reader (Molecular Devices). All western blot images were recorded on a Typhoon Trio Variable Mode Imager (GE Healthcare). Proteomics of peptide fragments were performed

using a RSLCnano U3000 HPLC coupled to a QExactive plus mass spectrometer via a nano-electrospray ion source. Separation of peptides was on an Acclaim PepMap RSLC C₁₈, 25 cm x 75 μm x 2 μm particle size, 100 Å pore size column.

Synthesis of norb-bio 1

Norb-bio 1 was synthesized as previously described.^{32,37}

Cell culture of HeLa cells

HeLa cells (derived from cervical cancer cells, ATCC) were maintained in a humidified atmosphere of 5% CO₂ at 37 °C and cultured in high glucose DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), and 1% penicillin-streptomycin (PS) (Gibco). Cells were grown to ~80-90% confluency, trypsinized (0.25%, trypsin-EDTA, Gibco), then neutralized with complete media prior to use.

Gel electrophoresis and western blot

Samples were separated by SDS-PAGE using NuPage 4-12% Bis-Tris protein gels (Invitrogen) with MES running buffer (200 V) and transferred to a polyvinylidene difluoride (PVDF) membrane (0.2 μM, iBlot, ThermoFisher) at 20 V. After transfer, the PVDF membrane was first washed with water (3 × 5 min) then blocked with BSA (3% in tris-buffered saline Tween-20 (TBST)) for 1 h at room temperature with gentle rocking. The membrane was washed with TBST (3 × 5 min) then incubated with 1:1,000 Alexa Fluor® 555 streptavidin (1 mg/mL stock in PBS, Invitrogen) in TBST for 30 min at room temperature with gentle rocking. The PVDF membrane was washed with TBST (2 × 5 min), water (2 × 5 min), and imaged by fluorescence (filter: 580, laser: 532 nm, PMT: 550 V) using Typhoon Trio imager. To assess equal protein loading, PVDF membranes were stained with Sypro Ruby blot stain (Invitrogen) according to manufacturer's instructions and imaged by fluorescence (filter: 610, laser: 532 nm, PMT: 550 V) using Typhoon Trio imager.

Preparation of stock solutions

Norb-bio (74 mg, 0.15 mmol) was dissolved in DMSO (500 μL) to give a final concentration of 300 mM. H₂O₂ (34 μL, 50 wt. % in H₂O, 17.6 M) was diluted in H₂O (966 μL) to give a final concentration of 600 mM. Dithiothreitol (DTT) (30 mg,

0.19 mmol) was dissolved in H₂O (982 μ L) to give a 200 mM stock solution. *N*-Ethylmaleimide (NEM) (54.5 mg, 0.44 mmol) was dissolved in PBS (4.355 mL) to give a 100 mM solution. Biotin (24 mg, 0.1 mmol) was dissolved in DMSO (1 mL) to give a 100 mM stock solution.

Labeling experiment on HeLa cells

Cells were cultured as described above. Cells were seeded in 6 \times T75 flasks and grown until \sim 90% confluency (approximately 8.4×10^6 cells per flask at confluency). Prior to experiment, the media was removed and cells washed with DMEM low glucose 0.5 % FBS media (2 \times 8 mL). A stock solution of norb-bio probe in media was prepared by adding norb-bio (500 μ L of a 300 mM stock in DMSO) to DMEM low glucose 0.5 % FBS media (50 mL). After removing wash media from cells, the norb-bio (3 mM) media (8 mL) was added to each flask and incubated at 37 $^{\circ}$ C for 2 h. After this time, H₂O₂ (27 μ L, 600 mM stock) was added to three flasks (oxidized samples) and further incubated at 37 $^{\circ}$ C for 2 h. The remaining three flasks were also further incubated for 2 h at 37 $^{\circ}$ C. After 2 h, the media was removed and trypsin (4 mL) added for 5 min. Trypsin was then neutralized with complete DMEM 10 % FBS media (6 mL). Cells were collected and gently centrifuged (160 \times g, 5 min) and trypsin removed before washing with cold PBS (3 \times 10 mL). Cell extraction buffer with protease inhibitor (500 μ L, Pierce) was added to the pellet and incubated on ice for 45 min with regular vortexing. After this time, cell lysis was centrifuged (10000 \times g, 5 min) then purified with a zeba spin column (7 kDa MWCO, 2 mL) pre-equilibrated with PBS and stored at -80° C until further use.

Affinity purification

Reactions were conducted according to the above labeling protocol. After removing unbound probe (zeba spin column), cell lysis (500 μ L) was collected and incubated with pre-washed (3 \times 1 mL PBS) streptavidin agarose bead conjugates (200 μ L, of 1.2 mg/mL as a 50% slurry in PBS, Merck). The beads were left to incubate for 2 h at 4 $^{\circ}$ C with gentle rocking. The beads were collected by centrifugation (14,000 g, 30 sec) and the supernatant removed and retained for analysis. The beads were washed (PBS 2 \times 1 mL, cell extraction buffer 1 \times 1 mL, PBS 2 \times 1 mL) each for 5 min with mixing between steps. After the final wash, a solution of DTT (50 μ L, 200 mM stock in water) was added to the beads with 450 μ L PBS to give 20 mM DTT. The beads were incubated at 37 $^{\circ}$ C for 1.5 h with shaking in the dark. The supernatant was removed and retained, then a solution of NEM (500 μ L, of 100 mM in PBS) was added to each sample and incubated at 37 $^{\circ}$ C for 1.5 h with shaking in the dark. The supernatant was removed and retained and the beads washed with PBS (1 mL). Biotinylated proteins were then eluted in 50 μ L elution buffer (prepared PBS (160 μ L), LDS (160 μ L), sample reducing agent (36 μ L), 2 mM free Biotin (8 μ L from 100 mM stock in DMSO)) by first incubating at room temperature for 20 min, followed by heating to 95 $^{\circ}$ C for 10 min. The supernatant was collected and stored at -80° C until further use.

LC-MS sample preparation

20 μ L protein eluate per sample were desalted through electrophoretic migration at 50 V for 10 min on a 4–12 % Bis-Tris polyacrylamide gel (Novex NuPAGE, Thermo Scientific, Darmstadt, Germany). After silver staining, protein bands were cut out, reduced, alkylated and digested with trypsin

before peptide extraction via sonication. Peptides were dissolved and diluted with 0.1 % TFA (v/v).

LC-MS analysis

For mass spectrometric analysis, 15 μ L peptide solution per sample were analyzed on a nano-high-performance liquid chromatography electrospray ionization mass spectrometer. 20 fmol Pierce Peptide Retention Time Calibration Mixture (Thermo Fischer Scientific, Bremen, Germany) were added to each sample before injection. The analytical system was composed of a RSLCnano U3000 HPLC coupled to a QExactive plus mass spectrometer via a nano-electrospray ion source (Thermo Fischer Scientific, Bremen, Germany). Injected peptides were concentrated and desalted at a flow rate of 6 μ L/min on a trapping column (Acclaim PepMao C₁₈, 2 cm \times 100 μ m \times 3 μ m particle size, 100 Å pore size, Thermo Fischer Scientific, Bremen, Germany) with 0.1 % TFA (v/v) for 10 min. Subsequently, peptides were separated at a constant flowrate of 300 nL/min over a 60 min gradient on an analytical column (Acclaim PepMap RSLC C₁₈, 25 cm \times 75 μ m \times 2 μ m particle size, 100 Å pore size, Thermo Fischer Scientific, Bremen, Germany) at 60 $^{\circ}$ C. Separation was achieved through a gradient from 4 to 40% solvent B (solvent A: 0.1% (v/v) formic acid in water, solvent B: 0.1% (v/v) formic acid, 84% (v/v) acetonitrile in water). Afterwards, peptides were ionized at a voltage of 1,500 V and introduced into the mass spectrometer operated in positive mode. MS scans were recorded in profile mode in a range from 200–2000 m/z at a resolution of 70,000 while tandem mass spectra were recorded at a resolution of 17,500. Tandem mass spectra were recorded with a data dependent Top20 method and 30% normalized collision energy. Dynamic exclusion was activated.

Computational mass spectrometric data analysis

Proteome Discoverer (version 2.1.0.81, Thermo Fisher Scientific, Bremen, Germany) was applied for peptide/protein identification with Mascot and MS Amanda as search engines employing the UniProt database (human; including isoforms; date 2019-02-13). A false discovery rate of 1% ($p \leq 0.01$) on peptide level was set as identification threshold. Proteins were quantified with Progenesis QI for Proteomics (Version 2.0, Nonlinear Dynamics, Waters Corporation, Newcastle upon Tyne, UK).

Analysis of proteins

All pie charts, Venn diagrams, and comparison lists were generated using FunRich analysis tool.^{46,47} The UniProt database (human; Swiss-Prot; isoforms not included) was used for all individual protein comparison and compartment assignments within the FunRich tool. The FunRich database was used for biological pathway assignments. Data from previously published surveys were taken from provided supplementary files and converted to their corresponding UniProt accession for use in comparison studies. The converted UniProt accession of each study is provided in supplementary information (Spreadsheet S3).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website. Spreadsheet S1 file contains the full list of quantified and enriched protein hits with statistical analysis. Spreadsheet S2 file contains the full list of 148 newly identified

proteins, including the 30 from Table 1 with additional information, and the full list of 109 proteins identified in all studies. Spreadsheet S3 file contains the full lists of proteins from previous surveys using probes 2-7 after conversion to UniProt accession that were used for the comparison.

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Author Contributions

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L.J.A carried out all probe synthesis, cell culture, pull-down studies, and assisted with proteomics analysis. M.L. completed all mass spectrometry, proteomic, and bioinformatics analysis of enriched proteins. K.S. and M.R. contributed to project supervision of proteomics analysis and evaluated data. M.V.P. contributed to project supervision of chemical synthesis and use of probes. G.J.L.B and J.M.C. conceived of the project and directed the research. L.J.A. and J.M.C. wrote the manuscript and all authors contributed to its evaluation and revision.

Notes

The authors declare no competing financial interest.

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Table of Contents Graphic:

