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Article

Keywords: sulfinic acid, redox modification, CysOx

Posted Date: October 19th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-956877/v1

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Version of Record: A version of this preprint was published at Nature Communications on September 21st, 2022. See the published version at https://doi.org/10.1038/s41467-022-33124-z.
Reaction-based fluorogenic probes for selective detection of cysteiny1 oxidation in living cells

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Measuring reactive oxygen, nitrogen and sulfur species in cells is established technology, but turn-on fluorescence tools for detecting the products of their reaction with protein cysteines remain essentially unknown. Toward this goal, here we describe fluorogenic probes for sulfenic acid, a redox modification of protein cysteines inextricably linked to signaling and oxidative stress. The probes, called CysOx1 and CysOx2, are reaction-based, exhibit excellent cell permeability, rapid reactivity, and high selectivity with minimal cytotoxicity. We applied CysOx2 in a cell-based 96-well plate assay to determine whether kinase inhibitors modulate protein S-sulfenylation as well as O-phosphorylation. Analysis of these data revealed an unexpected positive association of S-sulfenylation and inhibition of select kinases within the TK, AGC, and CMGC families including GSK3, a multitasking Ser/Thr kinase and emerging therapeutic target for neurodegenerative and mood disorders. Chemoproteomic mapping of sulfenic acid-modified cysteines in GSK3 inhibitor-treated cells shows that sites of S-oxidation localize to regulatory cysteines within key components of antioxidant defense systems. Our studies with CysOx probes offer up new insights into kinase-inhibitor dependent modulation of sulfenylome dynamics and should accelerate future efforts in the modern era of translational redox medicine.
Redox reactions and oxidative stress have been implied in the etiology of numerous diseases as well as in the aging process. The modern era in translational redox medicine seeks to identify types, sources, metabolizers, and targets of oxidants in order to develop effective drugs and therapies for ROSopathies.

In this context, turn-on fluorescence probes also referred to as fluorogenic probes that measure oxidative stress in living cells have proven invaluable for redox-related biomedical research. Analogous tools for detecting the reaction products between biological oxidants and proteins are grossly underdeveloped, a point exemplified by the lack of fluorogenic probes for detecting protein cysteine (i.e., cysteinyl) oxidation. The latter issue is especially striking since protein cysteines are the major target of oxidants originating from both endogenous and exogenous sources.

Sulfenic acid (Cys-SOH) is a central redox modification of protein cysteines and is inextricably linked to oxidant signaling and stress. Sulfenic acid is generated by oxidation of a thiolate by reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂) produced during cellular signaling and metabolism or by hydrolysis of sulfenyl halides, and very polarized nitrosothiols and disulfides. If stabilized by the protein microenvironment, the thiol-sulfenic acid pair can operate as a switch that is triggered by redox changes to regulate protein function, structure, and localization. The electrophilic sulfur atom in sulfenic acid can also react with a protein or low-molecular-weight thiol to form a disulfide or, under conditions of excess oxidative stress, can be oxidized further to sulfinic and sulfonic acids. In either scenario, stabilized or as transient intermediate, sulfenic acids are key modifications in the domain of biological redox regulation.

Selective chemical detection of sulfenic acid is predicated on the chemical nature of this moiety in which the sulfur can behave as both a nucleophile and electrophile. The latter reactivity has been exploited by numerous groups to develop carbon-based nucleophilic probes for detecting sulfenic acid in proteins and cells. The first fluorescent probe for sulfenic acid was reported in 2007, consisting of fluorescein.
attached at the C-4 position on 1,3-cyclohexanedione (Supplementary Fig. 1a). The nucleophilic C-2
carbon reacts selectively with sulfinic acid, but its application has been limited by the absence of “turn-
on” fluorescence or fluorogenic response. In 2016, our group identified phenaline-1,3-dione as a
chemical scaffold having fluorogenic potential vis-à-vis its reaction with sulfinic acid\textsuperscript{17} that was
subsequently adapted in the design of a ratiometric fluorescent probe\textsuperscript{18} (Fig. 1a and Supplementary Fig.
1b). However, exceedingly slow reaction rates of less than 0.01 M\textsuperscript{-1}s\textsuperscript{-1} and the absence of turn-on
fluorescence signal precluded any practical application.

To address the significant gap in chemical tools available that report on cysteinyl oxidation in real-time in
living cells, here, we describe fluorogenic probes for detecting sulfinic acid (Fig. 1b and Supplementary
Fig. 1c). The probes are reaction-based, exhibit excellent cell permeability, rapid reactivity, and high
selectivity with minimal cytotoxicity. The chemical tools have enabled live-cell imaging studies to detect
redox-dependent changes in cysteinyl oxidation. Adaptation to a 96-well plate format enabled high-
throughput analysis of sulfinic acid in cells and this platform was used to screen a curated inhibitor
library, targeting major families of the human kinome, as potential modulators of cysteinyl oxidation.
These studies identified a cohort of GSK3 kinase inhibitors that elicited significantly increased sulfinic
acid modifications that localized to the regulatory cysteines of proteins involved in the response to
oxidative stress. Collectively, these studies report on first-in-class tools for facile fluorogenic detection of
cysteinyl oxidation in a variety of formats with broad application in future studies of redox biology and
drug pharmacology.

Results
To create a fluorogenic probe for sulfinic acid, three key alterations of the phenaline-1,3-dione scaffold
were envisioned: (i) replacement of a carbonyl group with the more electron withdrawing sulfonyl group
to increase the rate of reaction; (ii) appendage of an amino group to create an electron “push-pull system”
system, and (iii) fluorination of the aromatic core to enhance absorption and fluorescence wavelengths
Optimized synthetic schemes for the resulting compounds is described under Synthetic Procedures. In brief, 6-bromo-2H-naphtho[1,8-bc]thiophen-2-one was prepared from naphthalene-1-thiol in three steps as previously reported. The thiophenone core was hydrolyzed and dimethylated to produce both thioether and ester functional groups and the thioether was oxidized to a sulfone group with mCPBA. The brominated sulfone intermediate was then used in amination reactions, followed by condensation in presence of NaH to produce the C-nucleophile center. Finally, a fluorination step provided the desired compounds.

**Kinetic and fluorescence characterization of phenaline-1,3-dione derivatives.** We first sought to assess the reactivity of phenaline-1,3-dione (1) and its analogs with an established small-molecule model for cysteine sulfenic acid, known as CSA. Pseudo first-order rate constants were obtained for compound 1 and related analogs designed to evaluate features that modulate reactivity and fluorescence properties. Compound 1 exhibited modest kinetics of 0.002 s⁻¹ and fluorination of this scaffold at the C-2 position (2 and 3) decreased reactivity by more than two orders of magnitude. Replacement of one carbonyl group with a sulfonyl moiety (4) increased the reactivity of the phenaline-1,3-dione core by more than 1000-fold. C-2 fluorination (5) of the parent sulfonyl compound enhanced reactivity while introduction of a 7-amino group (6) decreased reaction rate. Although less reactive, the amino group enhanced the water solubility as compared to non-aminated analogs. Further modification by fluorination at C-6 (7) retarded kinetics by 10-fold. Finally, the 7-amino
group was replaced by the cyclic amine, azetidine (8) affording a compound with an observed reaction rate of approximately 0.1 s\(^{-1}\). Overall, the sulfonyl group dramatically increased nucleophilic reactivity towards the sulfenic acid electrophile, which was tempered somewhat by the installation of amino and fluorine groups, as expected.

In subsequent experiments, we evaluated the fluorescence properties of sulfonyl derivatives 4 through 8. For this purpose, the reaction product between each analog and CSA was prepared and isolated from milligram-scale reactions. Fluorescence spectra of the resulting adducts were then recorded in organic solvent or aqueous solution and compared to that of non-adducted compounds alone (Fig. 2c-d, and Supplementary Fig. 3). CSA adducts of 4 and 5 exhibited weak fluorescence signal and were essentially nonfluorogenic. Furthermore, compounds 4 and 5 were only sparingly soluble in water. Compound 6 showed improved solubility but gave only weak fluorescence signal before and after reaction with CSA.

By contrast, CSA adducts of compounds 7 and 8 were fully soluble in PBS, exhibited bathochromic shifts of excitation and emission maxima as well as increases in fluorescence intensity compared to 7 or 8 alone, indicating that these compounds are fluorogenic for sulfenic acid (Fig. 2c-d). Specifically, the CSA-7 adduct gave a slight bathochromic shift of excitation and emission maxima of 3 nm with no change in the Stokes shift. The bathochromic shift was more pronounced for the CSA-8 adduct (53 and 75 nm for excitation and emission maxima, respectively) accompanied by an increase in the Stokes shift of 22 nm. The increase in fluorescence intensity was 2.3- for CSA-7 and 11.4-fold for CSA-8 (quantum yields increased by 2.1- and 2.6-fold, respectively) compared to 7 and 8 alone. UV-Vis spectroscopy analysis of 7 and 8 with their respective CSA adducts showed an increase in extinction coefficient of 1.7- and 6.4-fold, respectively (Supplementary Table 1 and Supplementary Fig. 4-5). Finally, a bathochromic shift in the absorption maxima of CSA-8 was also observed, in agreement with the shift observed in the fluorescence spectra. Comparison of data obtained for compounds 7 and 8 to compound 6 demonstrates that fluorination is essential for their fluorogenic response.
Next, we measured the fluorescence intensity of 7 and 8 in real-time reactions with CSA. Reactions containing 7 or 8 were excited at 443 nm and 447 nm, respectively. Fluorescence emission intensity increased over time and was linear with probe concentration (Fig. 2e,f). Pseudo first-order rate constants were then obtained at different probe concentrations to obtain second-order rate constants, which were
91 ± 3 and 105 ± 2 M\(^{-1}\) s\(^{-1}\) for 7 and 8, respectively (Fig. 2g,h). To screen for potential side reactions, we evaluated the reactivity of 7 and 8 with a panel of potentially reactive biomolecules, such as aldehyde and disulfide electrophiles as well as related sulfur species including reduced glutathione, glutathione nitrosothiol, glutathione sulfenic and sulfonic acid. No significant reaction took place between 7 or 8 and any of the aforementioned compounds (Fig. i,j). Taken together, these studies indicate that compounds 7 and 8, referred to hereafter as CysOx\(_1\) and CysOx\(_2\), exhibit reaction-based turn-on fluorescence, rapid reactivity, and high selectivity when evaluated in a small-molecule model of cysteine sulfenic acid.

**Evaluation of CysOx reactivity and fluorescence in complex biological settings.** Encouraged by our success in the CSA model, we then moved on to more targets with greater biological relevance and complexity. Toward this end, we examined CysOx probe reactivity in C64,82S glutathione peroxidase 3 (Gpx3; Fig. 3). This Gpx3 variant has one redox-sensitive cysteine, C36 that is readily oxidized to sulfenic acid using stoichiometric amounts of hydrogen peroxide (H\(_2\)O\(_2\)) and has been well-validated as a model for the study of protein sulfenic acid reactivity\(^{19,20}\). Intact mass spectrometry (MS) analysis demonstrated that Gpx3 C36 sulfenic acid (Gpx3-SOH) formed the anticipated adduct with CysOx probes in high yield, while reduced Gpx3 (Gpx3-SH) was not modified (Fig. 3b,c). Having verified the correct protein adducts by MS, we next evaluated the fluorescence spectra of isolated Gpx3-CysOx adducts compared to that of free CysOx probes. Satisfyingly, CysOx\(_1\) and CysOx\(_2\) exhibited a “turn-on” fluorescence response after respective excitation at 357 nm and 394 nm (Fig. 3d,e). Of the two probes, CysOx\(_2\) showed a larger fluorescence enhancement of up to 4-fold. Fluorescence spectra of analogous real-time or in-situ reactions gave similar results and also demonstrate that fluorogenic response is only observed when all three components – CysOx, H\(_2\)O\(_2\), and Gpx3 – are present in the reaction (Fig. 3f,g). Compared to data obtained with CSA, the fluorescence spectra of Gpx3-CysOx adducts were blue-shifted by about 50 nm. Finally, the reaction products of CysOx probes and Gpx3-SOH were visualized by in-gel fluorescence (Fig. 3h,i). Intense signal was detected in CysOx-treated Gpx3-SOH but not in Gpx3-SH, consistent with the findings in our intact MS analysis.
Having established that CysOx probes are fluorogenic and selective in small-molecule and protein models of sulfenic acid, we next assessed their ability to enter live cells and provide a fluorogenic readout of endogenous S-sulfenylated proteins. For these experiments we employed glucose oxidase (GOX) at 0.2, 2, and 20 U/mL to provide continuous production of H$_2$O$_2$ in glucose-containing cell culture media. HeLa cells incubated with CysOx1 or CysOx2 showed significant time- and GOX-dependent increases in fluorescence intensity (Fig. 3a,b and Supplementary Fig. 6). Fluorescence was distributed throughout the cytoplasm with no apparent co-localization and minimal cytotoxicity (Supplementary Fig. 7-8). Live-cell labeling by non-fluorogenic analog 6 was also investigated. In contrast to CysOx probes, compound 6 failed to promote oxidant-dependent “turn-on” fluorescence, instead giving weak signal throughout the duration of the experiment (Supplementary Fig. 9), consistent with our CSA studies. In-gel fluorescence analysis of lysates derived from HeLa cells incubated with CysOx probes showed concentration- and redox-dependent protein labeling by CysOx2 (Fig. 4c); however, signal from CysOx1 was much weaker (Supplementary Fig. 6c), reflecting the inherent difference in their fluorogenic intensities. Collectively,
these studies indicate that the reaction between CysOx probes and the electrophilic sulfur in sulfenic acid represents a viable strategy for fluorogenic detection of cysteinyl oxidation in the test tube and in real-time in living cells.

Application of CysOx2 to identify kinase inhibitors that modulate cysteinyl oxidation in cells.

Crosstalk between cysteine oxidation and other post-translational modifications such as phosphorylation had been reported but the interplay between kinase inhibition and cysteinyl oxidation remains unclear. To further showcase the utility of fluorogenic probes in the detection of cysteinyl oxidation and address the question above, we subsequently adapted CysOx2 for use in a cell-based 96-well plate assay. After probe concentration and treatment times were optimized for this scale (Supplementary Fig. 10) we screened a curated library of kinase inhibitors was in HeLa cells. Analysis of the resulting data revealed a 3-fold or greater increase in fluorescence for 8% (12 out of 154 compounds) of kinase inhibitors as compared to vehicle-treated cells (Fig. 4a and Supplementary Table 2). Protein S-sulfenylation was modulated by inhibition of RTKs as well as select kinases from the AGC and CMGC families, including the multitasking Ser/Thr kinase GSK3 (Fig. 4b). Upon closer inspection, three of the ten compounds associated with the greatest increase in fluorescence intensity (SB-216763, BIM-IX, and BIO) were identified as GSK3 inhibitors (Fig. 4a-c), suggesting a relationship between this idiosyncratic kinase and protein S-sulfenylation. Underscoring this point, application of the most effective inhibitor identified in this screen, SB-216763 promoted a fluorescence increase equivalent to that of exogenously applied peroxide
The ability of SB-216763 to increase cellular protein S-sulfenylation was also confirmed through fluorescence imaging microscopy (Fig. e,f) and in-gel fluorescence analysis (Supplementary Fig. 11).

Site-specific proteomic ID of cysteines that undergo S-sulfenylation in GSK3 inhibitor-treated cells. Having identified an interesting connection between GSK inhibition and cysteinyl oxidation, we next sought to site-specifically and quantitatively profile dynamic changes in the HeLa S-sulfenylome induced by GSK-3 inhibitors using BTD-based chemoproteomics (Fig. 5a). BTD is a clickable chemical probe for sulfenic acid detection. In agreement with fluorescence screening data, SB-216763 treatment resulted in the most significant perturbation to the S-sulfenylome, with 56.3% measured S-sulfenylation events exhibiting at least a 1.5-fold change (Fig. 5b). Moreover, S-sulfenylation levels in nearly half of quantified sites with Uniprot functional annotations were changed by SB-216763 (Fig. 5c), implying broad regulation. Gene ontology classification further revealed that the sub-sulfenylome perturbed by GSK3 inhibition \( R_{LH}^{SB-216763} \leq 1.5, R_{LH}^{BIM-IIX} \leq 1.0, R_{LH}^{Bio} \geq 1.0 \) was enriched in many biological processes involved in antioxidant response, including regulatory cysteines of thioredoxin, thioredoxin reductase, glutaredoxin and glutaredoxin reductase (Fig. 5d,e). GSK3 inhibition also increased oxidation of PRDX6 C47 whose
catalytic cysteine is notoriously susceptible to overoxidation under oxidative stress, whereas exogenous H$_2$O$_2$ downregulates this redox event in the same cell line$^{14}$. GSK3 inhibition and H$_2$O$_2$ both decreased selenylation at C152 of GAPDH, which is also well-known redox sensitive site (Supplementary Fig. 12). These findings suggest that redox changes induced by GSK3 inhibition might be more specific as opposed to simple diffusion of H$_2$O$_2$.

**Discussion**

In contrast to cysteinylation oxidation products, turn-on fluorescent probes for the detection of low-molecular-weight thiols like cysteine and glutathione have enjoyed a lengthy history of development and research$^{25}$.  

![Fig. 6](https://example.com/fig6.png)

**Fig. 6** | GSK3 inhibition selectively perturbs the S-sulfenylome. a, Workflow for the BTD-based quantitative S-sulfenylome analysis. HeLa cells were treated with vehicle or with GSK3 inhibitors as indicated, and then labeled with 5 mM BTD in vitro. BTD-labeled proteomes with and without inhibitor treatment were digested into tryptic peptides, conjugated with heavy and light azido biotin reagents with a photocleavable linker, respectively. Light and heavy labeled samples then were combined, clicked, enriched with streptavidin beads. The photoreleased peptides were analyzed by LC-MS/MS for identification and quantitation. Heavy to light ratio calculated for each BTD-labeled cysteine residue provided a measure of its relative level in inhibitor-treated samples versus controls. b, Box plot showing the side-by-side comparison of the quantitation results from three GSK3 inhibitors. c, Ranking plots showing the distribution of Log2 R$_{HL}$ values for each GSK3 inhibitor. d, GO enrichment of the sub-sulfenylome perturbed by GSK3 inhibition. e, Representative extracted ion chromatograms (XICs) showing changes in BTD-labeled peptides from the proteins as indicated. The profiles for light- and heavy-labeled peptide are shown in red and blue, respectively. The average R$_{HL}$ values calculated from two biological replicates are displayed below each individual XIC.
Such reagents have utility in cells and often show a degree of discrimination among different biothiols. Despite such advances, the degree to which these tools report on cysteine residues in proteins is not clear. Furthermore, this general class of reagents only detects biothiol oxidation as a loss of fluorescence signal. To address this significant gap, this study presents the first instance of fluorogenic tools for direct detection of protein cysteine or cysteinylation oxidation, specifically monitoring the formation of sulfenic acid.

The fluorogenic character of CysOx probes stems, at least in part, from the fluorescence quenching effect of the phenaline-1,3-dione keto tautomer, in which nearly parallel carbonyl groups gives the largest large dipole moment\textsuperscript{26}. Reaction of the phenaline-1,3-dione scaffold at nucleophilic C-2 with the electrophilic sulfur atom in sulfenic acid serves to stabilize the fluorogenic enol tautomer. A second key contributor to the fluorescence of these probes is the electron-withdrawing fluorine atom attached to the naphthalene core, which serves to stabilize the excited state\textsuperscript{27}. A final feature of CysOx probes is the push-pull system wherein the electron-donating amino group is in conjugation with fluorine via the intervening $\pi$-system\textsuperscript{28}.

Another aspect of this study was to apply CysOx technology to identify kinase inhibitors that modulate S-sulfenylation in cells. The curated screening library used in this study includes inhibitors of lipid, receptor and non-receptor tyrosine, serine/threonine, and dual specificity kinases\textsuperscript{29}. Several interesting patterns emerged from this screen. For instance, among inhibitors associated with a significant increase in cellular S-sulfenylation, about 40\% targeted kinases in the RTK family. This is consistent with reports from our lab and others indicating that growth factor signaling is intimately linked to ROS metabolism in cells\textsuperscript{5,8}. The balance of “hits” within this cohort target members of the AGC and CMGC families and, of special interest, three of these inhibit GSK3 function\textsuperscript{30}. GSK3 in an unusual kinase in that it is constitutively active and its substrates (over 100 are known) need to be pre-phosphorylated by another kinase, and it is inhibited as opposed to activated by the two pathways known to converge on GSK3, the insulin and Wnt pathways\textsuperscript{31}. The elucidation of such patterns highlights an important strength of CysOx probes for
identifying agents or conditions that modulate protein S-sulfenylation, an area of central importance to many fields including covalent targeting of semi-conserved cysteines which is a growing strategy in drug design\textsuperscript{32,33}.

To independently verify the relationship between GSK3 inhibition and cysteiny1 oxidation we performed chemoproteomic analysis using the sulfenic acid-selective probe, BTD. We found that GSK3 inhibitors did indeed increase cellular S-sulfenylation with SB-216763 exhibiting the greatest effect, followed by BIM-IX and BIO, consistent with the findings from our kinase inhibitor screen. In this regard, we note that GSK3 inhibition activates Nrf2 for the transcription of antioxidant machinery\textsuperscript{34} and recent studies suggest that the Nrf2 pathway can also be triggered by inactivation of thioredoxin and glutathione-glutaredoxin systems, which are key reducing mechanisms in eukaryotes\textsuperscript{35}. Consistent with this idea, S-sulfenylation of regulatory redox-active cysteines in thioredoxin, thioredoxin reductase, glutathione reductase and glutaredoxin, all increased with GSK3 inhibition and may represent a KEAP1-independent means to activate Nrf2. Future research will be required to dissect this intriguing link in greater molecular detail.

As with all technologies, it is worth noting current limitations. At present, the CysOx fluorophore presents low brightness and quantum yields when compared to other dyes, such as fluorescein and BODIPY. This is reflected in the low quantum yields (0.9-2.4\%) observed for the reaction product of sulfenic acid and CysOx probes. This limitation does not hinder application of CysOx probes in the studies presented in this work. However, attempts to further miniaturize the cell-based assay to a 384-well plate using CysOx2 failed, which may be addressed in future iterations of these tools with brighter probes and enhanced dynamic range.

In summary, we have introduced a general chemical strategy for reaction-based fluorogenic detection of cysteiny1 sulfenic acid. Using this strategy we created two chemical probes, CysOx1 and CysOx2 that
have excellent cell permeability, rapid reactivity, and high selectivity with minimal cytotoxicity. The kinetic and spectroscopic properties of CysOx probes make them powerful first-in-class tools for live-cell fluorescence imaging studies of redox-regulated biology in physiology and disease.

Methods

General Spectroscopy. Fluorescence spectra for studies not in cell culture were recorded on an Agilent Technologies Cary Eclipse Fluorescence Spectrophotometer running the Cary Eclipse v1.2 software. Absorbance spectra were recorded on a Varian Cary 300 Bio UV-Visible Spectrophotometer using Cary WinUV v4.20 software. Stock solutions of chemical probes were prepared in DMSO and then diluted in PBS (pH 7.4) at the indicated concentrations.

Preparation of probe-CSA adducts. To a solution of dipeptide cyclic sulfonamide CSA (36.6 mg, 0.100 mmol) in DCM (3.0 mL) was added a C-nucleophile probe (0.100 mmol, 1 equiv), followed by Et₃N (13.9 µL, 0.100 mmol, 1 equiv). The reaction mixture was stirred at rt overnight. LC-MS analysis of the reaction mixture indicated complete consumption of the starting materials and the formation of the desired CSA-probe adduct as a single product. The reaction mixture was neutralized with TFA, diluted in DCM (20 mL), and washed with water (20 mL) and brine (20 mL). The organic phase was dried over MgSO₄, filtered and concentrated. The obtained products were purified via column chromatography (SiO₂, 0-100% EtOAc in hexanes) and used as is in subsequent experiments.

C64,82S Gpx3 labeling with CysOx1 or CysOx2. C64,82S Gpx3-SH was spin-filtered into labeling buffer (50 mM HEPES, 100 mM NaCl, pH = 7.4) using pre-equilibrated P30 columns (BioRad). The concentration of C64,82S Gpx3-SH was determined by measuring the absorptivity at 280 nm (ε = 24,410 M⁻¹cm⁻¹). Solutions of C64,82S Gpx3-SH (20 µM) and CysOx1 or CysOx2 (20 µM) were prepared in
labeling buffer and kept in ice. The Cary Eclipse software was set to Kinetics mode using the fluorescence
excitation and emission maxima observed for Gpx3-CysOx adducts (357/498 nm for Gpx3-CysOx1 and
394/535 nm for Gpx3-CysOx2). C64,82S Gpx3-SH (50 µL) and CyxOx probe (50 µL) were mixed in a
cuvette. Following sample insertion into the fluorimeter, H₂O₂ (100 µL of 30 µM made up in labeling
buffer) was fluorescence measurements were acquired. Three reactions were analyzed per chemical
probe. For control reactions, labeling buffer was added instead of selected components of the reaction.

In-gel fluorescence analysis of CysOx-labeled proteins. For Gpx3: C64,82S Gpx3 incubated with
CysOx1 or CysOx2 (21 µL) were boiled for 5 min in reducing loading buffer (7 µL). For HeLa lysates
derived from CysOx-treated cells: Lysate (20 µg protein) was diluted to a final volume of 30 µL and boiled
for 5 min in non-reducing loading buffer (10 µL). SDS-PAGE precast 4-20% or 4-15% gels (BioRad)
were electrophoresed in Tris-Glycine-SDS running buffer at 80 V for 10 min and then at 120 V for 60 min.
Gels were then washed in H₂O and imaged using the ChemiDoc MP imaging system (BioRad). Settings
were as follows: Fluorescein (epi-blue 460-490 nm excitation and 577-613 nm emission filter). After
fluorescence imaging, gels were stained in Commassie Brilliant Blue solution for 5 min at rt, washed in
destaining solution (H₂O/MeOH/AcOH 5:4:1) overnight, and then scanned by ChemiDoc MP to visualize
Coomassie staining.

Fluorescence cell imaging of live cells treated with CysOx probes. HeLa cells (seeding population:
8.0 x 10⁴ cells/well) were incubated in EMEM (+10% FBS) in 6-well glass bottom plates at 37 °C. At 70-
80% confluence, media was aspirated, and cells were washed twice with DPBS and serum-free EMEM
was added to wells. After incubation for 16 h at 37 °C media was aspirated, and cells were washed twice
with DPBS. For GOX treatment: Cells were treated with CysOx probe and GOX at the indicated
concentrations in DPBS containing 0.1% DMSO and glucose (1 mg/mL). At the indicated time points,
well were directly analyzed in an Olympus FluoView IX81 confocal microscope using the FV10-ASW 3.0
software. For tBOOH treatment: Oxidant was added at the indicated concentrations in EMEM for 10 min at 37 °C followed by the addition of CysOx probe at the indicated concentration for additional 15 min at 37 °C. The media was then aspirated, cells washed twice with DPBS, and fresh DPBS added. The wells were then analyzed in an Olympus FluoView IX81 confocal microscope using FV10-ASW 3.0 software. The 458 nm laser channel was used to excite incorporated CysOx probes. Fluorescence signal was filtered using a SDM560 dichroic mirror, followed by BA505-605 band pass filter to observe the CysOx fluorescence. Five frames were recorded per condition. Images were analyzed using ImageJ software, where the pixel intensity of the cellular cytoplasmic regions was measured with at least 10 measurements per condition.

**Multi-well cell-based screening assay to identify kinase Inhibitors that modulate S-sulfenylation.**

HeLa cells were incubated in EMEM supplemented with 10% FBS in black 96-well plates with clear bottoms (seeding population: 2 x 10^4 cells/well) at 37 °C. At 90% confluence (48 h), media was aspirated, cells washed twice with PBS and serum-free EMEM was added to the wells. After incubation for 16 h at 37 °C media was aspirated, cells were washed twice with PBS, and then DPBS (90 µL) was added to each well. CysOx2 (50 µM) and kinase inhibitor (10 µM, Kinase Screening Library - Cayman Chemical, item no. 10505) in DPBS containing 1% DMSO was added each well for a total volume per well of 100 µL. Cells were then incubated at 37 °C for 60 min. Selected control wells did not receive kinase inhibitor and were instead treated with either PBS (negative control) or tBOOH (200 µM, positive control). Following the incubation period, DPBS (100 µL) and extracellular fluorescence quencher (20 µL, Solution C of Beta-Lactamase Loading Solutions Kit from Life Technologies, part number K1048) were added to each well prior to measurement in a Molecular Devices SpectraMax M5 plate reader and analyzed using SoftMax Pro v5.4 software. The fluorescence parameters of CysOx2 were selected according to the maxima measured in the fluorescence spectra (excitation/emission: 447/ 606 nm). Preliminary screening to identify hits was performed in duplicate and compounds exhibiting more than a 2.5-fold increase in fluorescence were subsequently confirmed in quintuplicate according to the same procedure.
Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data associated with this study are available in the published article and its Supplementary Information.

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Acknowledgements

We thank Dr. Louis Scampavia and Dr. Timothy Spicer (The Scripps Research Institute) for helpful discussions in high-throughput screening and providing the fluorescence quencher used in the cell-based microplate assay. We thank Professor Laura Bohn and Dr. Edward Stahl (The Scripps Research Institute) for training in fluorescence microscopy and providing technical support and maintenance to the microscope. We thank Alta Johnson (The Scripps Research Institute) for training in flow cytometry. We thank Longqin Sun and Tuo Zhang (Beijing Qinglian Biotech Co., Ltd) for their help and technical supports. The work was supported by grants from the Natural Science Foundation of China (21922702)
and the State Key Laboratory of Proteomics (SKLP-K201703 and SKLP-K201804) to J.Y., and NIH (GM102187 and CA222849) to K.S.C.

Author Contributions

R.B.F, K.S.C. and J.Y. designed the experiments, analyzed data and all authors wrote the manuscript and provided input. R.B.F. synthesized probes, performed rate studies, compound characterization, acquisition of UV-Vis and fluorescence spectra, fluorescence microscopy for live-cell imaging, fluorescence microplate assays, intact MS, SDS-PAGE, and in-gel fluorescence analysis of CysOx-Gpx3 adducts. J.Y. and L.F. performed chemoproteomic experiments and analyzed the data.

Competing Interests statement

The authors declare no competing financial interests.
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