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Optimization of Caged Electrophiles for Improved Monitoring of Cysteine Reactivity in Living Cells

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Abstract

Cysteine residues serve critical roles in protein function and are susceptible to numerous posttranslational modifications (PTMs) that serve to modulate the activity and localization of diverse proteins. Many of these PTMs are highly transient and labile, necessitating methods to study these modifications directly within the context of living cells. We previously reported a caged electrophilic probe, CBK1, which can be activated by UV for temporally controlled covalent modification of cysteine residues in living cells. To improve upon the number of cysteine residues identified in cellular cysteine-profiling studies, the reactivity and uncaging efficiency of a panel of caged electrophiles were explored. We identified an optimized caged electrophilic probe, CIK4, which affords significantly improved coverage of cellular cysteine residues. The broader proteome coverage afforded by CIK4 renders it a useful tool for the biological investigation of cysteine-reactivity changes and PTMs directly within living cells and highlights design elements that are critical to optimizing photoactivatable chemical probes for cellular labelling.

Graphical Abstract

Monitoring cysteine reactivity directly in living cells allows for elucidating sites of cysteine modification by oxidants and endogenous and exogenous thiol-reactive agents. We evaluated a panel of caged electrophiles as live-cell compatible cysteine-reactive probes and report an optimized probe for enhanced labelling of cellular cysteines.

Keywords

cage compounds; cysteine; electrophilic addition; proteomics; photochemistry

Cysteine plays critical roles in various families of enzymes such as thiol oxidoreductases, proteases, and phosphatases.¹ The thiol group of cysteine has high nucleophilicity and redox sensitivity, which facilitates protein functions including nucleophilic and redox catalysis, and metal binding.² Cysteine residues also regulate protein function through post-

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translational modifications (PTMs) such as oxidation,³ nitrosation,⁴ palmitoylation,⁵ prenylation,⁶ and Michael additions to oxidized lipids.⁷ These PTMs result in a loss in cysteine nucleophilicity that can be quantitatively monitored using cysteine-reactive electrophilic probes.⁸ For example, in a method termed isoTOP-ABPP,⁹ an iodoacetamide (IA)-alkyne probe is coupled with quantitative mass spectrometry (MS) to monitor reactivity changes in hundreds of cysteine residues within a proteome. Importantly, isoTOP-ABPP and its derivatives¹⁰ have been applied to identify cysteine residues susceptible to oxidation¹¹ and *S*-nitrosation,¹² modification by endogenous lipid-derived electrophiles,¹³ thiol-reactive small-molecule fragments,¹⁴ and zinc chelation.¹⁵ These previous applications demonstrate the versatility of coupling reactive electrophilic probes with MS to identify cysteine residues that are key sites of protein regulation.

Although the IA-alkyne probe used in these previous studies is cell-membrane permeable and enables cysteine labelling in living cells,¹⁶ the high cytotoxicity of this probe (LC50 of 16 μ M)¹⁷ prohibits use at the high concentrations needed to achieve expansive coverage of cellular cysteines. To address this issue we developed a caged electrophilic probe, CBK1, for UV-triggered electrophile activation directly in living cells.¹⁷ CBK1 contains an α -bromomethyl ketone (BK) electrophile that is protected with 1-(*o*-nitrophenyl)-1,2-ethanediol (NPE) as a photoremovable caging group, and a terminal alkyne for downstream conjugation to reporter tags via copper-catalyzed azide-alkyne cycloaddition (CuAAC). In its caged form, CBK1 is unreactive and therefore can be accumulated in living cells at high concentrations with negligible cytotoxicity. UV irradiation liberates the electrophilic BK to initiate protein labelling *in situ* with spatial and temporal resolution (Scheme 1). Application of CBK1 coupled with quantitative MS enabled the identification of cysteine residues subject to oxidation upon growth factor-stimulation in A431 epidermoid carcinoma cells. However, the number of cysteine residues identified as covalently modified by CBK1 in living cells (~300 cysteines) was lower than that of typical IA-alkyne labelling in cell lysates (~1000 cysteines).^{9, 14} To increase the number of cysteine residues identified, we sought to optimize CBK1 to arrive at a caged electrophile with improved cysteine labelling properties in living cells.

To optimize CBK1, derivatives with modifications to the linker unit (R^2) between the electrophile and alkyne, the BK electrophile itself (X), as well as the caging group (R^1) (Scheme 1) were synthesized and evaluated. Initially, probes CBK2-4 with amide and ether-containing linkers between the NPE-caged BK electrophile and the alkyne were synthesized. Labelling efficiencies of CBK2-4 were evaluated in HeLa cell lysates (Figure S1), whereby lysates treated with CBK2-4 (5 μ M) were irradiated (365 nm, 80 W) on ice, and incubated at 25 °C for an hour. A fluorescent rhodamine group was incorporated using CuAAC, followed by SDS-PAGE separation and visualization of protein labelling by in-gel fluorescence. Similar studies were performed to evaluate CBK2-4 protein labelling in living cells upon treatment of cells (250 μ M to 1 mM) for 60 mins prior to irradiation, cell lysis and in-gel fluorescence analysis (Figure S2). Although CBK2-4 showed cysteine labelling in lysates upon UV irradiation (Figure S1), minimal cysteine labelling was observed in living cells, indicating poor membrane permeability of these probes (Figure S2).

The BK electrophile was then converted to an iodomethylketone (IK) electrophile, under the hypothesis that IK would be more reactive than BK. An IK electrophile protected with the NPE caging group (CIK1) was synthesized (Scheme 2). Briefly, 6-heptynoic acid (**1**) was converted to the α -hydroxymethyl ketone (**11**) through acid chloride and diazomethyl ketone intermediates. Next, the ketone group of **11** was transformed to a ketal containing the NPE caging group to generate **13**. Finally, the hydroxyl group of **13** was triflated, and substituted by iodide to afford CIK1. Protein labelling by CBK1 and CIK1 were evaluated in lysates. Minimal protein labelling was observed without UV irradiation, and the labelling intensity reached a maximum after 3 min of irradiation. Importantly, CIK1 demonstrated higher fluorescence intensity than CBK1 (Figure S3 and S4), which is in accordance with the higher reactivity of the IK electrophile. The ability of iodine to form stronger halogen-bonding interactions with side-chain residues within the protein binding pocket could also be a potential contributor to the observed increase in labelling.¹⁸

Linker optimization of CIK1 generated CIK-a, a derivative containing an amide linker, which demonstrated the most potent labelling in lysates among the NPE-bearing caged probes (Figure 1A, S6). CIK-a was membrane permeable and displayed robust protein labelling in living cells (Figure S7). Furthermore, in cell-viability assays, CIK-a did not display any apparent cytotoxicity up to 100 μ M (Figure S8). Protein labelling by CIK-a was then evaluated by LC/LC-MS/MS analysis of HeLa cell lysates labelled with CIK-a (100 μ M). Briefly, the MS workflow comprised of incorporation of a chemically cleavable linker (Azo-tag) to CIK-a-labelled proteins using CuAAC, streptavidin enrichment, on-bead trypsin digestion and subsequent release of CIK-labeled peptides for MS analysis.¹¹ Analysis of the resulting fragmentation (MS2) spectra allows for identification of the sites of protein labelling by CIK-a, based on the presence of peptide fragments containing the mass of the IK-probe modification. Despite the in-gel fluorescence data, in which CIK-a showed higher protein labelling relative to CBK1, fewer cysteine residues were identified for CIK-a relative to CBK1 by MS (Table S1). Further evaluation of the generated MS2 spectra showed the presence of a predominant fragmentation event at the amide bond of CIK-a, which precludes subsequent identification of the resulting peptides based on backbone amide fragmentation (Figure S9). These MS data suggested that an amide linkage may not be optimal for future iterations of these caged electrophilic probes.

Notably, signals from lysates labelled with caged probes after irradiation were weaker than that obtained for the pre-uncaged electrophilic probes, signifying relatively low uncaging efficiency of the caged probes in lysates (Figure S4, S5). To improve upon uncaging efficiency, the NPE caging group of the CIK probe was replaced by a 1-(4,5-dimethoxy-2-nitrophenyl)-1,2-ethanediol (DMNPE) group (CIK4, 5; Scheme 1). DMNPE is known to exhibit greater absorption at 365 nm compared to the NPE group.¹⁹ As before, uncaging of these probes were evaluated in HeLa cell lysates, which revealed that probes with DMNPE showed enhanced labelling of cysteine in lysates (Figure 1B, C). The CIK derivatives (50 μ M) were also evaluated in living cells and CIK4 showed the most efficient labelling (Figure 1D).

Given the preliminary cell lysate and live-cell labelling profiles of the panel of caged electrophiles, we selected CBK1, CIK1, and CIK4 for detailed evaluation in living cells. Of

these probes, CIK4 demonstrated the most potent labelling of proteins in living cells by in-gel fluorescence analysis (Figure 2A). Importantly, according to cell-viability assays, these caged probes displayed low cytotoxicity (Figure 2B). MS analysis identified 820 and 669 cysteine residues for CBK1 and CIK1, respectively (average of three replicates). In contrast, CIK4 treatment resulted in the identification of 1287 cysteine residues, demonstrating that CIK4 detects cysteines with significantly greater efficiency (Figure 2C). Among the identified cysteine residues were known functional cysteines involved in catalysis, redox activity and metal and nucleotide binding as well as sites of PTMs such as *S*-nitrosation, oxidation and disulfide bond formation (Tables S2–S10). Notably, cysteine residues identified in the CBK1, CIK1 and CIK4 samples displayed substantial overlap, suggesting that the BK and IK electrophiles target a similar subset of cysteines upon uncaging (Figure 2D).

In summary, we designed, synthesized and evaluated a series of caged electrophiles with variations to the linker region, electrophile and caging groups to identify an optimized probe for monitoring cysteine reactivity in living cells. Our studies revealed that CIK4, containing an IK electrophile and DMNPE caging group, was the most effective for labelling and MS-based identification of reactive cysteine residues in lysates and living cells. This optimized probe enables the identification of ~1300 cysteine residues in living cells, which is a significant improvement on the first generation CBK1 probe.¹⁷ The broader proteome coverage afforded by CIK4 renders it a useful tool for the biological investigation of cysteine-reactivity changes and post-translational modifications directly within living cells.

Experimental Section

Complete experimental details for the synthesis of caged probes, protein labelling in lysates and living cells, click chemistry, and mass spectrometry experiments are provided in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Evaluation of caged probes in vitro and in living cells. (A, B) In-gel fluorescence images of cysteine labelling in HeLa cell lysates treated with the indicated probes (5 μ M). (C) Mean in-gel fluorescence across the entire gel lane was quantified by the ImageJ software. (D) In-gel fluorescence image of cysteine labelling in live HeLa cells treated with the indicated probes (50 μ M). Coomassie Brilliant Blue (CBB) images of gels are shown in Figure S10–S12..

Figure 2.

Evaluation of CBK1, CIK1 and CIK4 in living cells. (A) In-gel fluorescence image of cysteine labelling in live HeLa cells treated with the three probes (50 and 100 μ M). A CBB image of the gel is shown in Figure S13. (B) Cell-viability assays of HeLa cells treated with 1.95 to 1000 μ M of the three probes. (C) Number of probe-labelled cysteine residues identified by LC/LC-MS/MS analysis (200 μ M probe concentration). In-gel fluorescence analysis of the samples is shown in Figure S14. (D) Venn diagram demonstrating overlap between cysteine residues identified with the three probes.

Caged probe	X	R ¹	R ²
CBK1	Br	H	-CH ₂ -
CBK2	Br	H	-CONH-
CBK3	Br	H	-CONH(CH ₂) ₂ O-
CBK4	Br	H	-CONH((CH ₂) ₂ O) ₂ -
CIK-a	I	H	-CONH(CH ₂) ₃ -
CIK1	I	H	-CH ₂ -
CIK3	I	H	-(CH ₂) ₅ -
CIK4	I	OMe	-CH ₂ -
CIK5	I	OMe	-(CH ₂) ₅ -

Scheme 1.

Structures of caged electrophilic probes for cysteine labelling in living cells.

Scheme 2.

Synthesis of CIK derivatives. Reaction conditions: (a) oxalyl chloride, dichloromethane; (b) (trimethylsilyl)diazomethane, acetonitrile; (c) aqueous sulfuric acid; (d) 1-(o-nitrophenyl)-1,2-ethanediols, pyridinium p-toluenesulfonate, magnesium sulfate, acetonitrile or tetrahydrofuran; (e) trifluoromethanesulfonic anhydride, pyridine, dichloromethane; (f) sodium iodide, acetone.