Cu(II)-Assisted Novel Covalent Warheads for Proteome-wide Cysteine Profiling

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Abstract

In the realm of residue-specific labeling of amino acids within the proteome, cysteine (-SH) emerges as a particularly nucleophilic residue and plays a pivotal role in covalent drug discovery. Despite significant strides in targeting cysteine sites, there remains a substantial expanse of unexplored site space. We discovered that a nucleophilic phenol (DBA)/Cu^{2+} 'complex' serves as a potent warhead for traditionally "inert" cysteines. In the context of a general chemoproteomics profiling, the DBA-1 probe exhibits higher reactivity than IAA toward approximately 45% of quantifiable Cys sites. Dose-dependent profiling experiments suggest that Cu^{2+} may function not only as an oxidizing agent of phenol but also as a chelating agent of polar residues in proteins. Given its reliance on structural characteristics of proteins for site targeting, this novel (DBA)/Cu^{2+} 'complex' fits the scope of 'structure-based protein profiling' (SBPP), highlighting the role of metal ion in the design of effective warheads and the virtue of new proteomic microenvironmental chemistry 'hunting'.

Introduction

Cysteine stands out among amino acids due to its high nucleophilicity and vulnerability to oxidation[1,2]. It plays a crucial role in governing various biological processes, such as catalysis[3,4], redox[2], and structure stabilization[5,6]. Activity-Based Protein Profiling (ABPP) employs active site-specific chemical probes to monitor enzyme functional states in biological systems[7-9]. It also facilitates the analysis of active residues and modifications in proteomes[10-13]. Given cysteine's unique

reactivity, ABPP strategies have led to the development of various chemical warheads [14-18]. Examples include maleimides[19,20], α, β-unsaturated ketones[21,22], and haloacetamides[10,23-26]. Iodoacetamide alkyne (IA-alkyne)[10,24] and its desthiobiotin counterpart[27,28] are highly reactive and the most widely used probes for proteome-wide cysteine analysis in situ and in lysate, respectively. However, Iodoacetamide warhead only labels a fraction of functional cysteines in proteomes. To address this issue, novel cysteine-reactive labeling reagents like benziodazolone[29], fluorobenzene[30], dinitroimidazole[31], heteroarene sulfoxide[32], acetylated sugars[33,34], sulfonamide[35], ethynyl heteroarene[36], heteroarene thiol ether[37], and recently reported N-Acryloylindole (NAIA)[38], have been developed, expanding the detectable space of cysteine.

Considering there are approximately 300,000 cysteines in the human proteome, with only ~40,000 currently being detected[39], there is still vast room for exploration. Despite the diverse chemical structures of the mentioned probes, their labeling mechanisms commonly involve electrophilic substitution or addition. To effectively explore undiscovered cysteine sites, covalent probes based on novel labeling mechanisms are needed. Recently, we serendipitously discovered a metal-activated warhead capable of labeling proteomic cysteines and metal is the cupper (II) from the click chemistry 'cocktail'. Although the detailed mechanism of new warhead requires further investigation, its distinct mechanism will inspire the development of novel warheads.

Results

We previously tested a number of terminal alkynyl containing probes through gel-based screening in Hela cell (data not shown). Among them, a dihydroxybenzene alkyne (DBA-1), derived from dihydroxybenzoic acid, stand out for its efficient labeling capacity. We then conducted a time-dependent labeling test using DBA-1 on pure BSA protein and whole proteome (HeLa cell lysate) levels, with monohydroxybenzene alkyne (BA) serving as a control (**Fig. 1A**). The protein samples were incubated with the probes for the indicated time, followed by click reaction with TAMRA-N_3 and fluorescence scanning. To our surprise, there was no apparent correlation between labeling intensity and protein/probe incubation time (**Fig. 1B, C**), which suggests that the labeling events were achieved during the click step.

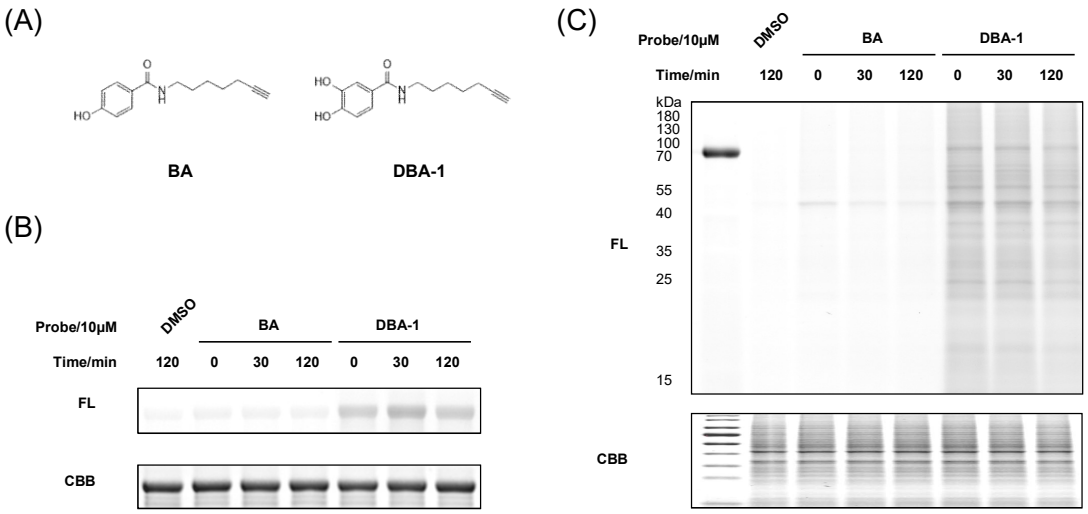

Fig. 1 (A) Chemical structure of BA and DBA-1. Time-dependent labeling of BSA (B) and HeLa lysate (C) by BA and DBA-1.

To determine which component in the click mixtures is responsible for activating DBA-1, protein was incubated with DBA-1 and individual reagents (component X) from the click mixtures. Then a typical click chemistry was performed in presence of 10x competitor (3,4-Dihydroxybenzoic acid). The fluorescent image clearly demonstrates that $CuSO_4$ acts as the key activator (**Fig. 2A**). This conclusion was further validated using a fluorescent probe, DBA-Cy3 (**Fig. 2B**), without involving the click conditions. To check whether other metal ions or oxidants could surpass Cu^{2+} in activation, common metal ions (Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Co^{2+}, Ni^{2+}, Zn^{2+}), along with the oxidant hydrogen peroxide (H_2O_2) and the reduced state Cu^{+}, were sceened. The results revealed that Cu^{2+} stood out among all other reagents (**Fig. 2C**), and the very mild effect from Copper(I) suggested that the activation of DBA-1 by Cu^{2+} may involve a redox process.

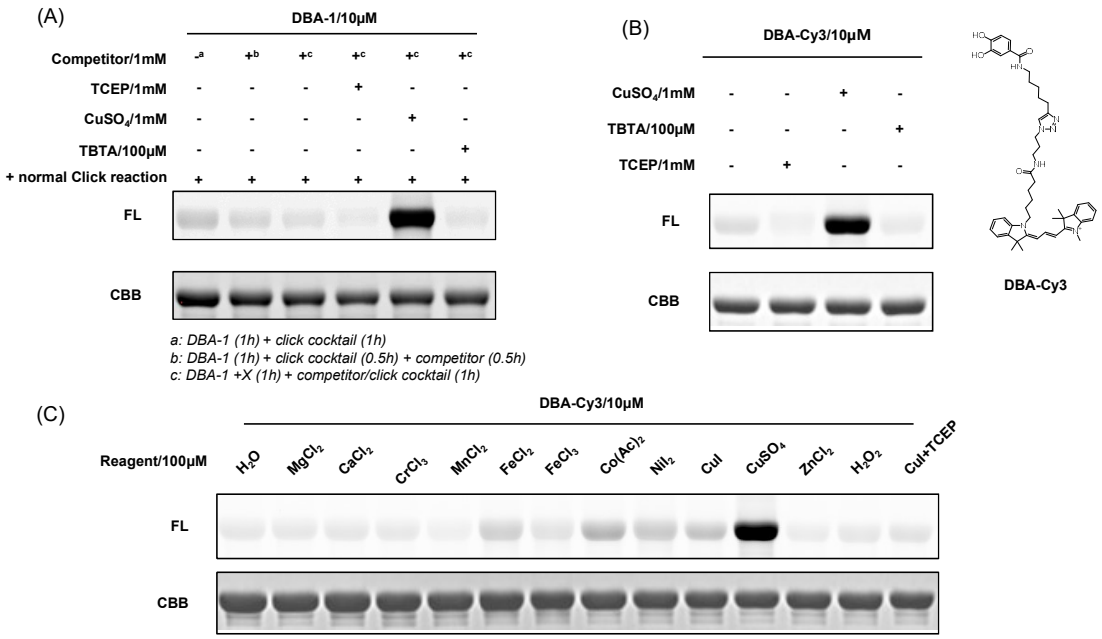

Fig. 2 (A) Investigation on click component with DBA-1. (B) Investigation on click component with DBA-Cy3. (C) Investigation on various reagent with DBA-Cy3.

Furthermore, we sought to identify the crucial moiety for the labeling ability of DBA-1. Various regional isomers of DBA-1 were synthesized (**Fig. 3A**). DBA-2 with ortho-dihydroxyl groups facilitated labeling, whereas the meta-dihydroxy analogue DBA-3 did not exhibit labeling ability (**Fig. 3B**). Without Cu^{2+}, DBA-1 and DBA-2 showed weak labeling, presumably due to autoxidation of dihydroxyl benzene to the Cys-reactive quinone (**Fig. 3C**).

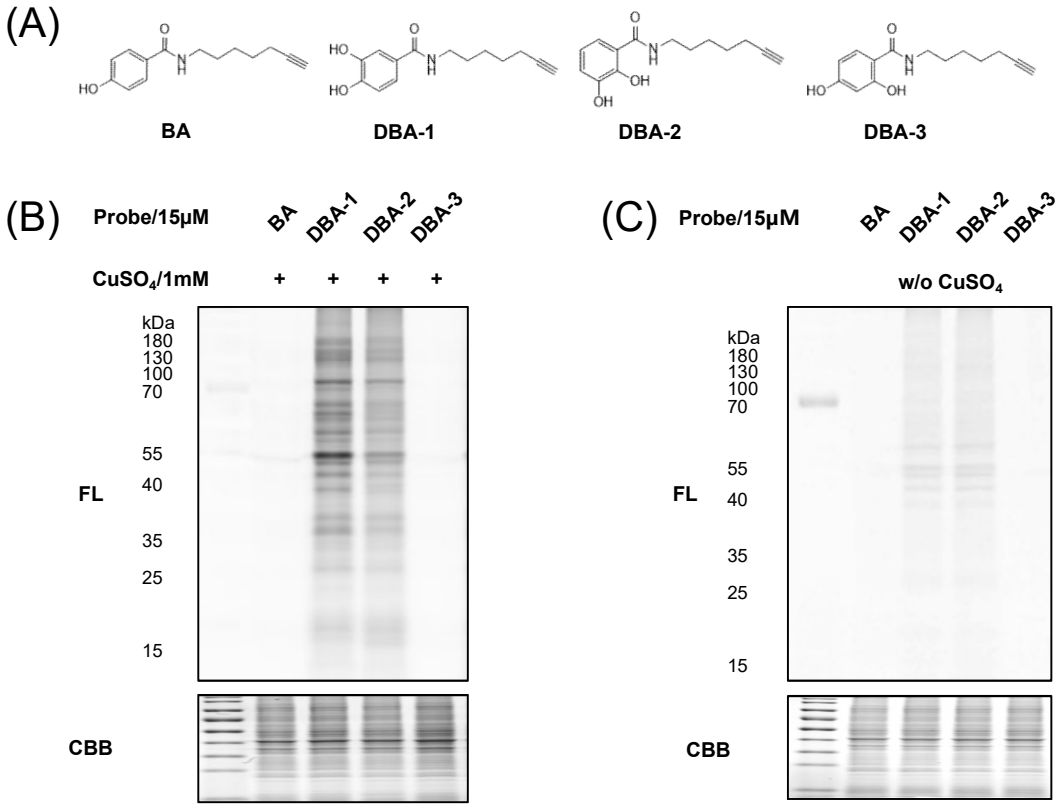

Fig. 3 (A) Chemical structures for DBA-1 analogues. (B) Gel-based fluorescent imaging with various probes in the presence of Cu^{2+}. (C) Gel-based fluorescent imaging with various probes in the absence of Cu^{2+}.

We next investigated the dose-dependent response of the whole proteome to each component of the probe (DBA-Cy3/Cu^{2+}) using quantitative gel-based imaging. To explore the dose-dependence of DBA-Cy3, the concentration of Cu^{2+} was fixed at a high level (1 mM). The results revealed that the labeled EC_{50} of DBA-Cy3 was 31.57 μM, with a saturation labeled concentration of approximately 125 μM (**Fig. 4A**). Similarly, when DBA-Cy3 was fixed at a high level (200 μM), the EC_{50} of Cu^{2+} was determined to be 293 μM, and the saturation labeling concentration was around 1 mM (**Fig. 4B**). Interestingly, the curve indicates minimal changes in the labeling strength when Cu^{2+} is within a concentration range of 0-62.5 μM, which could be presumably attributed to the consumption of Cu^{2+} by small molecules in the lysate or its strong binding pockets on the protein since there is no such a case in the labeling of pure BSA. These observations establish a foundation for the design of subsequent mass spectrometry experiments.

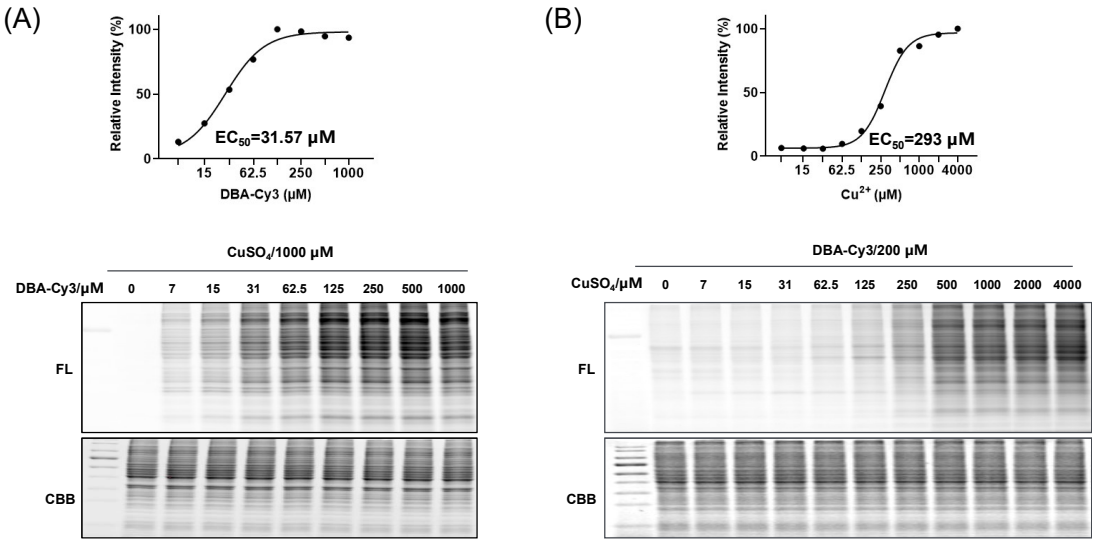

Fig. 4 (A) Dose-dependent labeling investigation of DBA-Cy3. (B) Dose-dependent labeling investigation of Cu^{2+}.

Subsequently, we aimed to identify the targeting amino acid residues of the probe by determine the addon moiety's molecular weight on the modified peptides. We treated the proteome with the probe, performed click chemistry to attach isotopically labeled desthiobiotin azide (isoDTB), and then enriched proteins on avidin beads. The enriched proteins underwent digestion, washing, elution, and the peptides were analyzed using LC-MS/MS. Unbiased analysis revealed a major shift in mass at 726.38 and 732.39 (**Fig. 5A**), corresponding to the quinone state of DBA-1 plus a light or heavy isotopic label (**Fig. 5B**). Statistical analysis demonstrated that DBA-1/Cu^{2+} exclusively labels cysteine residues on proteins (**Fig. 5C**).

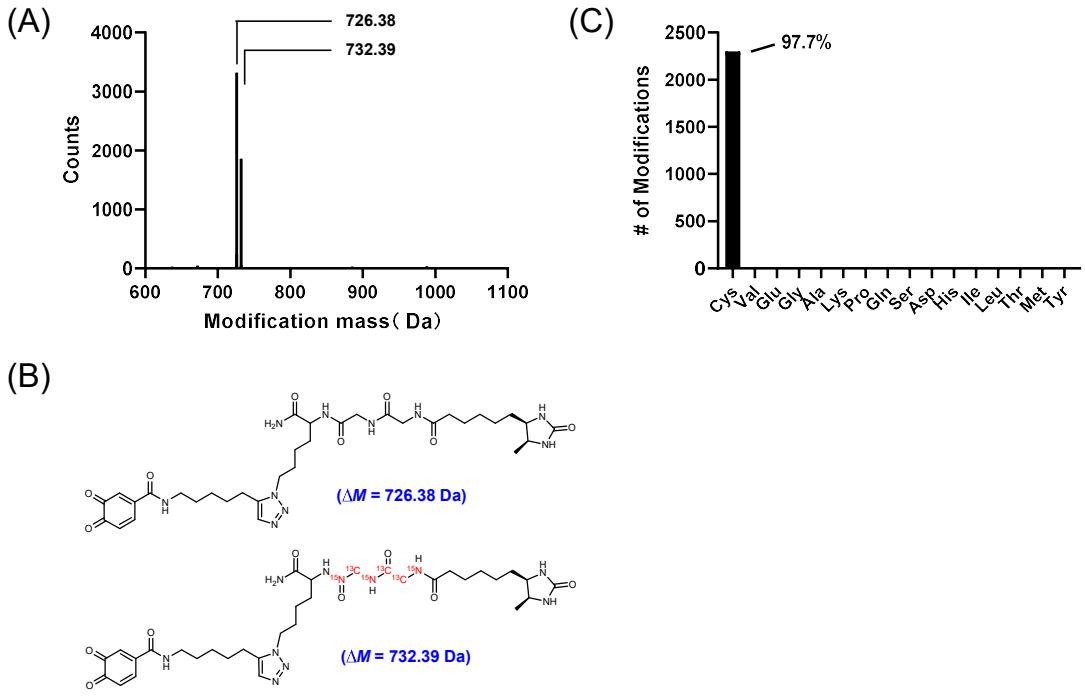

Fig. 5 (A) Mass shift of modified peptides. (B) The structures corresponding to the mass shift. (C)

Selectivity of amino acid residues targeted by DBA-1/Cu^{2+}.

We then investigated the targeting site space of our probe, in comparison to the classical iodoacetamide (IAA) warhead. To unbiasedly assess this, we utilized IAA as a competitive agent to monitor changes in the labeling intensity of the probes. The HeLa proteome was treated with DMSO or IAA (200 μM), followed by incubation with our probe (200 μM DBA-1, 1 mM Cu^{2+}). Subsequently, click chemistry to isoDTB, enrichment, digestion, washing, elution, and mass spectrometry analysis were conducted. Two biological replicates and two technical replicates were quantified using LFQ. As we expected, IAA was incapable of blocking all the labeling sites of DBA-1/Cu^{2+}. For approximately 45% of the sites, DBA-1 exhibited higher reactivity than IAA (DBA-1 > IAA) (**Fig. 6A, B**). In some extreme sites, DBA-1 overwhelms IAA (R≈1), where we found surrounding polar amino acids such as Asp, Glu, His, Tyr, Asn, etc (**Fig. 6C**). This suggests that Cu^{2+} not only act as an oxidizing agent but also as a chelator for specific regional functional groups.

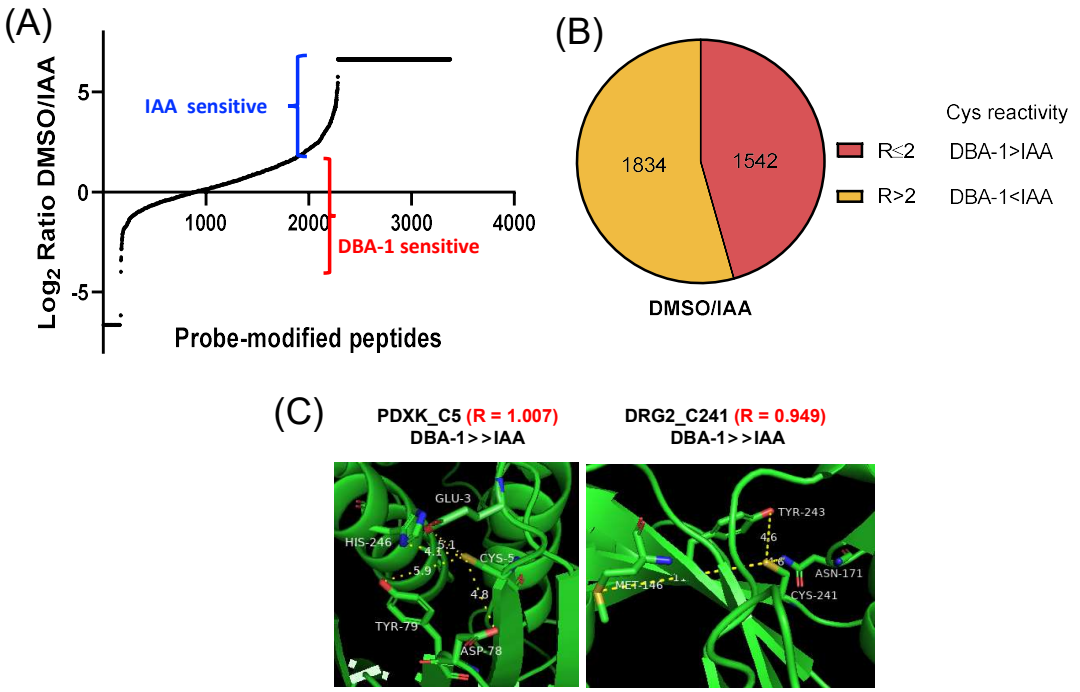

Fig. 6 (A) LFQ of probe-modified peptides by IAA and DBA-1/Cu^{2+}. (B) Classification of the Cys reactivity according to the ratio. (C) Representative 3D structure view of DBA-1/Cu^{2+} favors Cys sites (DBA-1 >> IAA)

Additionally, we explored the labeling differences at high and low concentrations of probes (20 vs 200 μM) to evaluate the reactivity of the probe at various labeling sites. The results indicated that 87% of the sites belonged to low-reactive cysteines (**Fig. 7A, B**), contrasting to the minor but hyperreactive sites such as SEC31A_C787 and GIPC3_C152 (ratio ≈ 1) where the crystal structure revealed the presence of more than one basic amino acid around cysteine (**Fig. 7C**). In conclusion, Cu^{2+} catalyzed the labeling of less reactive cysteines by DBA-1.

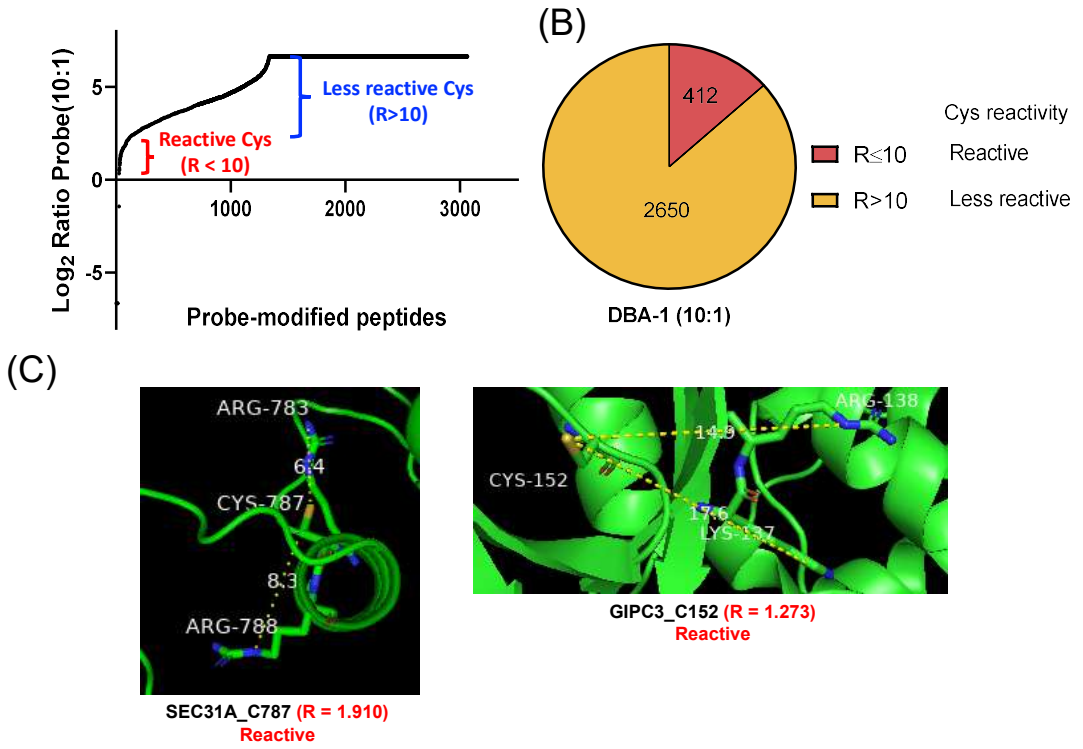

Fig. 7 (A) LFQ of probe-modified peptides by high dose and low dose DBA-1. (B) Classification of the cys reactivity according to the ratio. (C) Representative 3D structure view of Cys sites with hyperreactivity to DBA-1.

We also investigated labeling feature at high and low concentrations of Cu^{2+} (200 vs 1000 μM) to assess the impact of Cu^{2+} on cysteine reactivity. The results revealed that, in comparison to low Cu^{2+} concentration, the labeling intensity of 91% of the sites was enhanced under high Cu^{2+} concentration (R≥1), with approximately 20% that could only be labeled with high Cu^{2+} concentration (R=100) (**Fig. 8A, B**). Some of the sites with ratios close to 1 may be favored by Cu^{2+}, such as POR_C569 and EPPS1_C1076, where polar amino acids typically surround the cysteines, the presence of basic amino acids is not necessary (**Fig. 8C**). In summary, Cu^{2+} significantly broadens the labeling site space of DBA-1, possibly by leveraging the presence of polar amino acids around the cysteine sites.

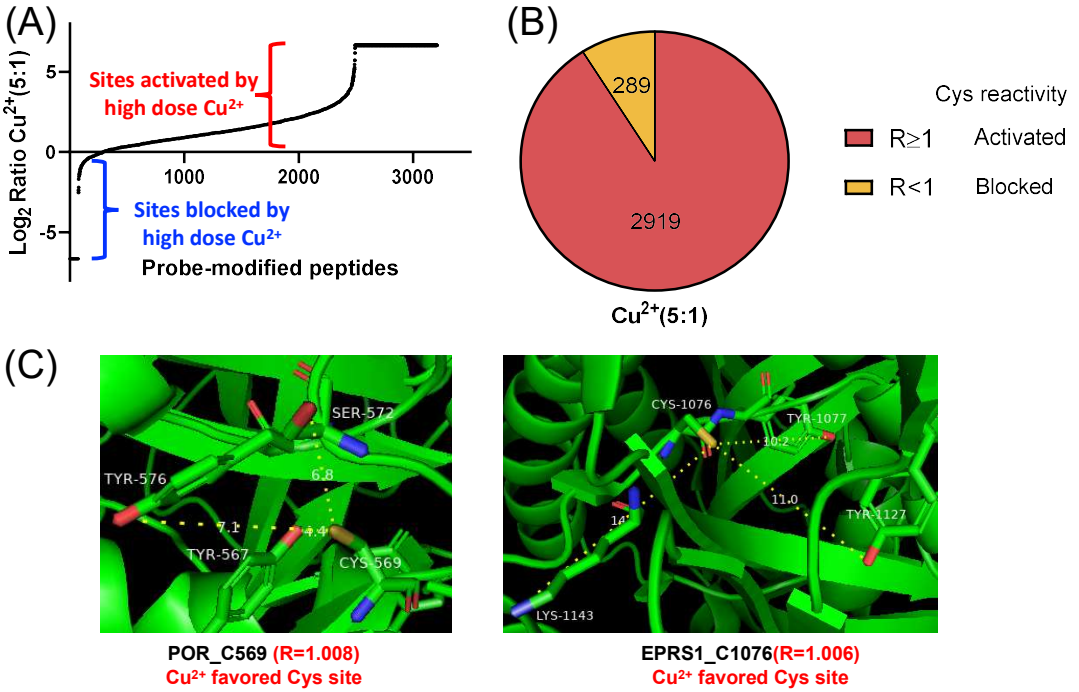

Fig. 8 (A) LFQ of probe-modified peptides at presence of high and low dose Cu^{2+}. (B) Classification of the Cys reactivity according to the ratio. (C) Representative 3D structure view of Cys sites that favored by low concentration Cu^{2+}.

As a tool probe for Activity-Based Protein Profiling (ABPP), our probe might support broad applications, including identifying targeted sites for active natural products and screening fragment ligands for currently unligandable sites. We selected several natural products containing dihydroxybenzene and fragments with acrylamide structures (**Fig. 9A**). These compounds were first incubated with living cells, followed by probe labeling of the lysed proteome and subsequent gel imaging. The results indicated that all compounds exhibited varying degrees of signal suppression and the phenolic natural products show a more pronounced effect (**Fig. 9B**).

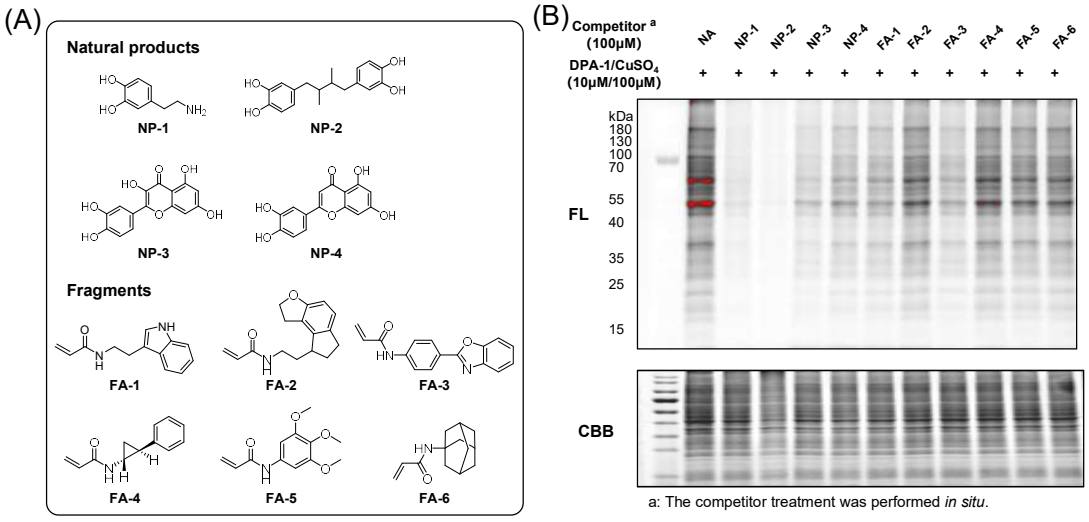

Fig. 9 (A) Structures of compounds for competitive ABPP with DBA-1/Cu^{2+}. (B) Gel-based

fluorescent imaging of competitive ABPP DBA-1/Cu^{2+}.

Building upon the experimental evidence presented above, we propose a mechanism as follows (**Fig. 10**). Cu^{2+} interacts with both the probe and the polar groups on the protein, including cysteine, and it subsequently facilitates oxidative cross-linking between the thiol group and the probe.

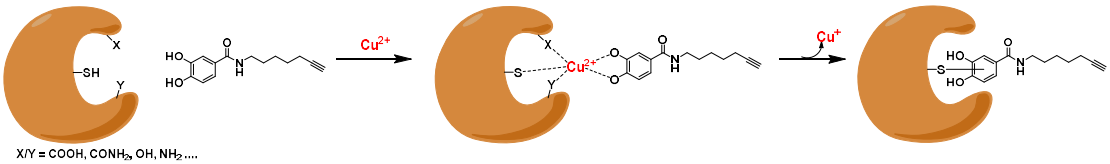

Fig. 10 Proposed mechanism for DBA-1/Cu^{2+} mediated Cys labeling.

Conclusion

In summary, the DBA-1/Cu^{2+} system reported here uniquely relies on a metal ion to activate the probe within the microenvironment of a protein, leading to the formation of a reactive warhead that marks cysteine thiol groups in the proximity. Beyond probe chelation and activation, the Cu^{2+} might also plays a crucial role in recognizing proteins via coordinating polar groups, thereby facilitating the selective targeting the specific microenvironments within proteins. Given its reliance on structural characteristics of proteins for site targeting, this novel (DBA)/Cu^{2+} 'complex' fits the scope of 'structure-based protein profiling' (SBPP), highlighting the role of metal ion in the design of effective warheads and the virtue of new proteomic microenvironmental chemistry 'hunting'.

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