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Expanding the Library of Covalent Cysteine Cathepsin Probes Featuring Sulfoxonium Ylide Electrophiles

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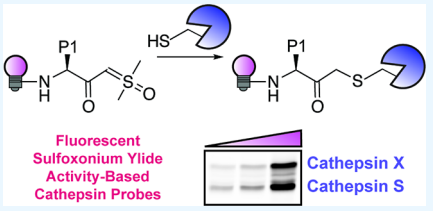
ABSTRACT: Covalent activity-based probes are invaluable tools to monitor protease activity in vitro and in vivo. We recently discovered that dimethyl sulfoxonium ylides (SYs) bind selectively to cysteine cathepsin proteases in a mechanism-dependent manner. Herein, we present the synthetic routes and characterization of an expanded library of SY probes with a greater diversity in recognition sequences. The probes exhibit a range of potency and selectivity for the cathepsin family members. We also investigated the impact of fluorophore positioning on probes bearing P1 lysine. When sulfonated cyanine 5 was attached via the lysine side chain, the resulting probe was selective for cathepsin S. When attached to the α-amine, with the side chain amine either free or Boc-protected, the probes reacted with both cathepsin S and X. Bulk in the P1 position is thus well tolerated by cathepsin S but not cathepsin X. We examined the impact of Cy5 sulfonation on probe properties, demonstrating that unsulfonated probes exhibit greater cellular uptake, which affects their relative selectivity. Finally, we demonstrated that SY probes exhibit minimal labeling of cathepsin S in freshly prepared lysates, but this increases during the prolonged incubation of lysates. This work extends our understanding of SY probes and informs future probe development.



INTRODUCTION

The last decades have seen a surge in covalent chemical probes to interrogate enzyme activity and function. Activity-based probes (ABPs) are equipped with an electrophilic moiety often referred to as a warhead, which can irreversibly bind to the enzyme active site in an activity-dependent manner. Selectivity can be conferred through incorporation of recognition sequences, often peptidic in nature but also nonpeptidic. ABPs can also include a range of tags, including fluorophores, radiotracers, biotin, or click chemistry handles, that permit the detection of binding using techniques such as imaging, flow cytometry, gel-based assays, and chemoproteomics. The covalent nature of ABPs enables unequivocal identification of their targets, which is essential in the study of promiscuous enzymes that have overlapping substrate preferences.

Cysteine and serine proteases have been attractive targets for covalent ABPs due their catalytic mechanisms affording reactivity with diverse electrophiles.[1,2] Among these are cysteine cathepsins, a family of clan CA proteases that contribute to cellular homeostasis and diverse pathologies. We recently identified the dimethyl sulfoxonium ylide (SY) as a new warhead capable of binding cathepsin proteases, with a preference for cathepsin X and cathepsin S (Figure 1a).[3]

We developed a suite of SY-containing ABPs with variable recognition sites and a sulfonated cyanine 5 (sCy5) fluorophore. When applied to living RAW264.7 macrophages, SY probes bearing Val, Ile, Leu, Nle, Trp, and Phe in the P1 position label cathepsin X and cathepsin S, with sCy5-Nle-SY (Figure 1b) emerging as the most potent. The identity of these proteases was verified by immunoprecipitation of probe-labeled lysates with cathepsin-specific antibodies, by loss of labeling in cathepsin X-deficient cells or tissue lysates,[3,4] or by competition with the cathepsin S-selective inhibitors MDV-590[5] and Z-FL-COCHO.[6] A probe bearing a P1 Lys with a fluorophore attached via the side chain amine specifically labeled cathepsin S (Cbz-Lys(sCy5)-SY; Figure 1c), while a dipeptide probe (Phe−Val) labeled cathepsins X, B, S, and L. Intriguingly, we observed that many of the SY probes were selective for cathepsin X in acidic lysates. This was despite clear activity of cathepsin S under the lysate conditions, as demonstrated by reactivity with the pan-cysteine cathepsin probe, BMV109.[7,8]

In the current study, we aimed to synthesize additional SY probes bearing new recognition sequences and examine the resulting impact on potency and selectivity toward cathepsin family members. We also sought to examine the consequences of fluorophore location and Cy5 sulfonation on reactivity profiles of SY-based ABPs and to investigate the differential labeling profiles between live cells and lysates.

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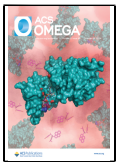

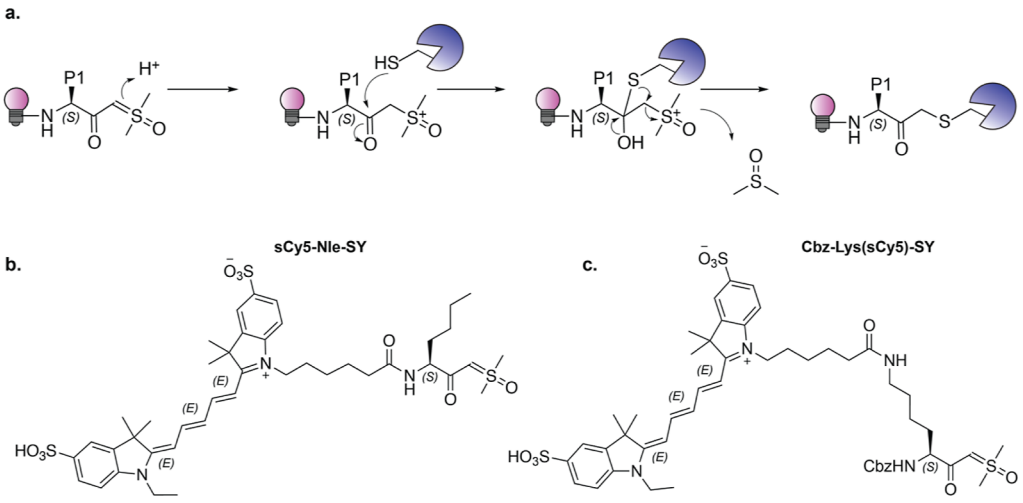

Figure 1. Mechanism and structure of SY ABPs. (a) Schematic of cysteine protease binding to SY. P1 refers to variable amino acid side chains at the P1 position. Structures of (b) sCy5-Nle-SY and (c) Cbz-Lys(sCy5)-SY, which were initially reported and characterized by Mountford et al.[3]

RESULTS

Synthesis of an Expanded SY Library. We expanded the library of SY probes by incorporating a variety of amino acids at the P1 position. Arg, Lys, and Glu were previously identified in a positional scanning substrate library as favorable P1 residues for cathepsin X cleavage.[9] We also elected to test Ser as an example of a smaller polar residue expected to drive specificity away from cathepsin X.[9] In our previous study, we reported that Cbz-Lys(sCy5)-SY, in which the sCy5 fluorophore was attached to the Lys side chain, was selective for cathepsin S over cathepsin X, especially in live cells.[3] We hypothesized that bulk in this position limited reactivity with cathepsin X. To address this, we synthesized two new derivatives of our P1 Lys-SY scaffold, in which sCy5 was attached via the α-amine of Lys, with the side chain amine either free (sCy5-Lys(NH_2)-SY) or Boc-protected (sCy5-Lys(Boc)-SY).

In order to incorporate a wider variety of amino acids at P1 in our probes (summarized in Table 1), we made use of commercially available *N*-Fmoc amino acids with associated side chain protection. This synthetic route is in contrast to the *N*-Boc amino acids we previously used and reported.[3] Activation of the carboxylic acids was achieved through either the nitrophenyl ester (Figure 2) or the succinimide ester (Figure 3). Formation of the SY under basic conditions also allowed for the deprotection of the Fmoc in one pot.[10] Labeling of the amino terminus with the desired fluorophore via amide bond formation was accomplished with either a preactivated NHS ester or activated in situ with PyClock for the carboxylic acid variants. Finally, if required, acidolytic deprotection of the side chain was achieved using TFA.

Table 1. Summary of Probes

probe #	sequence
3	sCy5-Ser-SY
6	sCy5-Lys(Boc)-SY
7	sCy5-Lys(NH2)-SY
9	sCy5-Arg-SY
13	sCy5-Pro-Nle-SY
14	Cy5-Pro-Nle-SY
15	Cy5-Nle-SY

Synthesis of a probe bearing P1 Arg (Figure 3) proved to be more challenging as initial attempts to isolate the activated amino acid resulted in a mixture of the desired product and a byproduct. Without extensive analytical investigation, the byproduct could be the 6-membered delta lactam, given the correct corresponding mass was observed. This would occur by cyclization with the side chain and such reactions have previously been reported to occur during Arg activation.[11] In our case, this issue was observed whether the Arg was activated as the nitrophenyl ester or the NHS ester and also whether Pbf or bis-Boc was used for side chain protection. Hence, conversion to the SY in one pot is suggested.

We also attempted to make an sCy5-Glu-SY probe. The synthesis was achieved; however, shortly after purification, the compound decomposed and hence was deemed too unstable for use. Conversion of the product to a peak associated with the loss of 78 was observed in the LC/MS, suggesting the loss of the ylide in the form of DMSO. Potentially, a side reaction with an O−H insertion of the carboxylic acid side chain across the SY could have been the cause. This type of insertion reaction has been reported as both Ru catalyzed[12] or metal free.[13]

Proline in the P2 position is known to limit reactivity of caspase- and legumain-targeted AOMK probes with cathepsin B,[14,15] as it is not a preferred P2 residue for cathepsin B at acidic or neutral pH.[16] As cathepsin X can cleave protein substrates with P2 proline residues,[17] we reasoned that sCy5-Pro-Nle-SY might have improved cathepsin X selectivity. Preparation of this probe (Figure 4) began with immobilizing Fmoc-Nle-OH on 2-chlorotrityl chloride resin. Using standard solid-phase synthesis conditions, the resin was deprotected, and Boc-Pro-OH was introduced. Cleavage from the resin was achieved with HFIP, and the resulting carboxylic acid was again activated via the nitrophenyl ester. Conversion to the SY and fluorophore labeling was achieved in a similar manner to the single amino acid probes.

Assessing the Reactivity of SY Probes. We first aimed to test the relative potency and selectivity of the probe series in cell lysates relative to our previously published probes sCy5-Nle-SY and Cbz-Lys(sCy5)-SY (Figure 1). RAW264.7 cells

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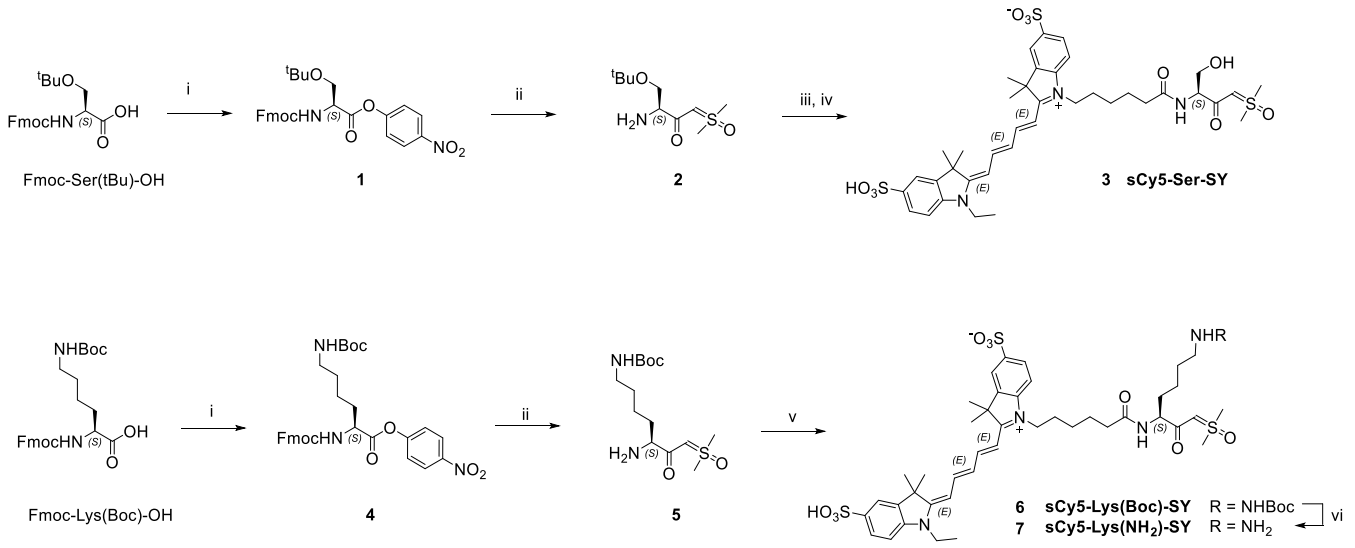

Figure 2. Synthesis of sCy5-Ser-SY, sCy5-Lys(Boc)-SY, and sCy5-Lys-sY. (i) 4-Nitrophenylchloroformate, Et_3N, DMAP, CH_2Cl_2, 0 °C, 4 h. (ii) $SOMe_3^+I^-$, KOtBu, THF, reflux, then cooled to 0 °C, and nitrophenyl ester was added. (iii) Sulfo-Cy5-NHS, Et_3N, DMF, r.t., 18 h. (iv) 90% TFA in CH_2Cl_2. (v) Sulfo-Cy5, PyClock, DIPEA, DMF, r.t., 18 h. (vi) 50% TFA in CH_2Cl_2.

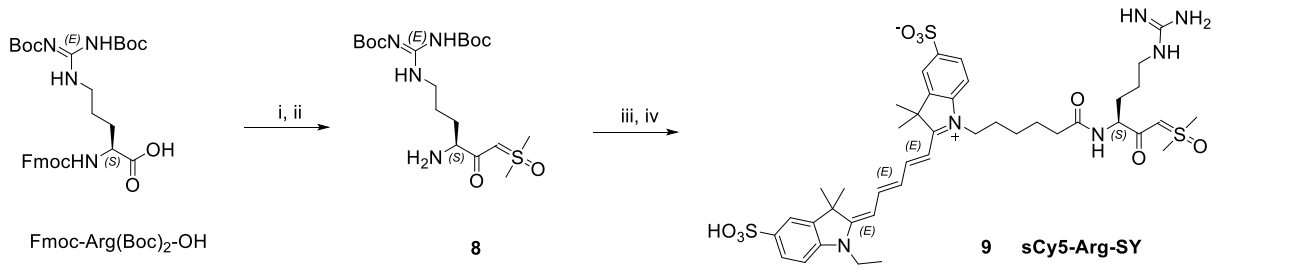

Figure 3. Synthesis of sCy5-Arg-SY. (i) NHS, DIC, THF, −10 °C, 1 h. (ii) $SOMe_3^+I^-$, KOtBu, THF, reflux, then cooled to −10 °C and activated amino acid was added, 2 h → 4 °C, 18 h. (iii) Sulfo-Cy5, PyClock, DIPEA, DMF, r.t., 2 h. (iv) 50% TFA in CH_2Cl_2.

were lysed in an acidic buffer optimized to maintain cathepsin activity (citrate buffer [pH 5.5]). Lysates were incubated with increasing probe concentrations (0.5, 1, and 5 μM), and proteins were resolved by SDS-PAGE. To visualize probe-labeled species, gels were scanned for sCy5 fluorescence. Probes containing P1 Ser and Arg exhibited a similar labeling profile to our previously reported sCy5-Nle-SY probe, albeit with slightly less potency (Figure 5a). When these probes were incubated with living cells, they also exhibited a similar binding profile to sCy5-Nle-SY, but with a slightly increased affinity for cathepsin S relative to cathepsin X (Figure 5b). Thus, P1 Ser and Arg both resulted in reduced cathepsin X affinity compared to Nle.

As we previously demonstrated, Cbz-Lys(sCy5)-SY was very selective for cathepsin S in living cells with minimal reactivity toward cathepsin X (Figure 5b). sCy5-Lys(NH_2)-SY and sCy5-Lys(Boc)-SY, however, exhibited similar reactivity to sCy5-Nle-SY in both lysates and living cells, with clear labeling of both cathepsin X and cathepsin S (Figure 5a,b). These results suggest that it is the bulk of sCy5 on the side chain that skews reactivity away from cathepsin X to enhance cathepsin S selectivity.

Compared with sCy5-Nle-SY, we observed less reactivity of sCy5-Pro-Nle-SY with cathepsin X in lysates. Contrary to our hypothesis that P2 proline would reduce labeling of cathepsin B, this probe exhibited significantly increased reactivity with cathepsin B in lysates (Figure 5a). In living cells, sCy5-Pro-Nle-SY was very similar to sCy5-Nle-SY at 5 μM (Figure 5b). At lower concentrations, the dipeptide probe favored cathepsins X and B over S.

Assessing the Impact of Cy5 Sulfonation on Reactivity Profiles of SY-Based Probes. We previously observed that sulfonation of the Cy5 fluorophore on cathepsin ABPs led to increased labeling in living cells compared to unsulfonated versions, although lysate reactivity was reduced.[18] We hypothesized that the sulfate groups may impair the permeability or limit uptake into the endolysosomal pathway. We thus synthesized Nle or Pro-Nle probes bearing unsulfonated Cy5 (Figure 4) and compared them to their sulfonated counterparts in both lysates and living cells. With both recognition sequences, the reactivity was similar in lysates, with slightly less intense labeling observed with the unsulfonated analogues (Figure 6a). By contrast, when applied to living cells, the unsulfonated probes exhibited more intense labeling, particularly of cathepsin B (Figure 6b). This was especially evident when analyzing residual cathepsin activity after SY probe treatment using the biotinylated cathepsin probe DCG-04.[19] In living cells, cathepsin B labeling by DCG-04 was only noticeably competed by the unsulfonated probes. Collectively, these results suggest that Cy5 enhances uptake compared to sCy5, and the higher local concentration of the probes leads to an enhanced reactivity with cathepsin B.

Investigating Cathepsin S Reactivity with SYs in Lysates. As shown in Figure 5 and our previous work,[3] most of the SY probes that we have tested are selective for cathepsin X when applied to RAW264.7 cell lysates, with very little

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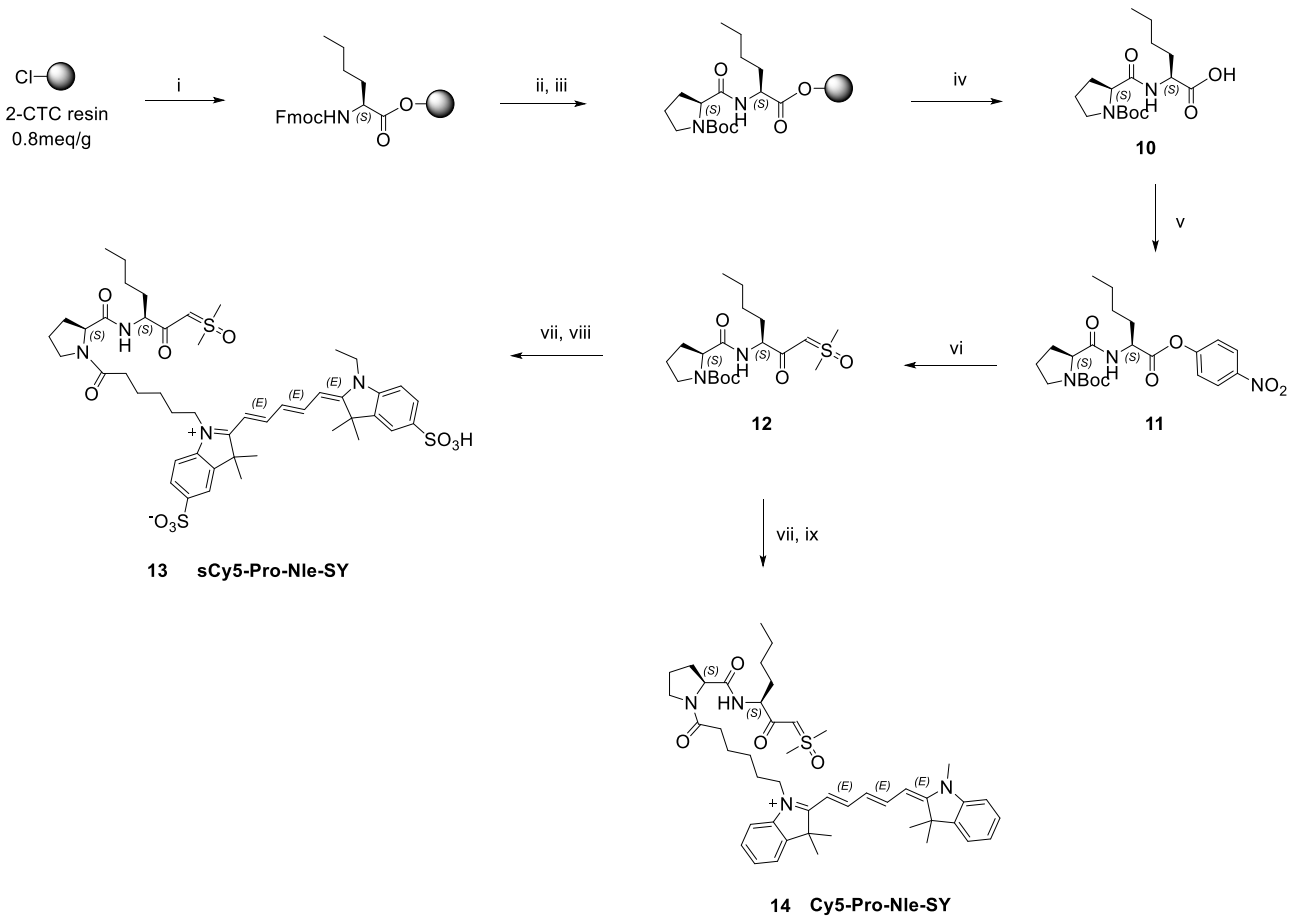

Figure 4. Synthesis of sCy5-Pro-Nle-SY and Cy5-Pro-Nle-SY. (i) Fmoc-Nle-OH, DIPEA, CH_2Cl_2, 2 h. (ii) 20% piperidine in DMF. (iii) Fmoc-Pro-OH, HCTU, DIPEA, DMF, 2 h. (iv) 20% HFIP in CH_2Cl_2. (v) 4-Nitrophenylchloroformate, Et_3N, DMAP, CH_2Cl_2, 0 °C, 4 h. (vi) $SOMe_3^+I^-$, KOtBu, THF, reflux, then cooled to −10 °C, and nitrophenyl ester was added. (vii) TFA/CH_2Cl_2 (1:1). (viii) Sulfo-Cy5, PyClock, DIPEA, DMF, r.t., 2 h. (ix) Cy5, PyClock, DIPEA, DMF, r.t., 4 h.

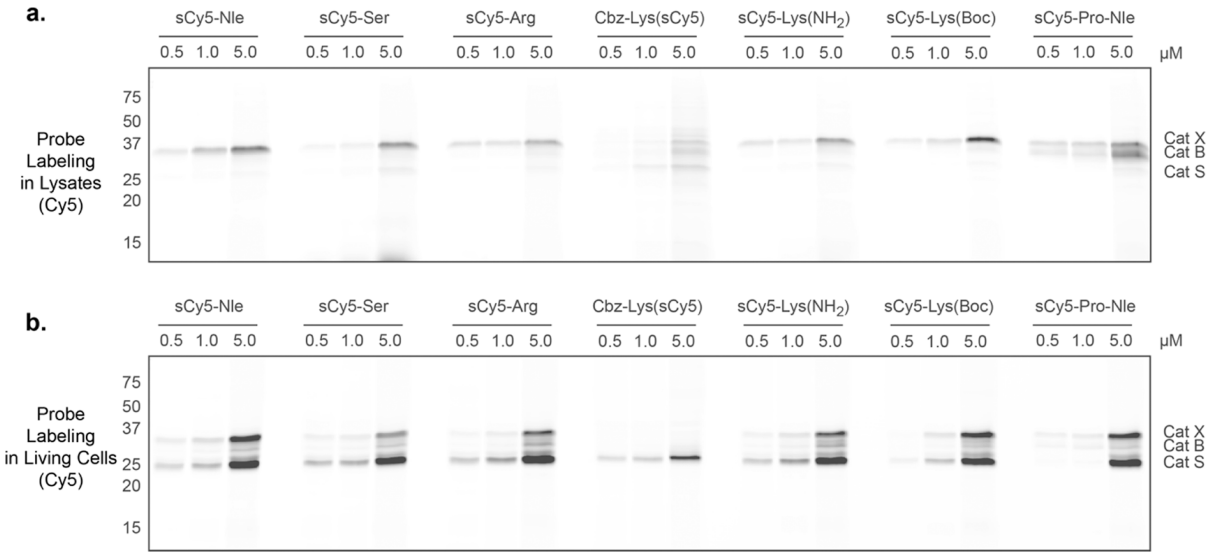

Figure 5. Assessing the impact of variable recognition sites on SY ABP specificity. (a) RAW264.7 cell lysates were labeled with the indicated ABP at the indicated concentration and analyzed by in-gel fluorescence. (b) Living RAW264.7 cells were incubated with the indicated probe at the indicated concentration, and labeling was assessed by in-gel fluorescence. This is a representative example from three independent experiments.

cathepsin S reactivity observed. This is despite clear labeling of cathepsin S observed with BMV109, a phenoxymethyl ketone-based pan-cathepsin probe, which suggests that cathepsin S is active in the lysate conditions.[3,8] By contrast, many of the SY probes (e.g., Ser, Lys(NH_2), and Arg) label cathepsin S to a greater extent than cathepsin X when applied to living cells.

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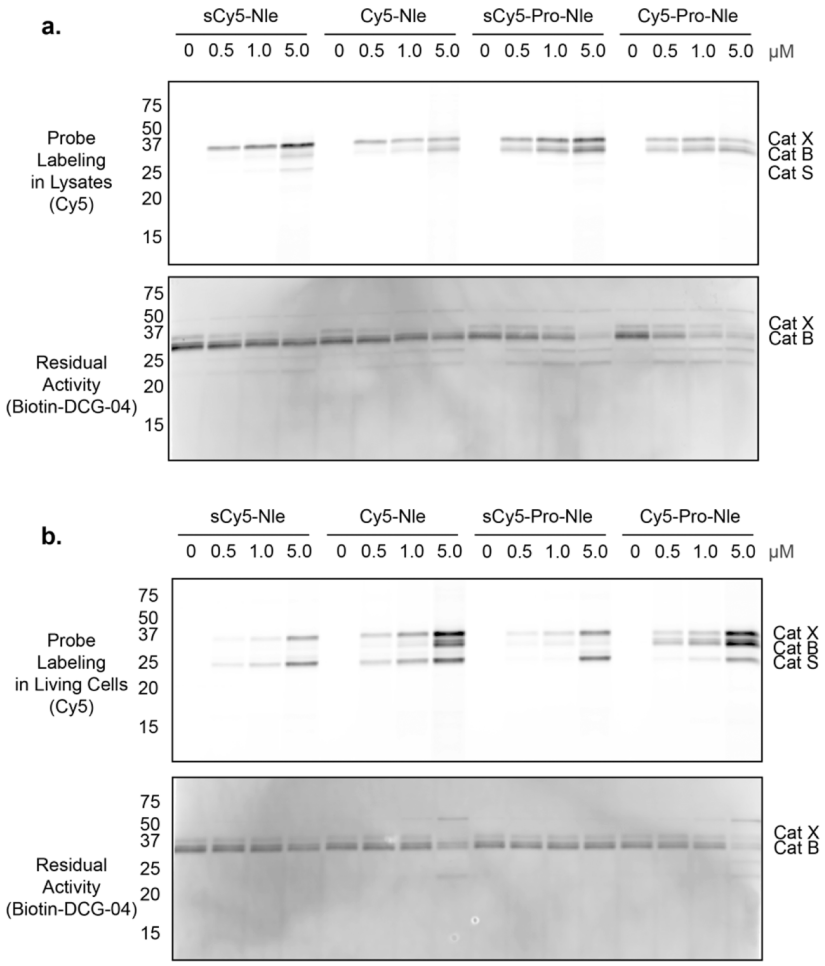

Figure 6. Cy5 sulfonation impacts ABP potency and selectivity. (a) RAW264.7 cell lysates or (b) living RAW264.7 cells were labeled with the indicated concentration of the indicated probes, and labeling was assessed by in-gel fluorescence (top panels). Residual cathepsin activity was assessed after probe treatment using the biotin-DCG-04 probe and streptavidin blotting (bottom panels). This is a representative example from three independent experiments.

Puzzled by this discrepancy, we previously tested a number of lysis conditions that might permit increased cathepsin S labeling in lysates (e.g., pH 5, 6, 7.2; citrate v acetate; ±4 mM DTT or 500 μM cysteine), but to no avail. We typically use freshly prepared lysates for ABP labeling assays. Serendipitously, we noticed that if the lysates had been permitted to incubate at 37 °C for 4 h prior to probe addition, sCy5-Nle-SY labeled significantly more cathepsin S, and this labeling was blocked when the cells were pretreated with the cathepsin S-specific inhibitor MDV-590 (Figure 7a). By contrast, cathepsin X reactivity was slightly reduced (Figure 7a), despite the total protein levels for both proteases remaining constant (Figure 7b, c). Cathepsin S labeling with the pan-cysteine cathepsin probe BMV109 did not change upon incubation at 37 °C for 4 h. Collectively, these results suggest that labeling of cathepsin S with the SY electrophile improves over time, while its labeling by phenoxymethyl ketone is constant.

DISCUSSION

Herein, we describe the synthesis and characterization of an expanded library of SY-based fluorescent probes targeting members of the cysteine cathepsin family. Probes bearing diverse P1 residues exhibit variable potency and selectivity toward cathepsins X and S, and to a lesser extent, B. Our limited attempts to introduce P2 residues into the SY probe scaffold resulted in loss in cathepsin selectivity. sCy5-Phe-Val-SY[3] labels cathepsin X, B, S, and L, while sCy5-Pro-Nle-SY labels cathepsin X, B, and S. Structural analysis suggests that cathepsin X can accommodate P2 residues with long side chains and hydrophilic tails.[20] The S2 pocket of cathepsin S, however, exhibits greater plasticity to accommodate bulkier P2 residues.[21−23] It may therefore prove difficult to develop SY probes that are selective for cathepsin X over S without exploiting additional residues. As C-terminomics methods to identify natural substrates of carboxypeptidases such as cathepsin X continue to improve, we will be better informed in the design of SY libraries with extended recognition sites. In any case, many of probes in this series permit simultaneous monitoring of cathepsin X and S, which is advantageous over pan-reactive probes such as BMV109 in cells that express high levels of cathepsin B and L. We have recently reported the application of sCy5-Nle-SY to study the regulation of cathepsin X activation during dendritic cell maturation and its contributions to cathepsin L processing and nuclear trafficking.[4,24]

Cbz-Lys(sCy5)-SY exhibits clear selectivity for cathepsin S over that for cathepsin X in live cells. Moving the sCy5 to the α-amine led to a significant increase in the level of labeling of both proteases. SY probes with Lys(NH_2) and Lys(Boc) exhibited a similar labeling profile; however, the Boc-protected

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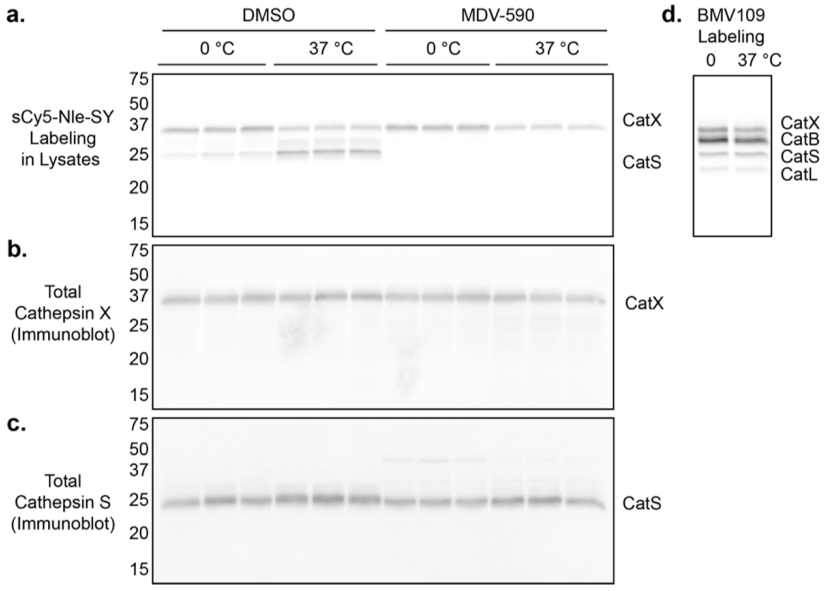

Figure 7. Prolonged lysate incubation increases SY reactivity with cathepsin S. (a) RAW264.7 cells were pretreated overnight with the cathepsin S-specific inhibitor MDV-590 (50 μM) or DMSO vehicle. Cells were lysed in citrate buffer and incubated at 0 or 37 °C for 4 h, followed by labeling with sCy5-Nle-SY at 37 °C for 20 min and detection by in-gel fluorescence. Immunoblots of gel shown in (a) with antibodies for (b) cathepsin X or (c) cathepsin S. Three replicates are shown. (d) RAW264.7 lysates were incubated at 0 or 37 °C for 4 h followed by labeling with the pan-cysteine cathepsin probe, BMV109, at 37 °C for 20 min. Labeling was assessed by in-gel fluorescence, and a representative example from three experiments is shown.

probe exhibited higher cathepsin X binding in both lysates and live cells. These results suggest that reducing bulk in the P1 position is permissive for cathepsin X binding and that a positive charge on the side chain does not confer significant advantages. Blum and colleagues recently reported that acyloxymethyl ketone (AOMK)-based ABPs bearing a free Lys in the P1 position exhibited increased affinity for cathepsin B, S, and L compared to those with a fluorophore attached via the side chain amine.[25] They speculated that it was the positive charge on the free amine that mediated this effect, although their study did not discriminate whether it was the charge or the associated reduction in steric hindrance. Cathepsin X affinity was not altered by this change, but this is in line with AOMK probes having limited reactivity with cathepsin X.[3,26]

Our data reaffirm that the choice and position of tags, fluorescent or otherwise, can have major impacts on ABP selectivity. This can be either through directly changing affinity for the protease through steric effects, as observed with our Lys-SY probes, or by influencing the uptake of probes to impact their local concentrations. As previously observed with cathepsin-targeted vinyl sulfone probes,[18] SY ABPs bearing Cy5 exhibited increased labeling in living cells compared to sCy5-tagged probes, despite similar affinities in lysates. These results suggest that the negative charge and hydrophilicity of the sulfonate groups limit the uptake of the probes into cells. Similar observations were recently reported with cell-penetrating peptides[27] and DNA nanostructures.[28] Cy5-CPP exhibited significantly greater uptake into cells than sCy5-CPP, and it was largely localized to endolysosomes. When introduced to tumor-bearing mice, the two CPPs exhibited very different pharmacokinetic profiles, with sCy5-CPP showing significantly greater tumor and renal uptake than Cy5-CPP. Thus, the choice in fluorophore must be carefully considered and fit for purpose. A critical advantage of covalent probes is that the impact of the fluorophore on protease binding, including potency and specificity, can always be directly evaluated by in-gel fluorescence.

We remain perplexed by the apparent differences in cathepsin S labeling by the SY electrophile in lysate conditions and living cells, while cathepsin X binding is similar in both contexts. We observed increased labeling of cathepsin S by sCy5-Nle-SY if lysates were incubated at 37 °C prior to addition of the probe as opposed to used immediately after lysis. This was also evident when lysates were subjected to freeze−thaw cycles. Disruption in compartmentalization of cystatin B was recently observed to yield differential labeling of cathepsin K in live cells and lysates.[29] If cathepsin S were inhibited by an endogenous inhibitor in lysates (e.g., cystatin C), and if that inhibitor were degraded, then cathepsin S would appear more active over time. BMV109 is significantly more potent than sCy5-Nle-SY, labeling cathepsin S in fresh lysates as low as 10 nM,[3] while sCy5-Nle-SY does not label it below 1 μM. It is therefore conceivable that BVM109 is better able to compete with the endogenous inhibitor than the SY probes. Regardless of the explanation, these results reinforce the need to use fresh lysates for all protease assays that preclude live cell analysis. Measuring protease activity in situ, where the compartmentalization of proteases and their inhibitors is maintained, often provides a more biologically relevant result. On the other hand, delivery of the probes to the correct intracellular locations can pose a different challenge. This is especially important to consider in light of increasing reports of extra-lysosomal cathepsin activity (e.g., in the cytosol and nucleus).[4,30]

In summary, we have extended the characterization of SY-based cathepsin probes by diversifying recognition sites and examining the impact of the fluorophore location and composition on probe reactivity. Our work has recently inspired the development of sulfonium-based ABPs that permit lysine profiling in alkaline conditions[31] and photoclick reactions aimed to more broadly profile the druggable

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cysteinome.[32] It will no doubt be exciting to continue to explore the broader applications of SY probes in the future.

MATERIALS AND METHODS

Synthetic Methods. Detailed synthetic methods can be found in the Supporting Information. High-resolution mass spectra and HPLC traces for all final compounds are shown in Supporting Information Figures S1−S14.

Cell Culture. RAW264.7 murine macrophage cells were cultured in DMEM containing 10% fetal bovine serum (v/v) and 1% antibiotic/antimycotic (v/v). Cells were passaged by scraping with rubber policemen.

ABP Labeling in Lysates and In-Gel Fluorescence. Cells were harvested by scraping and washed with phosphate-buffered saline (PBS) before lysing in buffer containing 50 mM citrate (pH 5.5), 0.5% CHAPS (w/v), 0.1% Triton X-100 (v/v), and 4 mM DTT. Cells were incubated on ice for 10 min with frequent vortexing. Supernatants were cleared by centrifugation at 21 000*g* for 5 min at 4 °C. Protein concentration was measured using a BCA assay (Pierce), and total protein (80 μg) was aliquoted into tubes in a final volume of 20 μL lysis buffer. Where indicated, cells were pretreated with DMSO (0.1%) or MDV-590 (50 μM) overnight before lysates were collected and incubated at 0 or 37 °C for 4 h. Probes were added from a 100x DMSO stock to the indicated final concentration and incubated for 20 min at 37 °C. Where residual cathepsin activity was measured, Biotin-DCG-04 (2 μM) was added for a further 20 min.[19] Reactions were then quenched by addition of 5× sample buffer (200 mM Tris-Cl [pH 6.8], 8% SDS (w/v), 0.04% bromophenol blue (w/v), 5% b-mercaptoethanol (v/v), and 40% glycerol (v/v)). Samples were boiled for 5 min, and proteins were resolved on a 15% SDS-PAGE gel poured in-house. Gels were scanned on a Typhoon 5 instrument at 633/670 nm excitation/emission to detect Cy5 fluorescence. To detect biotin, gels were transferred to nitrocellulose membranes using the TransBlot system (Bio-Rad) and blotted with streptavidin-IR800 (LiCor; 1:10 000) overnight.

Labeling Live Cells with ABPs. RAW264.7 cells were plated in six-well plates and grown to 80% confluence. ABPs were added from a 1000x stock solution to the indicated final concentrations (0.1% final DMSO concentration). After 4 h, media were removed, and cells were washed with PBS. Cells were then scraped, transferred to tubes, and lysed in PBS containing 0.1% Triton X-100 (v/v). After the BCA assay, total protein was analyzed by in-gel fluorescence as above. Where residual cathepsin activity was measured, cells were instead lysed in citrate buffer, and lysates were labeled with Biotin-DCG-04 as above.

ASSOCIATED CONTENT

Data Availability Statement

All original data are provided in the manuscript or Supporting Information. Synthetic methods are available in the Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c07604.

Synthetic methods and characterization of all reported compounds (PDF)

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Author Contributions

[§]B.X. and S.J.M. contributed equally to this work. **B.X.**: data curation; formal analysis; investigation; methodology; validation; writing—review and editing. **S.J.M.**: data curation; methodology; writing—review and editing. **P.E.T.**: conceptualization; resources; supervision. **L.E.E.-M.**: conceptualization; funding acquisition; investigation; methodology; project administration; resources; supervision; writing—original draft.

Notes

The authors declare no competing financial interest.

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