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Design and synthesis of fluorogenic substrate-based probes for detecting Cathepsin B activity

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ABSTRACT

Cathepsin B plays key roles in tumor progression with its overexpression being associated with invasive and metastatic phenotypes and is a primary target of protease activated antibody-directed prodrug therapy. It therefore represents a potential therapeutic and diagnostic target and effort has been made to develop fluorescent probes to report on Cathepsin B activity in cells and animal models of cancer. We have designed, synthesized, and thoroughly evaluated four novel "turn on" probes that employ a lysosomotropic dansylcadaverine dye to report on Cathepsin B activity. Enzyme activity assays using a recombinant human enzyme and cancer cell lysates coupled with confocal microscopy experiments demonstrated that one of the probes, derivatized with the self-immolative prodrug linker *p*-aminobenzyl alcohol, can selectively report on Cathepsin B in biological samples including live cells.

1. Introduction

Cathepsin B (CTB) is one of eleven human cysteine cathepsin proteases that play a key role in basic recycling functions such as lysosomal protein turnover, autophagy, antigen presentation, and apoptosis [1]. Numerous studies have shown that the expression of CTB occurs in a variety of pathological processes at both the gene and protein levels, most notably in tumour invasion and arthritis [2,3] and CTB activity has been linked to tumour growth in various cancers, e.g. oral [4], oesophageal [5], gastric [6], prostate [7], and breast [8]. Furthermore, CTB is secreted into the extracellular matrix (ECM) by cancer cells and tumour-associated macrophage cells where it can activate the proteolytic cascade stimulating the degradation of the ECM during initial stages of invasion [9]. Thus, CTB is considered to be a potential diagnostic or therapeutic target for aggressive cancer [10,11]. CTB hydrolytic activity is often targeted to release drugs from antibody-drug conjugates for anti-cancer therapy [12].

Over the last decade, several groups have developed activity-based and substrate-based molecular imaging probes intended to study CTB activity in cells and animals [13–19] and as tools to guide surgical resection by identifying tumour margins [20]. Activity-based probes (ABP's) are "suicide inhibitors" that are processed by the enzyme into a reactive intermediate that covalently links a reporter group to the target protease. In contrast, others have developed substrates of CTB intended to exploit the catalytic power of the target enzyme. A dual targeted approach has also been developed where a peptide-based substrate first binds to the folate receptor followed by internalization and hydrolysis by CTB [14]. However, probes selective to CTB over other cysteine cathepsins have been rarely reported due to overlapping substrate preferences between CTB and most notably Cathepsin L (CTL) [21]. Thus, the design and synthesis of selective and sensitive CTB probes are urgently needed.

In this study, we report the design, synthesis and *in vitro* evaluation of several novel substrate-based fluorescent probes (substrates **1–4**) derivatized with the fluorescent dye dansylcadaverine and bearing 2,4-dinitrophenylamine to serve as a quencher moiety (Fig. 1).

Satisfyingly, each probe was effectively quenched in the intact substrate and was hydrolysed by CTB. In particular, prodrug inspired probe **4** showed specificity towards CTB and was successfully used for detecting CTB activity in cancer cells lysates and living cells using confocal microscopy. As designed, dansylcadaverine that was enzymatically released into the lysosomes of living cells appeared to be effectively trapped in these acidic organelles through the lysosomotropic effect. Consequently, substrate **4** may be useful to study CTB activity in cell lysates or cancer cells and represents a lead compound towards substrate-based radiotracers or optical probes for monitoring CTB.

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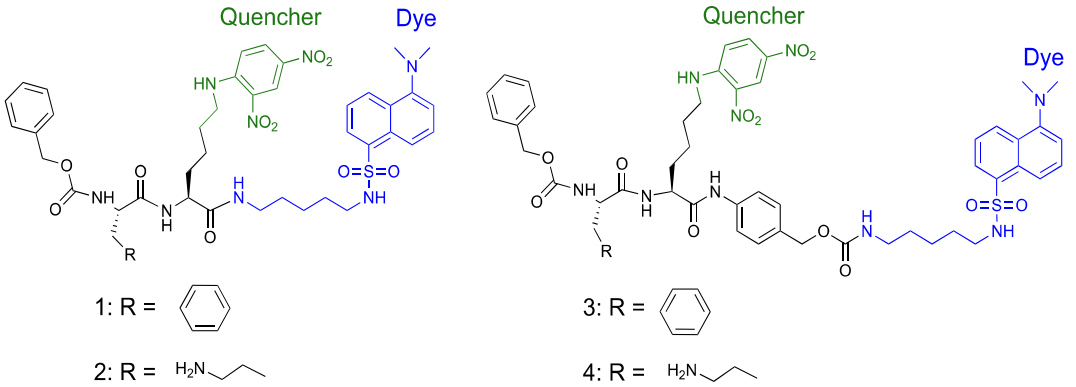


Fig. 1. The chemical structures of the intramolecular-quenched substrates.

2. Results and discussion

2.1. Probes design

In order to discover substrate-based probes that are efficiently and specifically hydrolyzed by CTB, we previously reported on pro-fluorogenic substrates based on a simple carbonyl benzyloxy (Cbz) protected dilysine peptide derivatized with the self-immolative prodrug linker *p*-amino-benzyl alcohol (PABA) and 7-amino-4-methyl-coumarin (AMC) as a fluorescent reporter group [22]. Upon selective hydrolysis by CTB to remove the peptide moiety, PABA spontaneously undergoes a 1,6-elimination reaction that simultaneously releases CO_2 and AMC thus providing a readout on enzyme activity. However, a substrate-based "turn on" probe that was not only cell permeable but which would also release a lysosomotropic fluorophore intended to remain trapped inside cancer cells after CTB hydrolysis would be preferable. Replacing AMC with a dye that would remain trapped inside live cells would be beneficial for cell imaging experiments using for example, confocal microscopy or flow cytometry. Additionally, an efficient and selective substrate would be useful for assessing CTB activity in cell lysates, evaluating novel CTB inhibitors or detecting CTB activity in various cells lines.

Several strategies have been developed to generate fluorescent substrates that are capable of revealing CTB activity in biological samples. Although elegant FRET-quenched substrate-based probes capable of revealing intracellular CTB activity have been developed, the use of large dyes and bulky quencher groups can limit the efficiency of hydrolysis by CTB [15]. In contrast, designing substrates using amino acids with flexible sides chains like (*S*)-lysine derivatized with a small quenching group located in close proximity to a reporter dye could be exploited to generate intramolecularly quenched substrates [23–25]. Although intramolecularly quenched probes often suffer from high background fluorescence of the intact substrate, this strategy is attractive due to straightforward synthetic routes and the potential to identify highly efficient, specific and cell permeable probes that can be used in simple, continuous, sensitive and rapid experiments to assess CTB activity (as illustrated in Fig. 2). To explore this approach for monitoring CTB activity in complex biological mixtures like cell lysates or living cells, we prepared several candidates consisting of three elements designed to exploit intramolecular quenching; a Cbz protected dipeptide to simultaneously serve as a selective CTB recognition sequence and scaffold bearing a small and efficient quenching moiety, a self-immolative linker known to impart selectivity towards CTB and a dye that becomes ionically trapped inside the acidic environment of the lysosome after CTB processing. In order for the intact probe to be non-fluorescent, the quencher and reporter pair must be carefully chosen. Using 2,4-DNP as a quencher was attractive since it is a relatively small group unlikely to interfere with CTB recognition, is non-fluorescent thus limiting background signal of the intact probe and is easily

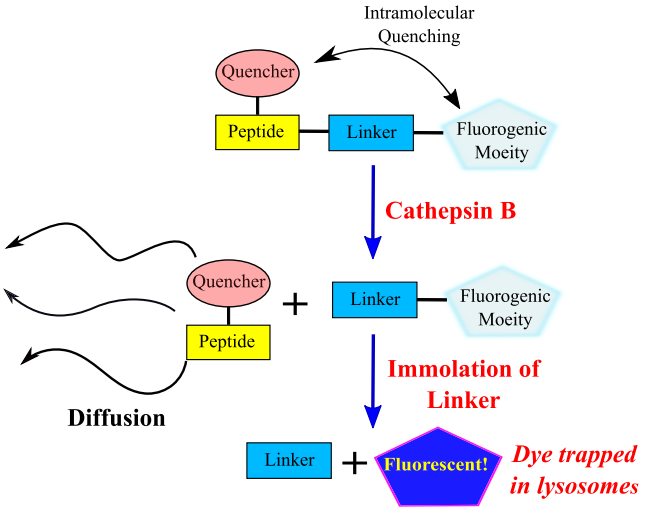


Fig. 2. Cartoon illustrating the design of and mechanism of an intramolecular quenched prodrug inspired substrate-based flurogenic probe for CTB.

incorporated onto the ε-amine of lysine within a peptide sequence. Introducing a linker into the substrate is important since these groups are known to impart enzyme selectivity as well as enable the conjugation of both alcohols and amines to the peptide moiety via stable ether and carbamate linkages. Moreover, using an efficient self-destructive linker like PABA would rapidly break down and release the fluorescent dye into the lysosomes prior to diffusion into the cytosol [26–31].

To develop substrates selective to CTB, probes were designed based on the tendency that most human cysteine cathepsins prefer a hydrophobic amino acid at P2 and a positively charged substituent at the P1 positions. Indeed, Cbz-(*S*)-phenylalanyl-(*S*)-lysine and Cbz-(*S*)-lysyl-(*S*)-lysine are established recognition elements leading to efficient hydrolysis by CTB [16,22]. However, the lysine at the P1 position also provided a convenient location for introducing 2,4-dinitro benzene (Sanger's reagent) into the peptide nearby the dansyl dye for effective quenching. To evaluate the effects of the linker and quencher moieties on hydrolysis rates and fluorescence quenching efficiency, we synthesized the probes **1** and **2** without the PABA linker whereas **3** and **4** contained the PABA linker. As the reporter dye, we chose dansylcadaverine because of its excellent photochemical properties, such as high quantum yield, large Stokes shift, and relative stability under physiological conditions. In addition, the free primary amine of the dansyl-cadaverine would become protonated once released into the acidic lysosomes and become trapped due to the lysosomotropic effect [32].

Importantly, intramolecular quenching requires that the dye and quencher must be in close proximity meaning that the 2,4-dinitrobenzene coupled to the P1 lysine side chain should effectively

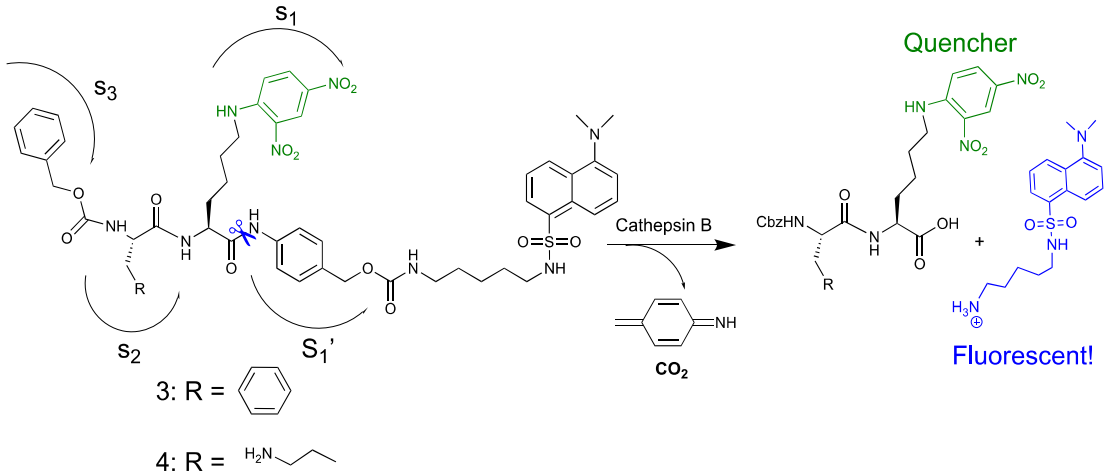


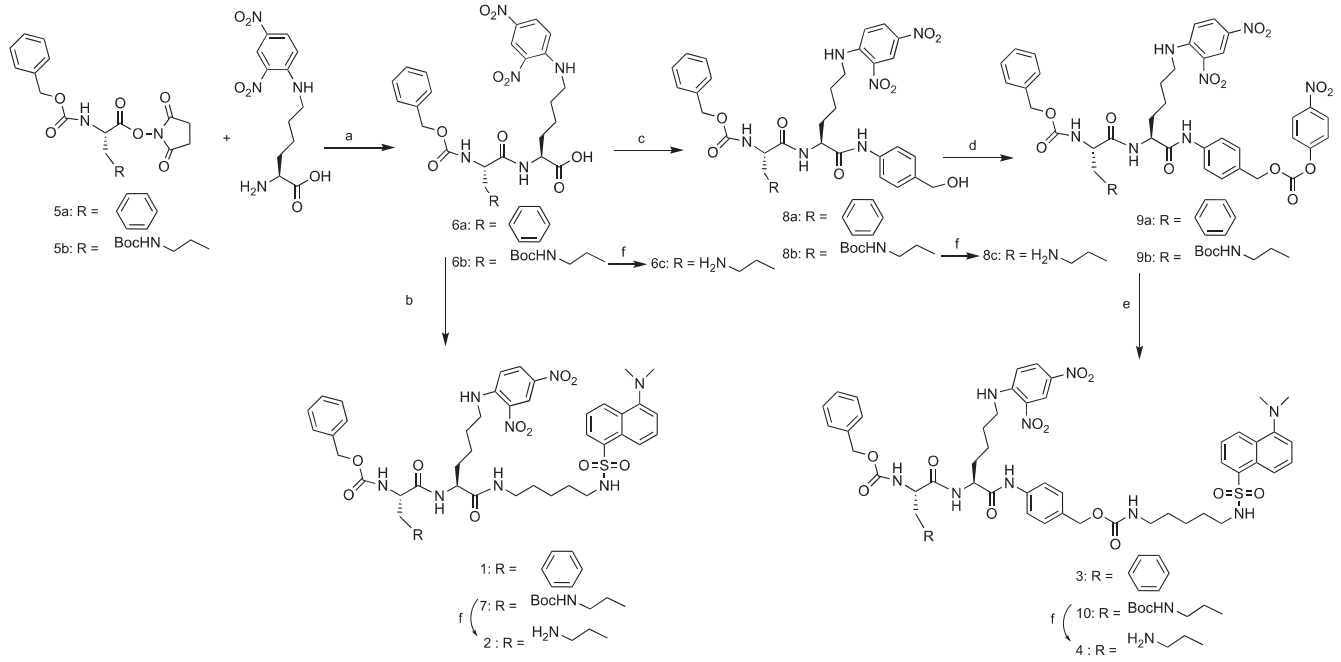
Fig. 3. Design and mechanism of a three-component substrate-based probe, hydrolysis, immolation and release of dansylcadaverine by CTB. Note that the CTB binding pockets are indicated by S3, S2, S1 and S1′.

quench dansylcadaverine within the peptide substrate. As shown in Fig. 3, hydrolysis of the probe by CTB would activate PABA self-immolation thus rapidly releasing dansylcadaverine from the peptide scaffold/quencher and allowing the reporter dye to "turn on" at full fluorescence intensity.

2.2. Synthesis and photochemical properties of the probe candidates

We prepared four probe candidates **1**, **2**, **3**, and **4** using solution peptide chemistry as presented in Scheme 1. The preparation of starting materials, compound **5a** (Cbz-Phe-O-N-hydroxy-succinimide) and **5b** (Cbz-Lys-N-ε-Boc-O-succinimide) followed our previous work [22]. Coupling of compound **5a** with N-ε-(2,4-dinitrophenyl)-(*S*)-lysine proceeded smoothly in anhydrous DMF in the presence of triethylamine to provide compound **6a** Cbz-Phe-Lys-N-ε-Boc-OH with 86% chemical yield. Using the same method, gram-scale amounts of **6b** were obtained by reaction of **5b** and N-ε-(2,4-dinitrophenyl)-(*S*)-lysine. The dipeptide **6a** was activated with *N*-hydroxysuccinimide in presence of DCC (N, N'-dicyclohexylcarbodiimide) in anhydrous THF, followed by removal of the urea by-product using filtration. Without further purification, the crude filtrate was used directly by adding dansylcadaverine to give the desired compound **1** with 66% chemical yield. Synthesis of compound **7** followed the same procedure with Boc deprotection achieved using 40% (v/v) TFA/CH_2Cl_2 for 10 min at 0 °C to provide probe **2** in good yields.

Efforts to synthesize the dipeptide-PABA conjugate **8a** and **8b** using the NHS and DCC method failed to provide the desired products in good yields. However, conjugation of compounds **6a** and **6b** to PABA proceeded smoothly using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) to obtain **8a** and **8b** in good chemical yields of 76% and



Scheme 1. Synthesis of the four probe candidates each with the DNP quencher. Reagents and conditions: (a) anhydrous DMF, triethylamine (TEA), 16 h, RT, 86% **6a**, 96% **6b**; (b) (i) *N*-hydroxysuccinimide (NHS), N,N'-dicyclohexylcarbodiimide (DCC), anhydrous THF, 1 h, RT, (ii) dansylcadaverine, TEA, 3 h, RT, 66% **1**, 63% **7**; (c) 4-aminobenzylalcohol, 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ), anhydrous THF, 48 h, RT, 76% **8a**, 56% **8b**; (d) 4-nitrophenyl chloroformate, anhydrous pyridine, anhydrous THF, 3 h, RT, 67% **9a**, 87% **9b**; (e) dansylcadaverine, TEA, anhydrous THF, 86% **3**, 74% **10**; (f) TFA/DCM solution, 0 °C, 10 min, 87% **6c**, 88% **8c**, 84% **2**, 87% **4**.

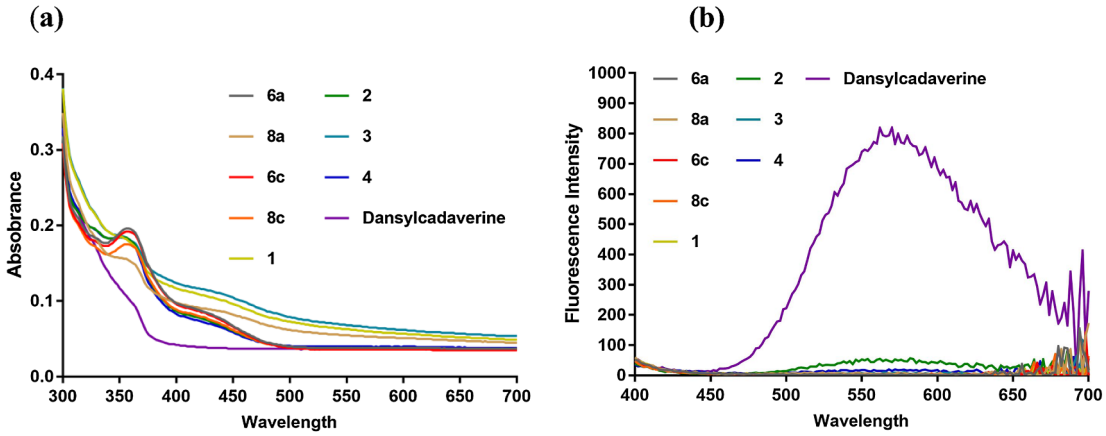


Fig. 4. Absorbance and fluorescence emission spectra of Dansylcadaverine, probe **1**, **2**, **3**, **4** and compounds **6a**, **8a**, **6c**, **8c**. All spectra were taken in acetate buffer (50 mM, pH 5.0) using 10 μM of each species. (a) absorption spectra; (b) emission spectra; λ_{ex} = 254 nm and λ_{em} = 400–700 nm.

56% respectively. The primary alcohol of **8a** and **8b** was then activated with 4-nitrophenyl chloroformate to afford carbonate **9a** and **9b** followed by direct coupling to dansylcadaverine to give carbamate probe **3** and intermediate **10**. Compound **10** was then deprotected using 40% (v/v) TFA/CH_2Cl_2 to generate probe **4** in good yield.

Once the purified substrates were prepared, we next determined the UV–VIS absorbance and fluorescence spectra of each compound and compared each to free dansylcadaverine as shown in Fig. 4. As expected, all compounds gave similar UV–VIS absorbance spectra (Fig. 4a). As shown in Fig. 4(b), the intact probes **1**, **2**, **3** and **4** had surprisingly low fluorescence intensity compared to the free dye indicating that the 2,4-DNP group at the P1 position effectively quenched the dansylcadaverine in each probe candidate. Ineffective quenching and high background fluorescence have limited the use of quenched substrates bearing a 4-nitrophenyl quenching moiety for other enzyme systems [33,34]. As shown in Fig. S4 in the supporting information, incubation of **6c** with dansylcadaverine did not result in a significant change in the emission spectra of the free dye. This confirms that intramolecular interactions lead to effective quenching of the intact substrate.

2.3. Kinetic evaluation of the probe candidates with CTB and CTL

To evaluate the ability of the probes to "turn on" after enzymatic hydrolysis, we monitored the onset of fluorescence of each probe candidate (25 μM) at 512 nm upon treatment with active and recombinant CTB (50 nM) in acetate buffer. As shown in Fig. 5, treatment of probes **1**–**4** led to a time dependent increase in fluorescence upon incubation with active CTB consistent with the release of dansylcadaverine from the peptides. Probes **1** and **2** that lacked the PABA linker and probe **3** with PABA were relatively poor substrates of the CTB. Encouragingly, probe **4** was hydrolysed much more efficiently. As an important negative control to demonstrate that each substrate was stable in assay buffer over the course of the experiment, minimal fluorescence was detected when CTB was omitted from the reaction mixture or when CTB was inactivated prior to the assay using commercially available irreversible inhibitors CA074 (40 μM, specific to CTB) and E64 (40 μM, a pan-cysteine cathepsin inhibitor). As shown in Fig. S3 in the supporting information, the onset of fluorescence intensity was proportional to the amount of recombinant CTB added at physiologically relevant concentrations of the protease. Based on these results, all substrates were stable to spontaneous hydrolysis over the course of the experiment and only active CTB led to the onset of a fluorescence signal proportional to the amount of enzyme present in the reaction mixture. In addition, probe **4**, bearing (*S*)-Lys at the P2 position and the PABA linker incorporated into the peptide acted synergistically significantly increasing the rate of hydrolysis by CTB.

To evaluate the hydrolysis of each probe candidate by a related lysosomal cysteine protease, recombinant human Cathepsin L (CTL) was activated and incubated with each substrate also shown in Fig. 5. Despite being a more hydrolytically potent endopeptidase, probes **1** and **3** were not efficiently hydrolysed by CTL. In addition, probe **2** was not a substrate of CTL since this enzyme does not usually tolerate a positively charged residue at the P2 position. Interestingly, probe **4** was a substrate of CTL but was not hydrolysed efficiently when compared to CTB at 25 μM of substrate.

After these assays confirmed that compound **4** is efficiently hydrolyzed by CTB and to a lesser extent CTL, we then determined K_M, k_{cat} and k_{cat}/K_M for each enzyme. Knowledge of these parameters for CTB and CTL would allow us to interpret enzyme activity data obtained from samples containing natural mixtures of the enzymes. As shown in Table 1 (and Fig. S1 in the supporting information), probe **4** had a K_M value of 1.50×10^{-2} mM and a k_{cat} of 2.4 s^{-1} which translated into a specificity constant of 160 $mM^{-1}\,s^{-1}$. Although probe **4** is a good substrate of CTB, others have used combinatorial libraries to identify fluorogenic tetrapeptides having much higher specificity constants (~7000 $mM^{-1}\,s^{-1}$) [35]. In contrast, probe **4** was a relatively poor substrate of CTL due to a slightly lower K_M of 1.24×10^{-2} mM but a significantly lower turnover number with a k_{cat} of 0.16 s^{-1} resulting in a modest k_{cat}/K_M value of 12.9 $mM^{-1}\,s^{-1}$. This demonstrates that probe **4** is hydrolysed ~12 times faster by CTB than CTL at saturating conditions. Taken together, these results suggest that probe **4** has the required kinetic efficiency for preferentially detecting CTB activity over CTL in cell lysates or living cancer cells especially considering CTB activity can be much higher than CTL in invasive cancers [36].

2.4. Hydrolysis of probe 4 in cancer cell lysates

To determine the ability of probe **4** to provide a readout on CTB activity in biologically relevant samples, cell lysates were prepared from DU145 (prostate) and MDA-MB-231 (triple negative breast) cancer cell lines. Cells lysates were obtained by sonication and centrifugation to remove the cellular membranes and insoluble proteins. As shown in Fig. 6, an increase in the fluorescence intensity at 512 nm was observed over time when probe **4** (25 μM) was incubated with cell lysates at pH of 5.0 in acetate buffer. To evaluate the specificity of probe **4** towards other cysteine cathepsin enzymes, cells lysates were pretreated with the CA074 or the pan-cysteine cathepsin inhibitor E64. Consistent with the kinetic assays that demonstrated rapid hydrolysis by CTB, lysates treated with CA074 and E64 had negligible substrate turnover over the course of the experiment. These data confirm "turn on" fluorescence and the selectivity of probe **4** towards CTB in a more

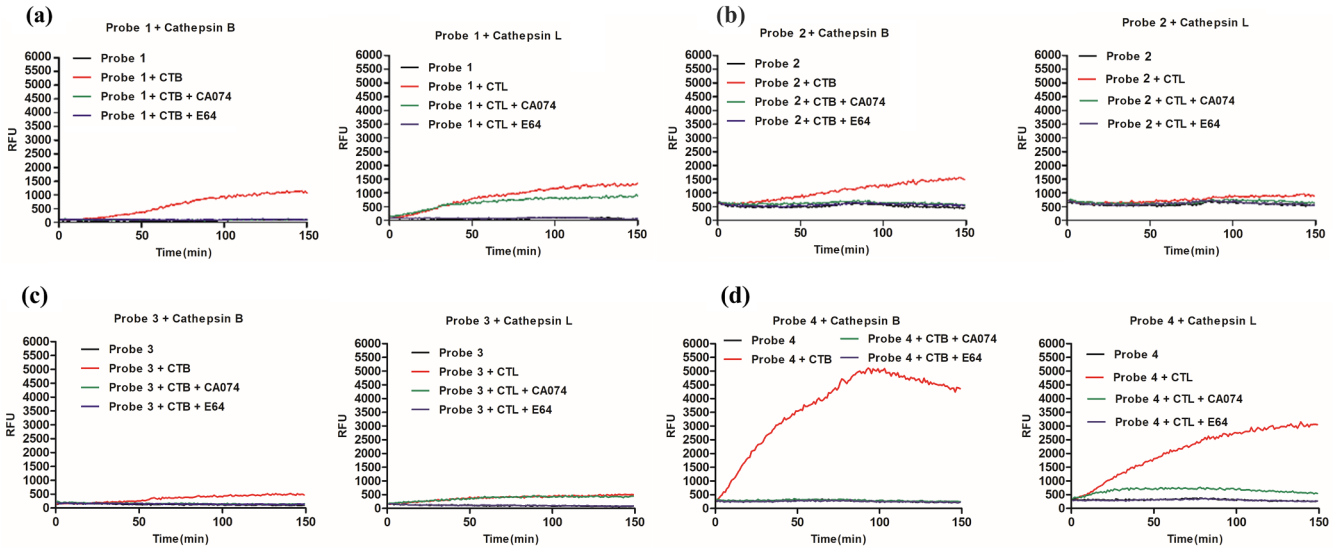


Fig. 5. Hydrolysis of probes **1**, **2**, **3** and **4** by Cathepsin B and Cathepsin L. All enzymatic hydrolysis reactions were conducted at 37 °C using pre-warmed solutions and in 96 well plates. Each well contained 50 µl of probe solution (100 µM stock in acetate buffer, 50 mM, pH 5.0, 1 mM EDTA, 4 mM DTT, and 20% v/v DMSO), 50 µl acetate buffer (50 mM, pH 5.0, 1 mM EDTA, 4 mM DTT) and 100 µl of activated CTB (100 nM, in acetate buffer, 50 mM, pH 5.0, 1 mM EDTA, 4 mM DTT). The final concentration of each probe and enzyme was 25 nM. For the control experiments using the enzyme inhibitors, 100 µl of recombinant enzyme (100 nM) in acetate buffer (pH 5.0, 1 mM EDTA, 4 mM DTT) containing 10 µM CA074 or E64 was incubated at 37 °C prior to each assay. The onset of fluorescence was monitored using λ_{ex} = 335 nm and λ_{em} = 512 nm in a Biotek Synergy 4 plate reader. (a) Represents hydrolysis of probe **1**; (b) probe **2**; (c) probe **3**; and (d) probe **4** respectively.

Table 1
Michaelis-Menten Kinetic Parameters Obtained for probe **4** as a Substrate of CTB and CTL. Each value was determined by three trials ± standard deviation.

	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M ($mM^{-1} s^{-1}$)
Cathepsin B	$1.5 \times 10^{-2} \pm 0.31 \times 10^{-82}$	2.4 ± 0.14	160 ± 0.21
Cathepsin L	$1.2 \times 10^{-2} \pm 0.15 \times 10^{-2}$	0.16 ± 0.01	13 ± 0.13

natural environment like cell lysates. It is important to note that the turnover of probe **4** is dependent upon the concentration and activity of CTB relative to other cysteine cathepsins, like Cathepsin S, present in the cell lysates as well as the expression of endogenous inhibitors [36].

2.5. Confocal microscopy experiments

Once we were confident that probe **4** demonstrated "turn on" activity and was preferentially hydrolysed by CTB in cell lysates, we next evaluated turnover by intracellular proteases in living cancer cells using confocal microscopy (Fig. 7). The cancer cell lines MDA-MB-231 (triple negative breast) and DU145 (prostate) were treated with 30 µM of probe **4** for 4 h. As a control experiment and to evaluate the lysosomotropism of the dye, cells were treated with 30 µM of dansylcadaverine. As expected, the free dansylcadaverine spontaneously accumulated in the lysosomes of the cancer cells visible as punctuate blue fluorescence. This behaviour is consistent with the spontaneous lysosomotropic effect. Importantly, the same cells treated with LysoTracker RED DND-99 displayed overlapping of the fluorescent signals. Since CTB hydrolysis of probe **4** releases dansylcadaverine directly into the lysosomes, the dye is expected become ionically charged and accumulate in these acidic organelles. As shown in Fig. 7 (and Figs. S11 and S12 in the supporting information), intracellular punctuate fluorescence was observed in both DU145 and MDA-MB-231 cells consistent with the accumulation of the enzymatically released dansylcadaverine dye inside the lysosomes. Importantly, this punctuate blue fluorescence also

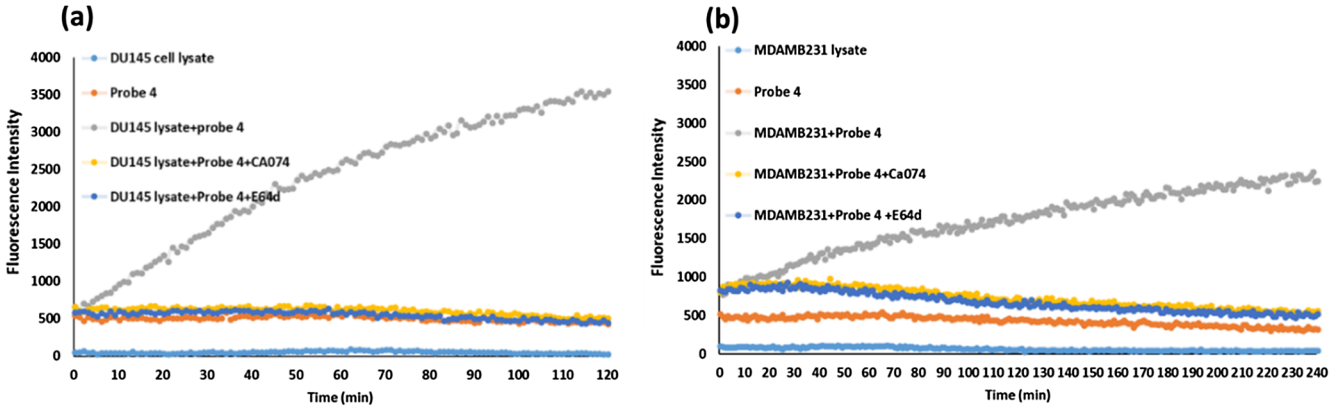


Fig. 6. Turnover of probe **4** in breast and prostate cancer cell lysates. (a) Data in cell lysates prepared from DU145 (prostate) cancer cells and (b) MDA-MB-231 (triple negative breast) cell lines. For each experiment, all solutions were prewarmed to 37 °C. To initiate the reaction, 100 µl of the cell lysate solution was added to a black 96-well plate and diluted with 50 µl of acetate buffer (pH 5, 1 mM EDTA and 4 mM DTT) and probe **4** in acetate buffer (pH 5, 1 mM EDTA and 4 mM DTT and 40% DMSO). For the inhibition experiments, we added 50 µl of acetate buffer containing 40 µM CA-074 or E64. Note that the final concentration of DMSO is 10%. Plots were generated by monitoring the onset of fluorescence (excitation λ_{em} = 335, λ_{em} = 512 nm) over time.

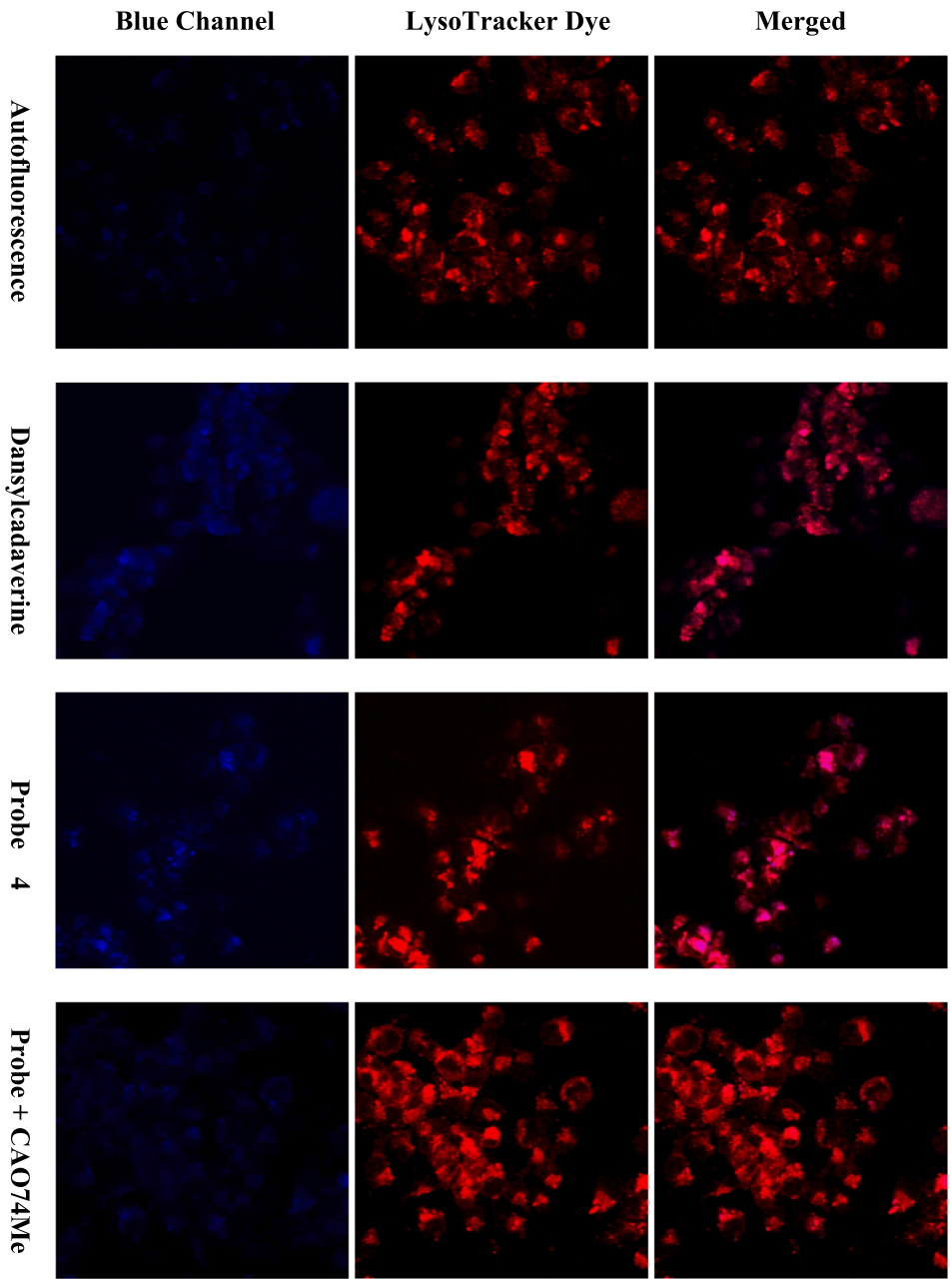


Fig. 7. Confocal microscope images of MDA-MB-231 breast cancer cells under various conditions. Images were taken using cells treated with vehicle for autofluorescence, dansylcadaverine, or probe **4**. As a control to evaluate probe specificity, cells were co-incubated with 30 uM CA-074Me or E64d with probe **4.** The merged images clearly indicate excellent overlap of the fluorescent signal from dansylcadaverine with lysotracker dye.

overlapped with the lysotracker dye (see "merged" in Figs. 7, S11 and S12). Collectively, these experiments demonstrate that dansylcadaverine is lysosomotropic and that cells treated with probe **4** results in substrate turnover, trapping and accumulation of the dye in the lysosomes. To show that the onset of the fluorescence was dependent on CTB activity, cells were simultaneously treated with either the membrane permeable CTB inhibitor CA074Me (30 μM) and the probe. CAO74Me treated cells had significantly reduced intracellular fluorescence suggesting that probe **4** has high specificity towards intracellular CTB. As expected, both cell lines treated with the membrane permeable pan-cysteine cathepsin inhibitor E64d and probe **4** also demonstrated significantly reduced intracellular fluorescence.

3. Conclusions

The goals of this study were to design, synthesize and evaluate novel "turn-on" fluorogenic probes that were efficient, selective and cell permeable substrates of CTB and that release a lysosomotropic reporter dye upon protease hydrolysis. To evaluate this approach, we prepared four probe candidates using straightforward solution peptide chemistry and obtained each in good chemical yields. Kinetic studies using recombinant enzymes revealed that probe **4** was an efficient substrate of CTB. Notably, the Cbz-Lys-Lys(2,4-DNP)-PABA scaffold was required for efficient recognition by CTB. Additionally, 2,4-DNP placed at P2 position was highly effective at quenching dansylcadaverine in the intact probe while maintaining efficient processing by human recombinant CTB and natural sources of CTB from human cancer cell lysates. Confocal microscopy experiments using prostate DU145 and

breast MDA-MB231 cancer cells treated with probe **4** revealed intracellular fluorescence that could be inhibited by CA074Me and E64d. Based on these encouraging results, we are currently developing next generation substrate-based fluorescent probes selective to CTB using different quencher/fluorophore pairs and lysosomotropic dyes with longer wavelengths for more sensitive CTB reporting in cells and animals.

4. Experimental section

4.1. Chemistry

4.1.1. General procedures

Moisture sensitive reactions were carried out in oven-dried glassware sealed with rubber septa under a positive pressure of dry argon. Similarly, air and moisture sensitive liquids and solutions were transferred via syringe. Reactions were stirred using Teflon-coated magnetic stir bars. All chemical reagents and anhydrous solvents were obtained from commercial sources and used without further purification. Analytical thin layer chromatography was performed with 0.25 mm silica gel 60F glass plates with F254 nm indicator. TLC plates were visualized by ultraviolet light. Flash chromatography was performced using Silica Flash P60 (Silicycle, Quebec City, Quebec). NMR Proton (^{1}H) and carbon (^{13}C) NMR spectra were acquired at the Saskatchewan Structural Sciences Centre using a Bruker Avance 500 MHz NMR in DMSO-d_6 with TMS as the internal standard. Coupling constants (J) are estimated in hertz (Hz). Mass spectra (MS) were obtained using an Advion Expression CMS and high-resolution mass (HRMS) was performed on JEOL JMS-T100GCv AccuTOF-GCv4G mass spectrometer in the Saskatchewan Structural Sciences Centre. All enzyme kinetics, absorbance and emission spectrum were performed on a Biotek Synergy 4 plate reader or a Biotek Cytation 5 imaging system. HPLC was performed on an Agilent 1200 Infinity LC with compounds separated on an Agilent Eclipse XDB-C18 column (4.6 mm, 150 mm, 5 μm) and detected using a 1200 series variable wavelength detector (G1314B). All fluorescence imaging was obtained by using Olympus FV300 Confocal Laser Scanning Biological Microscope.

4.1.2. Synthesis of the probe candidates

4.1.2.1. Cbz-Phe-Lys(2,4-DNP)-OH (6a). To a solution of Lys (2,4-DNP) (1.61 mmol, 500 mg) and **5a** (700 mg, 1.1 eq, 1.77 mmol) in 30 ml of anhydrous dimethylformamide (DMF) was added trimethylamine (1.5 eq, 2.4 mmol, 336 μl) under argon gas followed by stirring for 16 h at room temperature. To stop the reaction, 200 ml of ethyl acetate was added to the mixture and the organics were extracted successively with 10% critic acid (2x) and then saturated NaCl solution. The organic layer was separated, dried over anhydrous Na_2SO_4, filtered and the solvent removed under vacuum. Purification with flash chromatography (ethyl acetate) gave **6a** (900 mg, 86%) as yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.45–1.67 (m, 6H), 2.71 (d, J = 5 Hz, 1H), 3.99 (m, J = 5 Hz, 1H), 3.36(s, 2H), 3.47(s, 2H), 4.25–4.32 (m, 2H), 4.91 (t, J = 5 Hz, 2H), 7.14–7.31 (m, 10H), 7.50 (d, J = 2.5 Hz, 1H), 8.22–8.31 (m, 2H), 8.83–8.91 (m, 2H), 12.69 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 23.13, 28.01, 31.11, 33.82, 37.83, 43.11, 52.09, 56.48, 65.59, 115,71, 124.14, 126.70, 127.83, 128.09, 128.48, 128.70, 129.67, 130.01, 130.38, 135.02, 137.42, 138.59, 148.54, 156.31, 172.26, 173.99. ESI-Mass: calc. for $C_{29}H_{31}N_5O_9$, $[M]^-$, 592.2, found 591.7

4.1.2.2. Cbz-Lys-N-ε-Boc-Lys(2,4-DNP)-OH (6b). To a solution of Lys (2,4-DNP) (2.0 mmol, 620 mg) and **5b** (1.05 g, 1.1 eq, 2.20 mmol) in 30 ml anhydrous DMF was added trimethylamine (1.5 eq, 3.00 mmol, 418 μl) under argon gas followed by stirring for 16 h at room temperature. To stop the reaction, 200 ml of ethyl acetate was added to the mixture and the organics were extracted successively with 10% critic acid (2x) and then saturated NaCl solution. The organic layer was separated, dried over anhydrous Na_2SO_4, filtered and the solvent removed under vacuum. Purification with flash chromatography (ethyl acetate) gave **2b** (1.43 g, 96%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.22–1.75 (m, 21H), 2.71–2.87 (m, 3H), 3.34 (s, 2H), 3.44 (s, 2H), 3.44(s, 2H), 3.99 (s, 1H), 4.20 (d, J = 5 Hz, 1H), 4.98 (d, J = 5 Hz, 2H), 6.75(d, J = 5 Hz, 1H), 7.16(d, J = 5 Hz, 1H), 7.35 (d, J = 5 Hz, 1H), 8.09 (t, J = 5 Hz, 1H), 8.20 (q, J = 3 Hz, 1H), 8.22(s, 1H), 8.82 (d, J = 3 Hz, 1H), 8.86 (d, J = 5 Hz, 1H), 12.59 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 23.07, 23.27, 27.94, 28.71, 29.69, 31.05, 32.02, 43.11, 51.90, 54.91, 65.74, 77.78, 115.70, 124.12, 12.06, 128.17, 128.73, 130.01, 130.36, 135.02, 137.44, 148.52, 155.99, 156.37, 172.61, 173.99. ESI-Mass: calc. for $C_{31}H_{42}N_6O_{11}$, $[M]^-$, 673.3, found 672.8

4.1.2.3. Cbz-Lys-Lys(2,4-DNP)-OH (6c). Intermediate **6b** (36 mg, 0.05 mmol) was dissolved in 0.5 ml of a 40% v/v solution of TFA/CH_2Cl_2 followed by stirring for 10 min at 0 °C. After the reaction, 10 ml of diethyl ether was added and centrifuged at 1000 rpm/min for 10 min at 4 °C. The precipitate was dissolved in ethanol and passed through a silica gel pad yielding **6c** (25 mg, 87%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.23–1.93 (m, 12H), 2.74–2.76 (m, 2H), 3.34–3.47 (m, 2H), 4.02 (d, J = 8.4 Hz, 1H), 4.18 (s, 1H), 5.00 (s, 2H), 7.18–7.34 (m, 6H), 8.03 (d, J = 3.6 Hz, 1H), 8.24 (s, 1H), 8.84–8.87(m, 1H). ^{13}C NMR (DMSO-d_6) δ 22.71, 22.09, 27.03, 31.08, 31.71, 43.20, 54.75, 65.90, 115.73, 124.13, 12.06, 128.09, 128.21, 128.77, 135.07, 137.44, 148.56, 156.38, 172.20, 174.29. ESI-Mass: calc. for $C_{26}H_{34}N_6O_9$, $[M+H]^+$, 575.6, found 575.0; $[M+Na]^+$, 597.6, found 597.0.

4.1.2.4. Cbz-Phe-Lys(2,4-DNP)-Dansylcadaverine (1). A mixture of **6a** (0.2 mmol, 118 mg), *N*-hydroxysuccinimide (NHS, 1.1 eq, 0.22 mmol, 25 mg) and N,N'-dicyclohexylcarbodiimide (DCC, 1.1 eq, 0.22 mmol, 45 mg) were dissolved in 5 ml of anhydrous THF followed by stirring for 1 h at room temperature. A white precipitate formed and was removed by filtration. To the filtrate, dansylcadaverine (0.2 mmol, 76 mg) and TEA (2 eq, 0.4 mmol, 60 μl) were added followed by continual stirring for 3 h at room temperature. The solvent was removed under vacuum followed by purification using flash chromatography (ethyl acetate:hexanes 1:1 to 100% ethyl aceate) gave **1** (121 mg, 66%) as a brilliant yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.11–1.65(m, 12H), 2.70–2.86 (m, 11H), 3.37–3.42(m, 3H), 4.26 (dd, J = 5 Hz, 5 Hz, 2H), 4.92(s, 2H), 7.14–8.85 (m, 19H). ^{13}C NMR (DMSO-d_6) δ 22.90, 23.71, 28.09, 28.83, 29.21, 32.44, 37.81, 38.68, 42.76, 43.16, 45.50, 52.62, 56.62, 65.61, 115.52, 115.70, 119.61, 123.99, 124.12, 126.68, 127.79, 128.08, 128.20, 128.46, 128.61, 128.69, 129.48, 129.53, 129.64, 129.74, 129.95 130.36, 135.01, 136.58, 137.40, 138.50, 148.52, 151.76, 156.30, 171.49, 171.79. ESI-Mass: calc. for $C_{46}H_{54}N_8O_{10}S$, $[M+H]^+$, 911.3756; HRMS (ESI) found 911.3796 $[M+H]^+$.

4.1.2.5. Cbz-Lys-N-ε-Boc-Lys(2,4-DNP)-dansylcadaverine (7). A mixture of **6b** (0.3 mmol, 202 mg), NHS (1.1 eq, 0.33 mmol, 38 mg) and DCC (1.1 eq, 0.33 mmol, 68 mg) were dissolved in 5 ml of anhydrous THF followed by stirring for 1 h at room temperature. A white precipitate formed and was removed by filtration. To the filtrate, dansylcadaverine (0.3 mmol, 100 mg) and TEA (2 eq, 0.6 mmol, 85 μl) were added followed by continual stirring for 3 h at room temperature. The solvent was removed under vacuum, and purification with flash chromatography (ethyl acetate:hexanes 2:1 to 100% ethyl acetate) gave **7** (188 mg, 63%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.04–1.57 (m, 27H), 2.69–2.82 (m, 14H), 3.31–3.36 (d, 4H), 3.91 (s, 1H), 4.17 (s, 2H), 6.71–8.79 (m, 14H). ^{13}C NMR (DMSO-d_6) δ 22.91, 23.30, 23.68, 28.04, 28.68, 28.79, 29.17, 29.62, 31.94, 32.31, 38.66, 42.74, 43.16, 45.46, 45.50, 52.44, 55.23, 65.78, 77.76, 115.48, 119.59, 123.95, 128.01, 128.15, 128.59, 128.71, 129.46, 129.70, 130.32, 134.99, 136.56, 137.38, 148.48, 151.73, 155.98, 156.44, 171.55, 172.26. ESI-Mass: calc. for $C_{48}H_{65}N_9O_{12}S$, $[M+H]^+$, 992.5, found 991.6; $[M+Na]^+$, 1014.5, found 1013.5

4.1.2.6. Cbz-Lys-Lys(2,4-DNP)-dansylcadaverine (2). Compound **7** (80 mg, 0.08 mmol) was dissolved in 0.4 ml of a 40% v/v solution of TFA/CH_2Cl_2 followed by stirring for 10 min at 0 °C. After the reaction, 10 ml of diethyl ether was added and the mixture centrifuged at 1000 rpm for 10 min at 4 °C. The precipitate was dissolved in ethyl acetate/ ethanol (3:1) and purified using modified silica gel [37] (ethyl acetate: ethanol = 3:1) to obtain probe **2** (60 mg, 84%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.09–1.60 (m, 18H), 2.49–2.88 (m, 12H), 3.36–3.40 (m, 4H), 3.97 (d, J = 2.25, 1H), 4.19 (s, J = 2.75, 1H), 4.98 (s, 2H), 5.75 (d, J = 3.75, 2H), 6.71–8.82 (m, 14H). ^{13}C NMR (DMSO-d_6) δ 8.93, 14.42, 22.86, 23.70, 26.86, 28.02, 28.78, 29.19, 38.88, 42.75, 43.16, 45.50, 45.69, 52.61, 55.00, 55.39, 65.78, 115.51, 119.63, 123.99,124.09, 128.03, 128.18, 128.55, 128.74, 129.46, 129.52, 129.69, 129.95, 130.36, 135.00, 136.59, 137.38, 148.50, 151.73, 156.45, 171.65, 172.15. ESI-Mass: calc. for $C_{43}H_{57}N_9O_{10}S$, $[M]^-$, 892.4027; HRMS (ESI): found 892.4001 $[M+H]^+$.

4.1.2.7. Cbz-Phe-Lys(2,4-DNP)-PABA (8a). A mixture of **6a** (0.88 mmol, 520 mg), 4-aminobenzylalcohol (1.2 eq, 1.05 mmol, 129 mg) and 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ, 2 eq, 1.70 mmol, 430 mg) was dissolved in 20 ml of anhydrous THF followed by stirring for 48 h at room temperature. The solvent was removed under vacuum and purification using flash chromatography (ethyl acetate:hexanes 2: 1 to 100% ethyl acetate) gave **8a** (470 mg, 76%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.43–1.79 (m, 6H), 2.71(s, 1H), 3.01 (d, J = 10, 1H), 3.37–3.47 (m, 4H), 4.32–4.49 (m, 4H),4.94(d, J = 5, 2H) 5.14 (t, J = 5, 1H), 7.17–7.25 (m, 11*H*), 7.53–7.59 (m, 3H), 8.18–8.28(m, 2H), 8.80–8.89(m, 2H), 10.04 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 23.05, 28.18, 32.35, 37.85, 43.12, 53.52, 56.57, 63.03, 65.64, 115.73, 119.41, 124.14, 126.70, 127.34, 127.82, 128.10, 128.47, 128.71, 129.66, 129.96, 130.36, 135.01, 137.42, 137.87, 138.01, 138.51, 148.54, 156.34, 170.79, 172.12. ESI-Mass: calc. for $C_{36}H_{38}N_6O_9$, $[M]^-$, 697.3, found 696.7

4.1.2.8. Cbz-Lys-N-ε-Boc-Lys(2,4-DNP)-PABA (8b). A mixture of **6b** (0.46 mmol, 310 mg), 4-aminobenzylalcohol (1.5 eq, 0.69 mmol, 84 mg) and EEDQ (2 eq, 0.92 mmol, 227 mg) was dissolved in 20 ml of anhydrous THF followed by stirring for 48 h at room temperature. The solvent was removed under vacuum followed by purification using flash chromatography (ethyl acetate:hexanes 2: 1 to 100% ethyl acetate) to give **8b** (200 mg, 56%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.32–1.72 (m, 21H), 2.84 (s, 2H), 3.33–3.56 (m, 4H), 3.98 (s, 1H), 4.40 (s, 3H), 4.97 (s, 2H), 5.07 (s, 1H), 6.73(s, 1H), 7.14–7.50 (m, 10H), 8.06 (s, 1H), 8.16 (s, 1H), 8.78 (s, 1H), 8.83 (s, 1H), 9.94 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 23.03, 23.35, 25.56, 28.11, 29.69, 28.69, 29.65, 31.98, 32.23, 43.11, 53.34, 55.17, 63.00, 65.80, 77.77, 115.70, 119.33, 124.08, 127.30, 128.72, 135.00, 137.40, 137.94, 148.50, 155.98, 156.47, 170.82, 172.56. ESI-Mass: calc. for $C_{38}H_{49}N_7O_{11}$, $[M]^-$, 778.4, found 777.8.

4.1.2.9. Cbz-Lys-Lys(2,4-DNP)-PABA (8c). Compound **8b** (40 mg, 0.05 mmol) was dissolved in 0.4 ml of a 40% v/v solution of TFA/CH_2Cl_2 followed by stirring for 10 min at 0 °C. After the reaction, 10 ml of diethyl ether was added and the mixture was centrifuged at 1000 rpm for 10 min at 4 °C. The precipitate was dissolved in ethanol and passed through a silica gel pad to obtain probe **8c** (30 mg, 88%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.23–1.62 (m, 12H), 2.73–2.75 (m, 2H), 3.47 (m, 3H), 4.04 (q, *J* = 6 Hz, 1H), 4.44 (s, 2H), 5.02 (s, 2H), 7.18–7.59 (m, 12H), 8.14–8.21 (m, 2H), 8.83–8.86 (m, 2H), 10.03 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 22.91, 23.06, 27.25, 31.1, 32.21, 43.14, 53.49, 54.92, 63.03, 65.85, 115.76, 119.41, 124.13, 127.34, 128.23, 130.02, 135.07, 137.44, 137.90, 138.01, 148.57, 156.51, 158.54, 158.74, 170.93, 172.46. ESI-Mass: calc. for $C_{33}H_{41}N_7O_9$, $[M+H]^+$, 680.7, found 680.1; $[M+Na]^+$, 702.7, found 702.1.

4.1.2.10. Cbz-Phe-Lys(2,4-DNP)-PABA-PNP (9a). A mixture of **8a** (0.2 mmol, 140 mg), 4-nitrophenyl chloroformate (1.2 eq, 0.24 mmol, 49 mg) in 10 ml anhydrous THF was treated with anhydrous pyridine (1.2 eq, 0.24 mmol, 19 µl) under argon gas. After 3 h, the solvents were removed under vacuum followed by purification with flash chromatography (ethyl acetate:hexanes 1:1 to 3:1) gave **9a** (116 mg, 67%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.46–1.73 (m, 6H), 2.74 (s, 1H), 3.03 (s, 1H), 3.89–3.49 (m, 3H), 4.37 (s, 1H), 4.52 (s, 1H), 4.96 (s, 2H), 5.27 (s, 2H), 7.19–7.71 (m, 16H), 8.21–8.33 (m, 3H), 8.82(s, 1H).), 8.90 (s, 1H), 10.23 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 23.08, 28.20, 32.26, 37.88, 43.12, 53.64, 56.58, 65.66, 70.71, 115.72, 119.61, 123.06, 124.14, 125.86, 126.71, 127.84, 127.97, 128.11, 128.48, 128.71, 128.75, 129.68, 129.88, 129.92, 129.97, 130.36, 135.03, 137.43, 138.52, 139.76, 145.60, 148.55, 152.44, 155.76, 156.37, 171.15, 172.19. This compound did not provide a detectable molecular ion under ESI conditions due to the instability of the activated carbonate functional group under ionizing conditions.

4.1.2.11. Cbz-Lys-N-ε-Boc-Lys(2,4-DNP)-PABA-PNP (9b). A mixture of **8b** (0.45 mmol, 360 mg), 4-nitrophenyl chloroformate (1.2 eq, 0.54 mmol, 109 mg) in 10 ml anhydrous THF was treated with anhydrous pyridine (1.2 eq, 0.54 mmol, 46 µl) under argon gas. After stirring for 3 h, the solvents were removed under vacuum followed by purification with flash chromatography (ethyl acetate:hexanes 1:1 to 2:1) gave **9b** (370 mg, 87%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 0.79–1.95 (m, 21H), 2.84 (s, 2H), 3.32–3.43 (m, 3H), 3.98 (d, J = 3.5, 1H), 4.41 (d, J = 3, 1H), 4.98 (s, 2H), 5.21 (s, 2H), 6.73–8.84 (m, 16H), 10.10 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 14.38, 22.51, 23.03, 23.34, 28.12, 28.68, 29.64, 31.04, 43.09, 53.44, 55.13, 60.18, 65.79, 70.65, 77.75, 115.69, 119.52, 123.01, 124.08, 125.81, 128.04, 128.15, 128.71, 129.80, 129.86, 129.93, 130.32, 135.00, 137.41, 139.72, 145.56, 148.50, 152.39, 155.72, 155.98, 156.47, 171.17, 172.62. This compound did not provide a detectable molecular ion under ESI conditions likely due to the instability of the carbonate functional group under ionizing conditions.

4.1.2.12. Cbz-Phe-Lys(2,4-DNP)-PABA-Dansylcadaverine (3). A mixture of **9a** (0.08 mmol, 70 mg), dansylcadaverine (1.2 eq, 0.1 mmol, 33 mg) and TEA (1.2 eq, 0.1 mmol, 14 µl) was dissolved in 5 ml of anhydrous THF followed by stirring for 2 h at room temperature. The solvents were removed under vacuum followed by purification with flash chromatography (ethyl acetate:hexanes 2:1 to 5:1) gave **3** (73 mg, 86%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.08–1.98 (m, 12H), 2.74–2.89 (m, 9H), 3.00 (d, J = 15, 1H), 3.36–3.45(m, 4H), 4.02(d, J = 3.5, 1H), 4.34–4.89 (m, 2H), 4.93 (t, J = 5, 4H), 7.11–8.89 (m, 23H), 10.12 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 14.44, 21.23, 22.55, 23.06, 23.63, 28.18, 29.20 (d, J = 7.5), 31.44, 42.75, 42.96, 45.51, 53.54, 56.57, 60.23, 65.32, 65.63, 115.53, 115.73, 119.48, 119.61, 124.02, 124.13, 126.70, 127.82, 127.95, 128.09, 128.21, 128.42, 128.70, 129.48, 129.66, 130.36, 132.56, 135.01, 136.57, 137.41, 138.50, 138.90, 148.54, 151.76, 156.47, 170.97, 172.14. ESI-Mass: calc. for $C_{54}H_{61}N_9O_{12}S$, $[M+H]^+$, 1060.4239, HRMS (ESI): found 1060.4244 $[M+H]^+$.

4.1.2.13. Cbz-Lys-N-ε-Boc-Lys(2,4-DNP)-PABA-Dansylcadaverine (10). A mixture of **9b** (0.32 mmol, 300 mg), dansylcadaverine (1.2 eq, 0.38 mmol, 129 mg) and TEA (1.2 eq, 0.38 mmol, 53 µl) was dissolved in 5 ml of anhydrous THF followed by stirring for 2 h at room temperature. The solvents were removed under vacuum followed by purification with flash chromatography (ethyl acetate:hexanes 1:2 to 3:1) gave **10** (270 mg, 74%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 1.07–1.73 (m, 27H), 2.73–2.85 (m, 13H), 3.34–3.44(m, 6H), 4.00 (s, 1H), 4.43 (s, 1H), 4.90 (s, 2H), 4.99 (d, J = 2.75, 2H), 6.75–8.85 (m, 20H), 10.05 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 14.87, 23.04, 23.35, 23.60, 28.12, 28.70, 29.14, 29.20, 29.65, 31.98, 42.73, 45.48, 52.40, 53.42, 55.15, 65.34, 65.79, 77.78, 115.51, 115.70, 119.40, 119.59, 123.99, 128.05, 128.18, 128.66, 128.73, 129.02, 129.47, 129.51, 129.74,

129.94, 130.34, 132.49, 135.00, 136.55, 137.42, 138.88, 148.51, 151.75, 155.99, 156.48, 171.03, 172.60. ESI-Mass: calc. for $C_{56}H_{72}N_{10}O_{14}S$, $[M+H]^+$, 1141.5, found 1140.4. $[M+Na]^+$, 1163.5, found 1162.5.

*4.1.2.14. Cbz-Lys-Lys(2,4-DNP)-PABA-dansylcadaverine (**4**).* Compound **10** (100 mg, 0.088 mmol) was dissolved in 0.4 ml of a 40% v/v solution of TFA/CH_2Cl_2 followed by stirring for 10 min at 0 °C. After the reaction, 10 ml of diethyl ether was added and the mixture was centrifuged at 1000 rpm for 10 min at 4 °C. The precipitate was dissolved in ethyl acetate/ethanol (3:1) and purified using modified silica gel [37] flash chromatography (ethyl acetate:ethanol = 3:1) to obtain probe **4** (80 mg, 87%) as a yellow solid. ^{1}H NMR (DMSO-d_6) δ 0.80–1.97 (m, 18H), 2.72–3.15 (m, 10*H*), 3.42–3.44 (m, 2H), 4.03 (s, 1H), 4.42 (s, 1H), 4.89 (s, 2H), 4.99 (s, 2H), 7.10–8.86 (m, 20H), 10.23 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 14.42, 22.48, 22.89, 23.06, 23.61, 26.90, 28.08, 29.16, 31.41, 31.60, 42.74, 43.11, 45.63, 53.59, 54.91, 55.39, 66.31, 66.80, 115.77, 119.41, 124.10, 124.26, 128.06, 128.15, 128.74, 129.48, 129.96, 130.35, 132.42, 134.48, 136.66, 137.40, 138.99, 148.51, 156.45, 171.13, 172.46. ESI-Mass: calc. for $C_{51}H_{64}N_{10}O_{12}S$, $[M+H]^+$, 1041.4504; HRMS (ESI): found 1041.4495 $[M+H]^+$.

4.1.3. Photochemistry

A solution containing 10 μM dansylcadaverine, probe **1**, **2**, **3** and **4** and buffer (50 mM, pH 5.0) was prepared and a 100 μl pipetted into 96 well plates (Nunc, 96-well plates with a flat Bottom). The absorbance spectra were obtained for each probe by scanning from 300 nm to 800 nm. The emission spectrum was obtained by excitation at 254 nm and scanning from 400 nm to 700 nm in black plates (Costar 96 wells assay plate, flat Bottom). All spectra were obtained in in Biotek Synergy 4 or Cytation 5 plate reader.

4.2. Biology

4.2.1. General cell culture procedures

DMEM high glucose culture media, 0.25% Trypsin-EDTA solution, Dulbecco's phosphate buffered saline, Trypan blue (0.4%) solution, and fetal bovine serum were purchased from Fisher Scientific. LysoTracker@ RED DND-99 was purchased from ThermoFisher Scientific. DU145 prostate cancer cell and MDA-MB-231 breast cancer cells were obtained from the American Type Culture Collection (Manassas, VA).

DU145 and MDA-MB-231 cells were maintained in DMEM media supplemented with 10% v/v fetal bovine serum (FBS). On reaching desired confluency (1×10^6 cells/ml in a 25 ml flask), 1×10^6 cells were seeded into 25 ml of fresh supplemented media in 6 well plates. The cells were incubated under humidified air containing 5% CO_2 at 37 °C. The cell density was kept between passages 5 and 15, and below 1×10^6 cells/ml to ensure exponential growth and to avoid differentiation. Cell viability was above 95% for all experiments and was based on trypan blue exclusion from the cells. The assay was performed in a haemocytometer using a light microscope.

4.2.2. Hydrolysis of probes 1, 2, 3 and 4 using recombinant Cathepsin B and Cathepsin L

Prewarmed solutions containing a 50 μl aliquot of 100 μM of each probe (**1**, **2**, **3** and **4**) in acetate buffer (100 mM sodium acetate, pH 5.0, 1 mM EDTA, 4 mM DTT and 20% DMSO) was diluted in 50 μl of prewarmed acetate buffer (50 mM, pH 5.0, 1 mM EDTA, 4 mM DTT). In a separate reaction, a 100 μl solution of CTB or CTL (100 nM) in acetate buffer (50 mM, pH 5.0, 1 mM EDTA, 4 mM DTT) was incubated for 10 min at 37 °C. To initiate the enzymatic reaction, the 100 μl solution containing the active enzymes were added to the solution containing the desired probe. The final concentrations of each probe were 25 μm, active enzyme was 50 nm and DMSO 10% v/v. The onset of fluorescence was monitored using a Biotek Synergy 4 or a Cytation 5 plate reader (λ_{ex} = 335 nm, λ_{em} = 512 nm). To produce the inactivated cathepsin enzymes, solutions of CA074 (40 μM) and E64 (40 μM) were prepared in acetate buffer and incubated with CTB or CTL at 37 °C for at least 10 min prior to initiating each enzymatic reaction.

4.2.3. Kinetics parameters of probe 4 for recombinant human Cathepsin B and Cathepsin L

To obtain the K_M, k_{cat} and k_{cat}/K_M parameters for probe **4** as a substrate of Cathepsin B and L, solutions of probe **4** were prepared at concentrations ranging from 10 to 200 μM in acetate buffer (100 mM sodium acetate, pH 5.0, 1 mM EDTA and 4 mM DTT, 20% DMSO). A 100 μl solution of human recombinant Cathepsin B or Cathepsin L (10 nM) in acetate buffer (100 mM sodium acetate, pH 5, 1 mM EDTA and 4 mM DTT) was incubated at 37 °C for at least 10 min in black 96 well plates. To initiate the enzymatic reaction, a 100 μl of the prewarmed probe **4** solution was added to the well containing enzyme and monitored spectrophotometrically for the release of free dansylcadaverine by fluorescence (λ_{ex} = 335 nm, λ_{em} = 512 nm). In each reaction well, the final enzyme concentration was 5 nM, the substrate concentrations ranged from 5 to 100 μM and the reaction mixture contained 10% v/v DMSO. To determine the initial velocity of each enzymatic reaction, the concentration of released dansylcadaverine was determined by a standard curve measured in the same acetate buffer (pH 5.0, 1 mM EDTA, 4 mM DTT and 10% DMSO).

4.2.4. Cell lysate assays

MDA-MB-231 breast and DU145 prostate cancer cells were grown to 80% confluency on 10 cm culture dishes, washed with cold PBS buffer 3 times, then collected by centrifugation (3000 rpm, 5 min) to obtain a cell pellet. The pellet was resuspended in 10 ml of cold acetate buffer (100 mM sodium acetate, pH 5.0, 1 mM EDTA) and the cell membranes disrupted using sonication (10 s on, 10 s pause, for 1 min). During sonication, the cell suspension was kept on an ice bath. After cell membrane disruption, the mixture was centrifuged (14000 × g, for 20 min at 4 °C) to provide lysates that were stored at −80 °C until use. To evaluate CTB activity, 100 μl of the cell lysate solution was added to a black 96-well plate and diluted with 50 μl of acetate buffer (100 mM acetate, pH 5.0, 1 mM EDTA and 4 mM DTT). For the experiments requiring inactivated enzymes in lysates, the acetate buffer was prepared as described above but with CA074 or E64 so that the final concentration of inhibitors was 10 μM. For complete inhibition, the cell lysate solution and acetate buffer containing the inhibitors were incubated at 37 °C for 10 min prior to addition of probe. To initiate the enzymatic reaction, a 50 μl aliquot of probe **4** (100 μM stock) in acetate buffer (100 mM acetate, pH 5.0, 1 mM EDTA and 4 mM DTT, 40% DMSO) was added. In each reaction, the final concentration of probe **4** was 25 μm and 10% DMSO. The enzymatic reaction was monitored spectrophotometrically for the release of free dansylcadaverine by fluorescence (λ_{ex} = 335 nm, λ_{em} = 512 nm) in the plate reader at 37 °C.

4.2.5. Confocal microscopy using DU145 and MDA-MB-231 cancer cells lines and probe 4

A vial containing 1 ml of 100,000 cells/ml of DU145 or MDA-MB-231 was seeded onto glass coverslips in twelve-well plates. The cells were cultured in DMEM with 10% FBS and incubated at 37 °C in a 5% CO_2 atmosphere overnight. Cells were washed with 1 ml of PBS buffer two times, then a 30 μM solution of probe **4** in FBS free culture medium was added to each well. As a positive control, 30 μM of dansylcadaverine in FBS-free culture medium was added. For the inhibition experiments, cells were treated with CA074Me or E64d (30 μM) with probe **4** simultaneously. In each experiment, probe **4** was incubated with cells at 37 °C for 4 h, then LysoTracker RED DND-99 (100 nM final concentration) was added followed by continued incubation for an additional 30 min. The cover slips were removed from the wells,

washed with PBS buffer, and fixed with 0.5 ml of 4% formaldehyde solution for 10 min. The cover slips were washed with 1 ml of PBS, then slides were prepared using ProLong® Gold Antifade Mounting Media.

Declaration of Competing Interest

None.

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Appendix A. Supplementary material

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