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Prodrug-Inspired Probes Selective to Cathepsin B Over Other Cysteine Cathepsins

Morshed A. Chowdhury,[Ψ] *Ignace A. Moya,*[Ψ] *Shardul Bhilocha,*[ǂ] *Cody C. McMillan,*[ǂ] *Brady G. Vigliarolo,*[ǂ] *Ingeborg Zehbe,*[Ψ, ǂ, ◊] *and Christopher P. Phenix*[Ψ, ǂ, ◊, *]

[Ψ]Thunder Bay Regional Research Institute, 2321- 290 Munro Street, Thunder Bay, Ontario, Canada, P7A 7T1.

[ǂ]Department of Biology and Chemistry, Lakehead University, 955 Oliver Road, Thunder Bay, Ontario, Canada, P7B 5E1

[◊]Northern Ontario School of Medicine, Medical Sciences Division, 955 Oliver Road, Thunder Bay, Ontario, Canada, P7B 5E1

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ABSTRACT: Cathepsin B (CTB) is a cysteine protease believed to be an important therapeutic target or biomarker for several diseases including aggressive cancer, arthritis and parasitic infections. The development of probes capable of assessing CTB activity in cell lysates, living cells and animal models of disease are needed to understand its role in disease progression. However, discovering probes selective to Cathepsin B over other cysteine cathepsins is a significant challenge

due to overlap of preferred substrates and binding site homology in this family of proteases. Herein, we report the synthesis and detailed evaluation of two prodrug inspired fluorogenic peptides designed to be efficient and selective substrate-based probes for CTB. Through cell lysate and cell assays, a promising lead candidate was identified that is efficiently processed and has high specificity for CTB over other cysteine cathepsins. This work represents a key step towards the design of rapid release prodrugs or substrate-based molecular imaging probes specific to CTB.

INTRODUCTION

The papain family of cysteine proteases includes 11 human enzymes (cathepsins B, C, F, H, K, L, O, S, V, W and X) responsible for lysosomal protein degradation in many biological and pathological processes.[1] Cysteine cathepsins are synthesized as inactive "proenzymes" whose expression is controlled by transcriptional and translational mechanisms. The regulation of enzyme activity is accomplished through a variety of posttranslational processes, the most important being the protease activation of the proenzyme in the acidic environment of the endosomes/lysosomes and the expression of high affinity endogenous inhibitors from the cystatin family.[2] Significant increases in functional enzyme can result from alterations in posttranslational mechanisms[3,4] highlighting the need for techniques that reveal enzyme activity when evaluating cysteine proteases in disease promoting processes.

Cathepsin B (CTB, EC 3.4.22.1) is a promising diagnostic and prognostic biomarker of various cancers[5-9] and is a member of a tumor promoting proteolytic network.[10-12] CTB appears to affect cancer progression based upon its localization with aggressive cancers having high CTB activity at the invading edge of a tumor and in the extracellular matrix secreted by a variety of cell types.[13] In contrast, intracellular CTB appears to inhibit cancer by enhancing apoptosis.[14] Recent reports suggest CTB, together with the related enzyme cathepsin L (CTL, EC 3.4.22.15), may protect

against anticancer chemotherapies such as Taxol, meaning CTB and/or CTL activity may be a useful marker predictive of response to chemotherapy.[15] Sensitive and specific tools capable of assessing CTB activity in cell lysates, cell culture and animal models of aggressive cancer are needed to validate this enzyme as a high priority cancer marker or therapeutic target.

To provide information beyond protease expression and to instead directly evaluate CTB activity in complex mixtures, cells, and animals, two strategies have recently emerged. The first was developed by Bogyo and coworkers[16-18] who designed elegant activity-based probes that efficiently label CTB and CTL with a fluorescent tag. These optical probes are irreversible inhibitors that label active enzyme and are well-suited for detecting and localizing CTB and CTL activity *in vitro* and *in vivo*. Although specificity towards CTB over CTL was never achieved in the optical agents, derivatives of these compounds were radiolabeled for positron emission tomography (PET) studies to evaluate cysteine protease activity in animal models of cancer.[19] While modest tumour uptake was observed in the PET images, a high percentage of the injected dose was found in the liver and eliminated through hepatobiliary clearance. Indeed, some of the most well-established and promising PET probes for imaging enzyme activity in cancer are small molecule substrates that accumulate in the tumor and are rapidly cleared through the renal system.

The second strategy seeks to improve cellular and *in vivo* sensitivity by exploiting the catalytic power of CTB since a single active protease can amplify the fluorescent signal by continuous turnover of many substrate-based probes. To evaluate this approach, a synthetic graft copolymer consisting of poly-(*S*)-lysine modified with a protease releasable Cy5.5 near-infrared dye was assessed in cancer cells and tumor xenograft mice.[20] Although the polymer sensitively revealed protease activity *in vivo*, low specificity towards CTB was observed since serine proteases also activated this probe. Kim and co-workers[21] extended this strategy by developing a nano-sized

polymer decorated with peptides conjugated to a near-infrared fluorescent dye and a quencher molecule that is released upon CTB hydrolysis. Although strong fluorescence was observed in cancer cells and tumors that correlated with CTB expression, a polymer approach is largely dependent upon the enhanced permeability and retention (EPR) effect for efficient tumour delivery. As a consequence, polymer uptake and imaging performance can vary because the EPR effect is heavily influenced by the conditions of tumor growth in xenografts and the specific cancer cell lines used.[22] Although substrate-based polymers may offer higher sensitivity over the activity-based inhibitors, small molecule probes are easier to prepare, purify and usually have pharmacodynamic properties superior for clinically established molecular imaging techniques like PET.

Despite the potential benefits that small molecule substrates have over the activity- and polymer-based probes for molecular imaging, designing compounds specific to CTB over other lysosomal cysteine cathepsins is an enormous challenge since the S1, S1' and S2' recognition sites of these enzymes are almost identical.[23] Therefore, it is advantageous to thoroughly assess new compounds for CTB efficiency and specificity over the other cysteine cathepsins early on in the discovery process and prior to *in vivo* studies. A standard protocol used to evaluate the specificity of substrate-based fluorogenic probes involves the incubation of each probe candidate with a series of enzymes having similar substrate preference or related family members. A graph of fluorescence activation vs time is plotted to compare the relative rates of probe turnover by each protease as a qualitative measure of specificity. Unfortunately, these experiments can be misleading especially when saturating levels of probe are used which do not reflect intracellular or *in vivo* conditions where probe concentrations are expected to be much lower. In contrast, the Michaelis-Menton parameters K_M (defined as the concentration of substrate at which the initial velocity is one-half the maximal velocity) and k_{cat} (defined as the catalytic constant for the conversion of substrate to product)

provide detailed insight into the recognition and catalytic efficiency of a substrate or probe candidate towards the target enzyme. The ratio of these two parameters, the "specificity constant" (k_{cat}/K_M), in general, reflects enzymatic efficiency at low substrate concentrations by combining both affinity to the enzyme (K_M) and catalytic turnover (k_{cat}).[24] Values of k_{cat}/K_M may better predict probe performance inside living cells where the concentration of substrate is likely below the K_M value, meaning enzyme reaction rates are a second order process proportional to k_{cat}/K_M values. In addition, k_{cat}/K_M is useful to compare probe turnover by related proteases and to identify lead compounds as viable candidates for *in vivo* imaging using benchmark kinetic values established by PET tracers that image enzyme activity in humans.[25, 26]

To determine if CTB is a legitimate biomarker and/or therapeutic target of aggressive cancer, small molecule PET probes that are efficient and specific substrates of CTB would be a valuable tool for imaging studies on animal models of cancer. However, small molecule substrates that are cell permeable and selective to CTB have not been reported to date. In this study, we have synthesized and evaluated two-component (substrate-fluorophore) and prodrug inspired three-component (substrate-linker-fluorophore) fluorogenic peptides that release 7-amino-4-methylcoumarin (AMC) upon protease hydrolysis. Using an *in vitro* screening platform that includes kinetic studies, lysate and cell assays, we have identified one candidate that is simple to prepare, biocompatible, cell permeable and may be useful in the design of rapid release prodrugs or substrate-based PET molecular imaging probes specific to CTB.

RESULTS AND DISCUSSION

Probe Design. As a key step in identifying efficiently-processed CTB substrates that could be later adapted into probes useful for sensitive fluorescence microscopy or PET molecular imaging, we sought small-molecule lead compounds which are easily synthesized, fluorogenic, and specific

to CTB over other cysteine cathepsins. To develop an experimental platform for identifying lead candidates that meet the above requirements, we adapted the chemistry developed for activatable "profluorophore" probes based on a three-component, prodrug-inspired strategy. Imaging probes of this design consist of: a peptide portion that provides high affinity and specificity to CTB, a self-destructive linker that spontaneously releases a reporter upon its enzymatic removal of the peptide and a latent fluorophore which becomes highly fluorescent once enzymatically freed from the intact probe (Figure 1). The prodrug linker *p*-aminobenzyl alcohol (PABA), originally reported by Katzenellenbogan,[27] was chosen because it can be conveniently coupled to peptides through its amino group, allowing stable conjugation of alcohol and aniline-based fluorophores and drugs.[28-32] As the fluorescent reporter molecule, we chose AMC which is often used in the synthesis of fluorogenic peptides to evaluate the substrate specificities of cysteine cathepsins. In addition, AMC would be useful to evaluate probe specificity in cell lysate and preliminary fluorescence microscope assays.

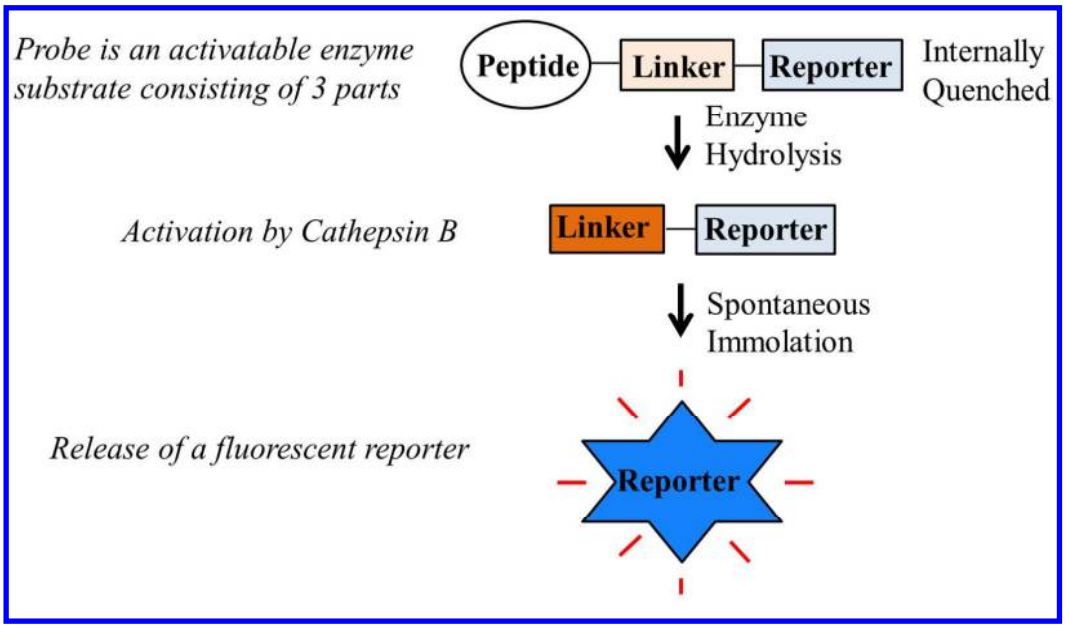


Figure 1: A prodrug inspired strategy for sensing CTB activity. To identify substrates that are efficient and selective substrates of CTB, we employed AMC as a latent fluorophore that becomes fluorescent once released from the intact probe.

It is well established that most human cysteine cathepsins prefer a hydrophobic amino acid at P2 and a positively charged substituent at the P1 positions.[33] Therefore, most two-component benzyloxycarbonyl (Cbz) protected substrates like Cbz-Phe-Arg-AMC cannot be used to analyze the activity of a specific cysteine cathepsin in samples having mixtures of these enzymes such as cell lysates or living cells. Although Cbz-Arg-Arg-AMC is the substrate of choice for assaying CTB activity in cell lysates under acidic and reducing conditions, it is not commonly used in cell assays presumably due to poor cell membrane permeability. The recognition of doubly cationic substrates such as Cbz-Arg-Arg-AMC by CTB has been attributed to a glutamic acid residue at the top of the S2 binding pocket.[34] Other related enzymes lack an equivalent glutamic acid residue and prefer hydrophobic amino acids at the P2 position of substrates, explaining the selectivity of Cbz-Arg-Arg-AMC for CTB. We decided to synthesize Cbz-Phe-Lys-AMC (**1**)[35] and Cbz-Lys-Lys-AMC (**2**) to evaluate how substitution of Lysine for Arginine would affect recognition and turnover by CTB and CTL and to serve as control substrates for the PABA-modified compounds described below. Using Lys instead of Arg is synthetically attractive given the convenience of installing and removing the *tert*-butoxycarbonyl (BOC) protecting group from the ε-amine of (*S*)-lysine.

Previous work has demonstrated that PABA-containing peptides modified into prodrugs are serum stable and efficiently processed by CTB to release a cytotoxic drug.[29,30] This suggests that PABA and a bulky moiety like a drug or fluorophore are well tolerated at the P1' and P2' positions respectively, and that a three-component prodrug inspired probe may be efficiently processed by CTB. In addition, three-component peptides may offer higher specificity towards CTB because the

probe extends into the core S3 - S2' regions (Figure 2a).[23] To evaluate the efficiency of CTB to activate the prodrug-inspired probes bearing the PABA spacer, we synthesized Cbz-Phe-Lys-PABA-AMC (**3)** and Cbz-Lys-Lys-PABA-AMC **(4)** (Figure 2b).

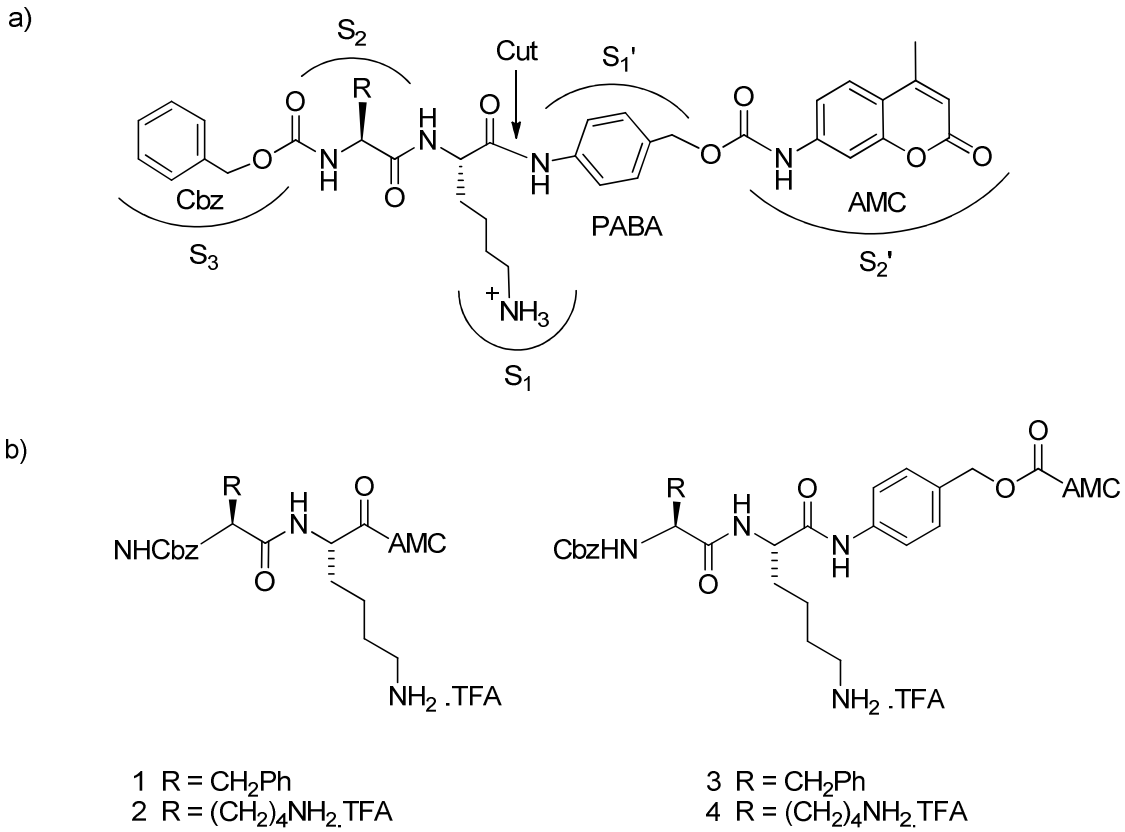
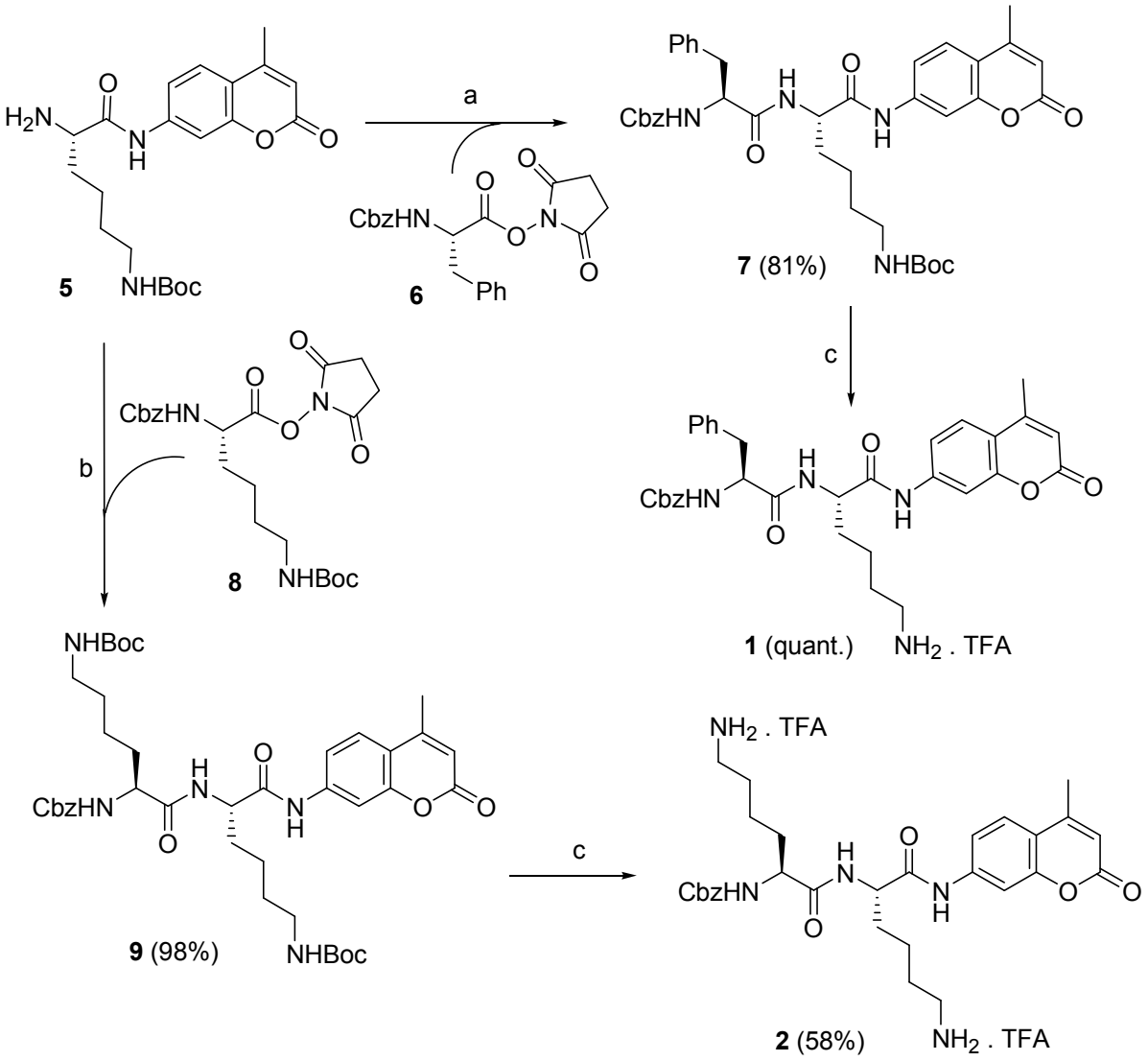


Figure 2. (a) The recognition of the three component peptides by cysteine cathepsins. Cysteine cathepsins bind to their substrates in an active site cleft where the well-defined S2, S1 and S1'sites primarily determine substrate preference. Additional interactions between the substrate and the S3 and S2' sites can influence recognition and must be considered when designing artificial substrates. The three component probes have phenylalanine or lysine occupying the S2 site and lysine at the S1 site while Cbz, PABA and AMC extend into the S3, S1' and S2' binding regions respectively. (b) The fluorogenic peptides synthesized over the course of this work.

Chemistry. The synthesis of the two component fluorogenic peptides was conducted using routine solution peptide chemistry as shown in Scheme 1. The synthesis of Cbz-Phe-Lys-AMC (**1**) began by the coupling H-Lys-*N*-ε-BOC-AMC (**5**)[36] with Cbz-Phe-O-sucinnimide (**6**) using sodium bicarbonate in aqueous THF[37] to produce Cbz-Phe-Lys-*N*-ε-BOC-AMC (**7**). Subsequent deprotection of the BOC group from **7** proceeded smoothly in 50% TFA/CH_2Cl_2 to yield **1**. Using a similar strategy, gram-scale amounts of Cbz-Lys-Lys-AMC (**2**) were readily prepared by coupling **5** with Cbz-Lys-*N*-ε-BOC-O-succinimide (**8**) in DMF in presence of triethyl amine to give Cbz-Lys-*N*-ε-BOC-Lys-*N*-ε-BOC-AMC (**9**) followed by BOC deprotection in a 50% TFA/CH_2Cl_2 (Scheme 1).

Scheme 1. Synthesis of the two component fluorogenic peptides[a]

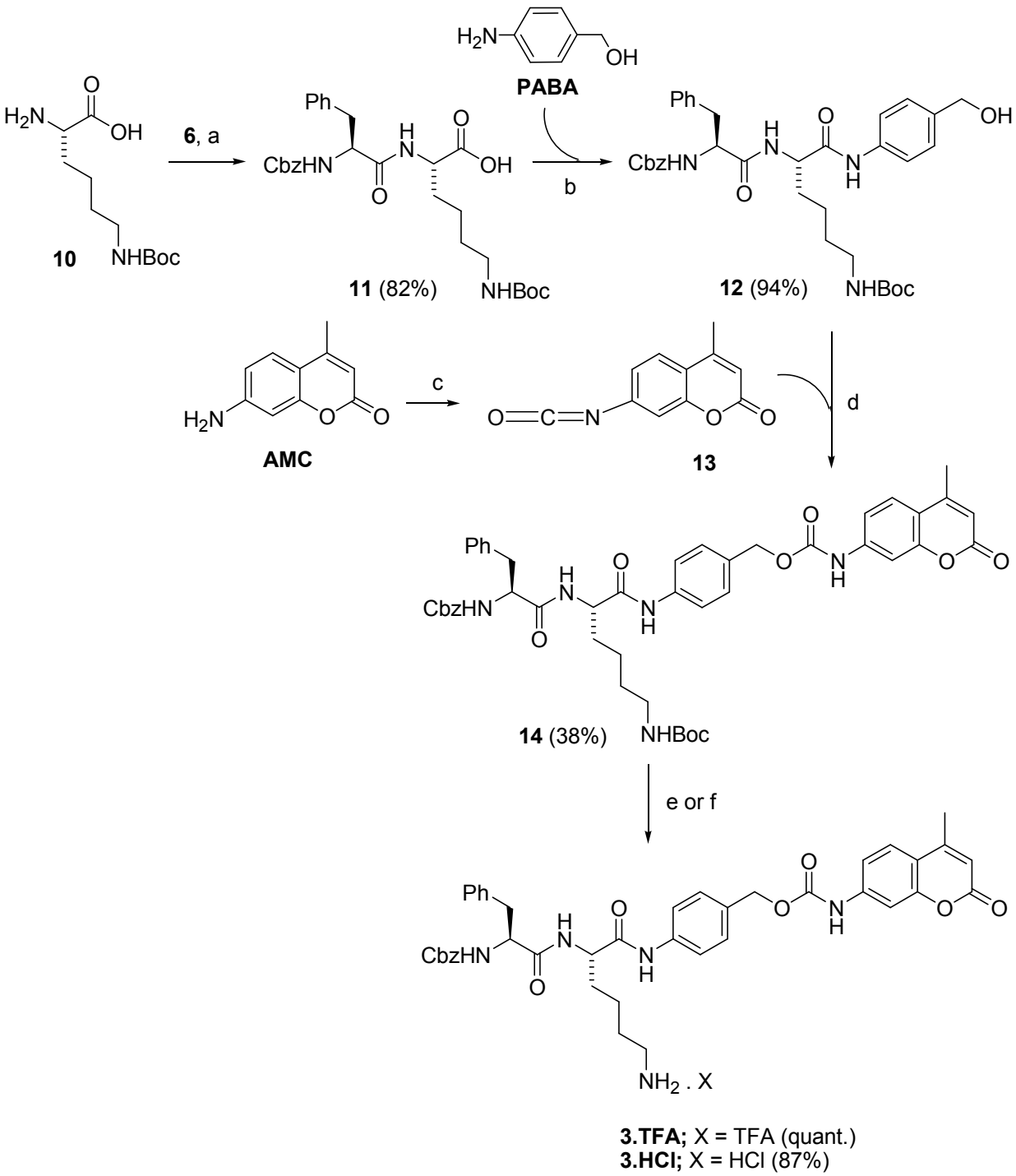


[a]Reagents and conditions: (a) $NaHCO_3$, THF-H_2O, rt, 16 h; (b) Et_3N, DMF, 0 °C to rt, 16 h; (c) TFA-CH_2Cl_2 (1:1, v/v), ice-bath, 15 min.

As shown in Scheme 2, the synthesis of the three component fluorogenic peptide Cbz-Phe-Lys-PABC-AMC (**3)** started with the coupling of H-Lys-*N*-ε-BOC-OH (**10)** with **6** to provide the dipeptide Cbz-Phe-Lys-*N*-ε-BOC-OH (**11**). Conjugation of the PABA linker with **11** was carried out in presence of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ)[38] to give Cbz-Phe-Lys-*N*-ε-BOC-PAB-OH (**12**). Efforts to prepare the AMC carbamate Cbz-Phe-Lys-*N*-ε-BOC-PABC-AMC (**14**) failed when treating peptide **12** with *p*-nitrophenyl chloroformate,[37] 1,1'-

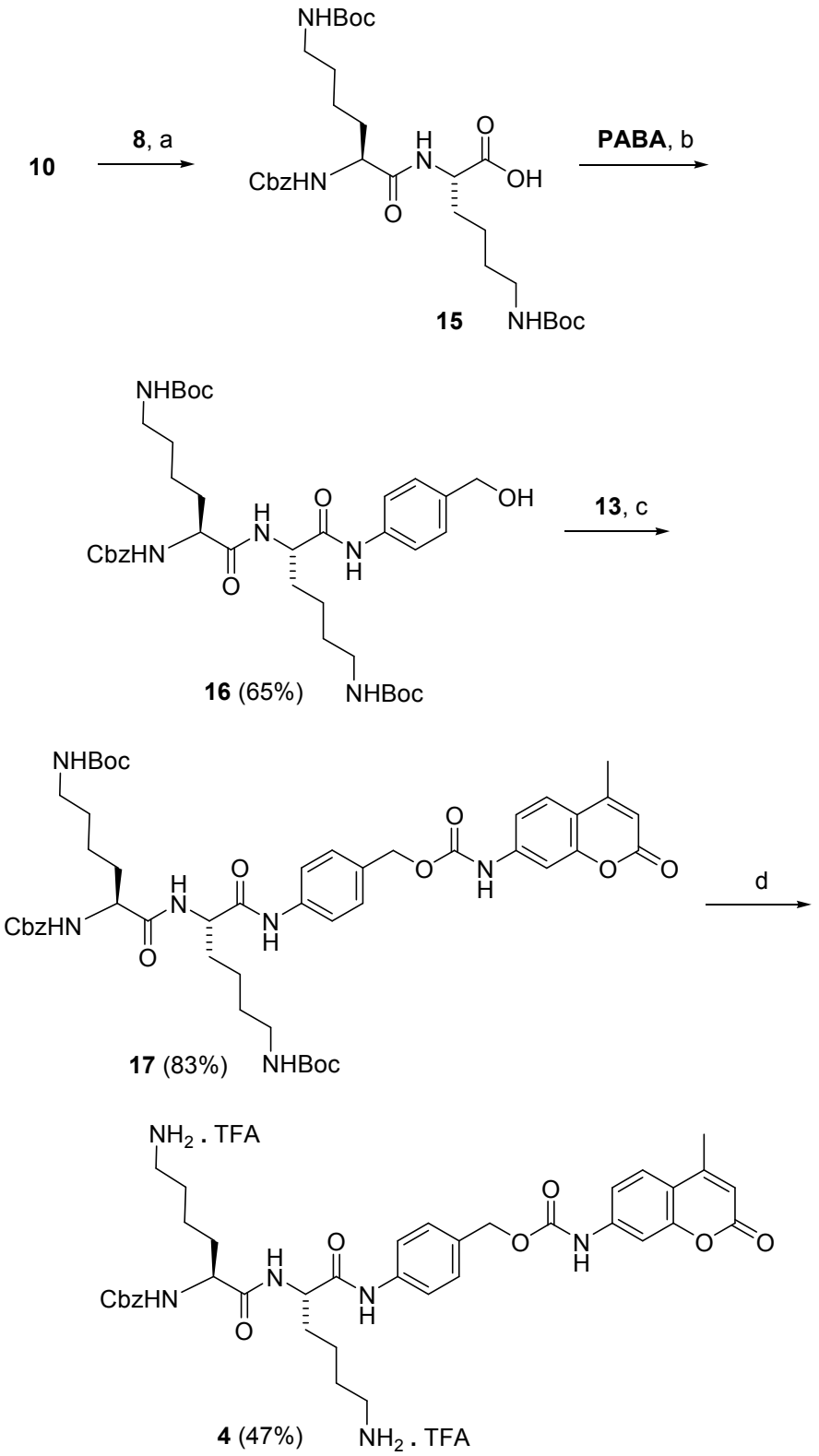
carbonyldiimidazole,[39] or phosgene, likely due to the low nucleophilicity of the nitrogen of AMC. Efficient incorporation of AMC to yield carbamate **14** was ultimately accomplished following the reaction of **12** with 7-isocyanato-4-methylchromen-2-one (**13**) which was generated by refluxing of AMC with phosgene. Removal of the BOC group from **14** with TFA/CH_2Cl_2 furnished the TFA salt of probe Cbz-Phe-Lys-PABC-AMC (**3**). To improve the aqueous solubility of probe **3**, deprotection of BOC from **14** with methanolic-HCl gave the HCl salt as shown in Scheme 2. Similarly, Cbz-Lys-*N*-ε-BOC-Lys-*N*-ε-BOC-PAB-OH (**16**) was prepared by conjugating the peptide Cbz-Lys-*N*-ε-BOC-Lys-*N*-ε-BOC-OH (**15**) with PABA following the same procedure as used for the preparation of **12**. Coupling of **16** with isocyanate **13** afforded the carbamate Cbz-Lys-*N*-ε-BOC-Lys-*N*-ε-BOC-PABC-AMC (17). The BOC deprotection from carbamate **17** with TFA/CH_2Cl_2 furnished the TFA salt of Cbz-Lys-Lys-PABC-AMC (**4**) (Scheme 3).

Scheme 2. Synthesis of the three component fluorogenic peptides **3**[a]



[a]Reagents and conditions: (a) $NaHCO_3$, THF-H_2O, rt, 16 h; (b) THF, EEDQ, rt, 16 h; (c) 15% phosgene in toluene, 120°C, 16 h; (d) THF, 80 °C, 2 h; (e) TFA-CH_2Cl_2 (1:1, v/v), ice-bath, 15 min; (f) Methanolic-HCl (0.5M), rt, 16 h.

Scheme 3. Synthesis of the three component fluorogenic peptide **4**[a]


10
8, a
NHBoc
CbzHN
15
NHBoc
PABA, b
16 (65%)
13, c
17 (83%)
d
NH2 . TFA
4 (47%)

[a]Reagents and conditions: (a) Et_3N, DMF, 0 °C to rt, 16 h; (b) THF, EEDQ, rt, 16 h; (c) THF, 80 °C, 2 h; (d) TFA-CH_2Cl_2 (1:1, v/v), ice-bath, 15 min.

Kinetic Evaluation of Probe Candidates. Benchmark kinetic values of K_M, k_{cat} and k_{cat}/K_M were established for the two-component fluorogenic peptides using recombinant CTB, CTL and cathepsin S (CTS). CTS, a lysosomal cysteine cathepsin also linked to cancer,[13] was chosen to evaluate probe specificity since the human enzyme is commercially available and has similar substrate preferences as CTB and CTL. As shown in Table 1, Cbz-Phe-Arg-AMC, Cbz-Arg-Arg-AMC, **1** and **2** were excellent substrates of CTB as judged by their k_{cat}/K_M values. In contrast, CTL and CTS efficiently hydrolyzed both Cbz-Phe-Arg-AMC and probe **1** but had little activity towards Cbz-Arg-Arg-AMC and **2**. These results indicate that Cbz-Lys-Lys, similar to Cbz-Arg-Arg, can offer efficient recognition and maintain improved CTB specificity over Cbz-Phe-Lys in selective substrates.

Prior to determining the kinetic parameters for the three component fluorogenic peptides, it was necessary to demonstrate that **3** and **4** are chemically stable towards spontaneous hydrolysis and AMC release under the assay conditions. As shown in the Figure 3, HPLC chromatograms demonstrate that probes **3** and **4** are chemically stable in assay buffer for at least two hours, indicating that the rates of spontaneous hydrolysis and AMC release are not significant and will not impact the initial velocity measurements of the enzyme catalyzed reactions. In addition, the chemical purity of the probes is high as only buffer components appear at early retention times in each chromatogram.

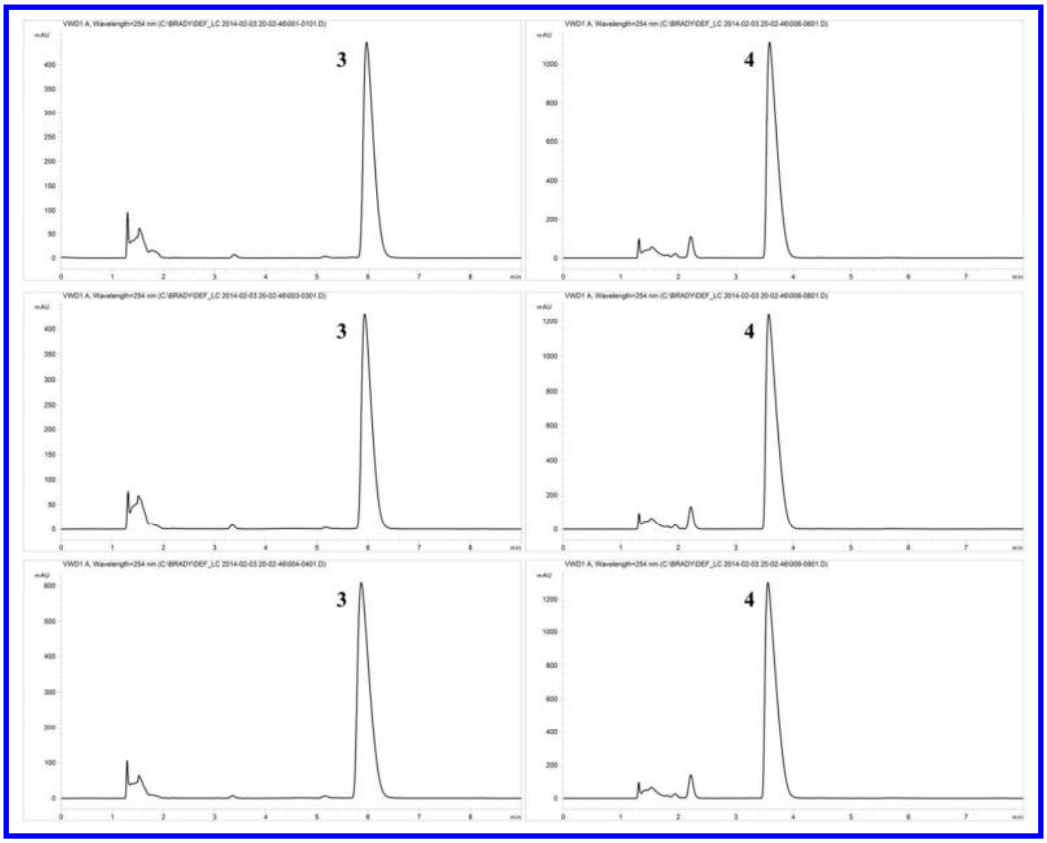


Figure 3: HPLC chromatograms demonstrating the chemical stability of the three component peptides 3 and 4. Each probe (120 μM) was dissolved in enzyme assay buffer and incubated at 37 ºC for the indicated length of time. An aliquot was taken and 90 μL injected into an HPLC and separated on a C18 reverse phase column. Over the course of two hours, the total peak area of probes 3 and 4 remained constant and no AMC was observed at the expected retention times (2.3 minutes and 2.9 minutes respectively). Note that the early peaks in all chromatograms are from buffer components and that the mobile phase used to separate 3 or 4 from AMC were different as described in the materials and methods sections.

Once we were confident that probes **3** and **4** were chemically stable for enzyme kinetic analysis, we determined how the PABA spacer affects recognition and turnover by CTB, CTL and CTS. The introduction of PABA into probes **3** and **4** resulted in a lower apparent K_M value for CTB when

compared to the corresponding two-component peptides. This suggests that introduction of the PABA linker results in higher affinity towards CTB and may improve probe performance *in cellulo*. The lower K_M value determined for **3** was partially offset by a lower k_{cat}, but an impressive specificity constant was maintained with k_{cat}/K_M = 61 mM^{-1} s^{-1} for CTB. Encouragingly, rapid turnover of **4** was observed (k_{cat}/K_M = 231 $mM^{-1}s^{-1}$) resulting in the highest specificity constant of the novel CTB substrates tested. For comparison purposes, ^{18}F-FHBG, with a k_{cat}/k_M = 43 $mM^{-1}s^{-1}$, is a substrate that has been successfully used to image herpes simplex virus thymidine kinase activity in humans. Surprisingly, CTL turnover of **3** suffered dramatically as no reliable kinetic data was obtained even when the concentration of enzyme was increased 30 fold higher than used is assays to evaluate compound **1**. As expected, CTS efficiently hydrolyzed **3** while neither CTS nor CTL efficiently processed probe **4**.

Table 1: The kinetic parameters obtained for each fluorogenic peptide as a substrate of CTB, CTL and CTS.[a]

	Cathepsin B			Cathepsin L			Cathepsin S		
Probe	K_M **(mM)**	k_{cat} **(s^{-1})**	k_{cat}/K_M **(mM^{-1} s^{-1})**	K_M **(mM)**	k_{cat} **(s^{-1})**	k_{cat}/K_M **(mM^{-1} s^{-1})**	K_M **(mM)**	k_{cat} **(s^{-1})**	k_{cat}/K_M **(mM^{-1} s^{-1})**
Cbz-Arg-Arg-AMC	0.34 ± 0.06	56 ± 3	165 ± 37	0.024 ± 0.005	0.22 ± 0.01	9.2 ± 2.3	n.d.	n.d.	n.d.
Cbz-Phe-Arg-AMC	0.09 ± 0.01	36 ± 2	400 ± 70	0.52 ± 0.2 x 10^{-3}	0.38 ± 0.01	730 ± 280	0.072 ± 0.01	1.3 ± 0.1	18 ± 2
(1)	0.19 ± 0.03	26 ± 2	137 ± 13	0.59 ± 0.12 x 10^{-3}	0.39 ± 0.01	661 ± 149	0.088 ± 0.02	1.1 ± 0.1	13 ± 3
(2)	0.25 ± 0.01	37 ± 1	148 ± 5	0.092 ± 0.001	0.14 ± 0.06	1.5 ± 0.6	n.d.	n.d.	n.d.

(3)	0.082 ± 0.003	0.50 ± 0.04	61 ± 7	n.d.	n.d.	n.d.	0.056 ± 0.02	0.39 ± 0.03	69 ± 25
(4)	0.16 ± 0.04	37 ± 2	231 ± 70	0.043 ± 0.01	0.11 ± 0.01	2.5 ± 0.8	n.d.	n.d.	n.d.

[a]All values were determined by an average of three trials ± standard deviation. Where indicated by n.d. no reliable kinetic data could be obtained using concentration of enzyme as high as 10 nM.

Cell Lysate Assays. Cell lysates were prepared from HeLa cervical cancer cells[40] and incubated individually with 100 μM of Cbz-Arg-Arg-AMC, **3**, or **4** under acidic and reducing conditions favoring lysosomal cysteine cathepsin activity. As shown in Figure 4, probe **4** was the most efficient substrate consistent with the highest k_{cat}/K_M value. In contrast, the relative hydrolysis of probe **3** was unexpectedly high with overall turnover similar to Cbz-Arg-Arg-AMC. The higher relative activity measured for probe **3** is likely due to the hydrolysis of this substrate by CTB together with the additive contributions from other proteases present in the cell lysates.

To evaluate the cysteine cathepsin specificity of Cbz-Arg-Arg-AMC, **3**, and **4**, cell lysates were prepared from HeLa cells treated overnight with the well-established CTB inhibitor CA-074Me and the broad spectrum cysteine cathepsin inhibitor E64d. Although CA-074Me is routinely used to efficiently inactivate CTB in living cells, it has been shown that CTL activity is also inhibited.[41] Nonetheless, assays using cell lysates prepared from CA-074Me-inhibited cells in combination with the *in vitro* kinetic data using purified enzymes is a concise way to assess probe specificity. As shown in Figure 4, CTB hydrolysis of Cbz-Arg-Arg-AMC or **4** was not observed in lysates obtained from cells treated with CA-074Me or E64d. This demonstrates that both inhibitors effectively inactivate CTB and that both Cbz-Arg-Arg-AMC and **4** are highly specific to CTB over the other cysteine cathepsins. In contrast, cell lysates prepared from cells treated with CA-074Me maintained about 35% of the relative activity towards probe **3** when compared to the lysates prepared from

HeLa cells without inhibitor treatment. This clearly demonstrates that probe **3** is hydrolyzed by CTB and one or more other enzymes. Lysates from HeLa cells treated with E64d were unable to efficiently hydrolyze **3** demonstrating that the remaining 35% relative activity observed in lysates prepared from the CA-074Me treated cells results from other cysteine cathepsin activity.

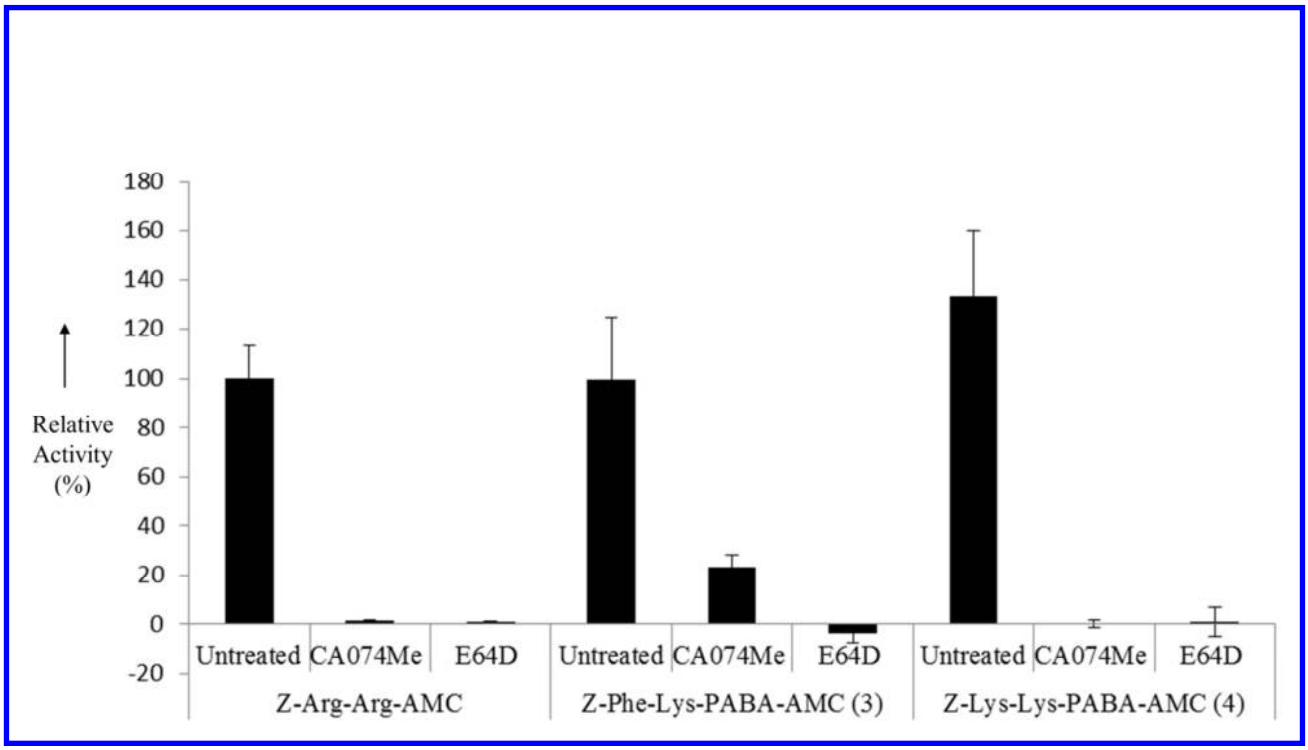


Figure 4: Cell Lysate assays to evaluate the specificity of 3 and 4 towards CTB. HeLa cell lysates incubated with Cbz-Arg-Arg-AMC, **3** and **4** using lysates prepared from untreated cells and cells treated with CA-074Me or E64d. The bars represent the relative activity (%) compared to Cbz-Arg-Arg-AMC incubated with untreated lysates adjusted to total protein concentration. For each experiment, background RFU due to spontaneous hydrolysis in wells containing no enzyme was subtracted from the total fluorescence and error bars depict standard deviation from 4 independent trials.

Cancer Cells Assays. Next, the effectiveness of the two-component fluorogenic peptides to detect intracellular protease activity over short incubation times was evaluated. In separate wells,

live HeLa cells were incubated with Cbz-Arg-Arg-AMC, **1**, or **2**. Following incubation, the cells were washed twice with phosphate buffered saline (PBS) to remove unreacted probe in the media or probe absorbed onto the surface of the cancer cells. The cells were then placed in a fluorescence microscope and images taken. As shown in Figure 5, none of the compounds tested resulted in the detectable accumulation of fluorescence inside of living cells over the two hour incubation time.

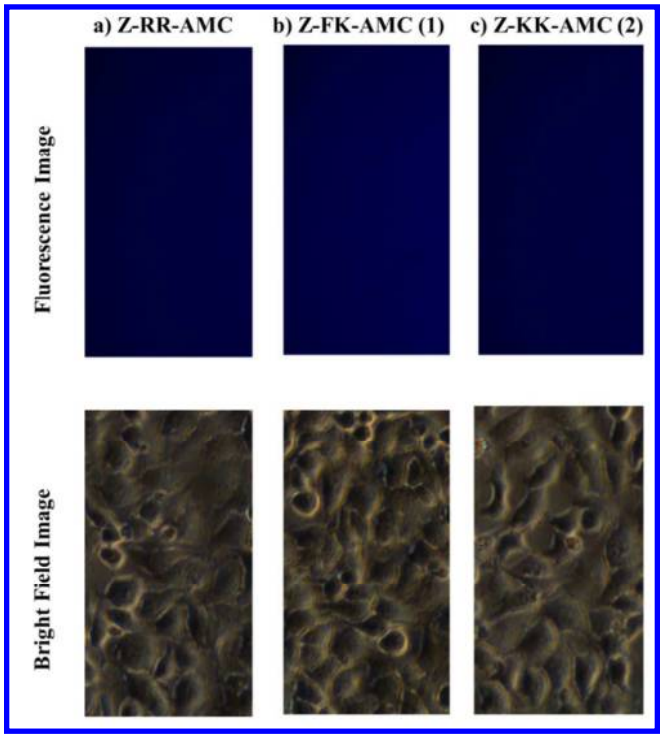


Figure 5: Fluorescence Microscope Images of HeLa cells incubated with the two-component peptides. Fluorescent and bright field microscope images taken of HeLa cells treated with 40 μM of a) Cbz-Arg-Arg-AMC b) two-component peptide (**1**) c) two-component peptide (**2**). Images were recorded using a fluorescence microscope after treatment of cells for two hours with the appropriate compound followed by two washes with PBS buffer to remove unreacted substrate in the media and absorbed onto the cell surface.

To evaluate the utility of the three component probe candidates for visualizing intracellular protease activity, each peptide was incubated at 40 μM in the presence of living HeLa cells

followed by washing twice with PBS buffer to remove unreacted probe and probe absorbed onto the cell surface. As shown in Figure 6 a) and b), strong fluorescence intensity was clearly visualized inside of HeLa cells. Interestingly, cells incubated with probe **3** underwent rapid morphological changes reminiscent of cell death while those treated with **4** appeared to tolerate this compound well**.** To evaluate the performance of each three component probe in another cancer type, a Her2-positive breast cancer cell line derived from MD-MBA-231 called H2N[42] was incubated with **3** or **4**. Intracellular fluorescence was observed in this cell line consistent with images taken of the HeLa cells (see Figures **6c** and **6d**).

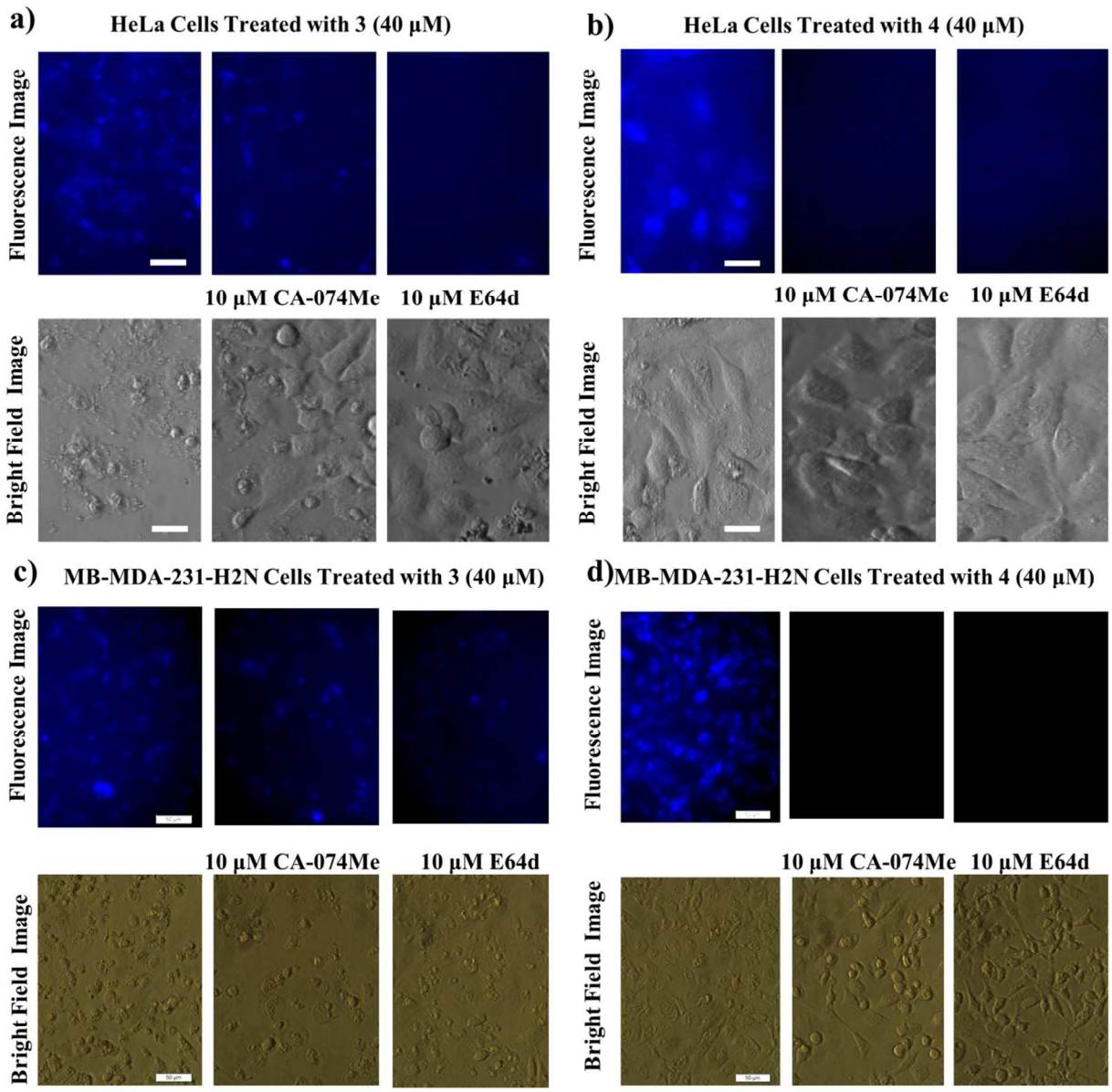


Figure 6: Bright Field and Fluorescence Microscope Images of HeLa cervical and MB-MDA-231-H2N breast cancer cells lines treated with probes **3** and **4**. Fluorescent microscope images taken of HeLa cervical cancer cells treated for 2 hours with a) 40 µM of (**3**) and b) 40 µM of (**4**) and MB-MDA-231-H2N breast cancer cells treated c) 40 µM of (**3**) and d) 40 µM of (**4**). Control experiments to evaluate probe specificity were conducted by overnight treatment with CA-074Me (10 µM CTB inhibitor) or E64d (10 µM broad spectrum cysteine cathepsin inhibitor) followed by

incubation with each probe for 2 hours, washed twice with PBS then imaged. White bars represent 50 μm.

To evaluate the specificity of probe candidates towards CTB in live cells, CA-074Me was used to treat both cervical and breast cancer cell lines. As shown in Figure 6, HeLa and H2N cells treated with CA-074Me or E64d were unable to efficiently activate probe **4,** suggesting high CTB specificity of this probe in live cancer cells. In contrast, cells treated first with CA-074Me followed by probe **3** showed residual intracellular fluorescence, indicating that **3** is hydrolyzed inside the CTB-inhibited cells consistent with the kinetic and lysate data that **3** is an efficient substrate of one or more other enzymes. Indeed, the broad spectrum cysteine cathepsin inhibitor E64d prevented the onset of fluorescence in HeLa and H2N cells, supporting the hypothesis that turnover of **3** results from another cysteine protease such as CTS.[43] Nonetheless, these experiments demonstrate that designing future CTB imaging probes based on Cbz-Phe-Lys-PABA may lack the desired specificity for selectively sensing CTB activity in living cells.

Cell Viability and Proliferation Assays.

To evaluate changes in cell viability of the HeLa and H2N cell lines induced by probes **3** and **4**, we used the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay. MTT is a yellow tetrazolium salt that is reduced to an insoluble purple formazan dye by reductase enzymes in viable cells. Therefore, cell death through necrosis and/or apoptosis induced by the probe candidates can be quantified using this convenient spectrophotometric assay. As shown in Figure 7, a significant decrease in cell viability was found in HeLa and H2N cells treated with probe **3** at 10 and 40 μM. Interestingly, the H2N cells had no decrease in viability at 1 μM of probe **3** while HeLa cell viability was reduced to approximately 50% compared to the untreated cells. This MTT assay confirms the high toxicity of probe **3** in these cells lines, as observed in the fluorescence microscopy

experiments, preventing its use for live cell applications. In contrast, HeLa and H2N cells treated with Cbz-Arg-Arg-AMC or probe **4,** no statistically significant differences in cell viability were observed at 1, 10 μM and 40 μM concentrations. This data confirms that the fluorescence microscopy images in Figure 6 were performed on a viable population of HeLa and H2N cells with no significant increases in necrosis or apoptosis induced by **4** throughout the two hour incubation times.

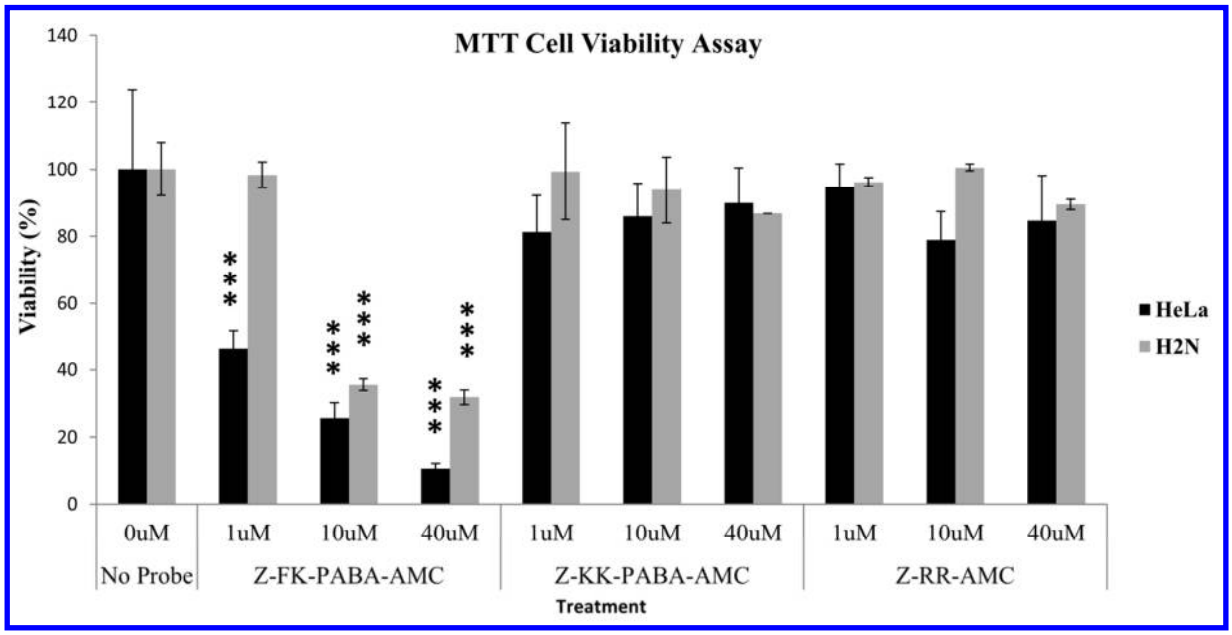


Figure 7: The effects of Cbz-Arg-Arg-AMC, 3 and 4 on cell viability. HeLa and H2N cells were treated with probes **3**, **4** or Cbz-Arg-Arg-AMC at 1, 10 and 40 μM. Cell viability was quantified using MTT and expressed as a % of the viability of untreated cells. Statistical differences in viability were determined by performing ANOVA Posthoc dunnett's test; $p<0.05$ was considered significant and indicated by ***.

CONCLUSION

The purpose of this study was to determine if PABA derivatized peptides were potentially useful in the development of molecular imaging probes specific to Cathepsin B. Kinetic studies using purified enzyme revealed that the incorporation of PABA into the fluorogenic peptides produced highly efficient three-component substrates of CTB. Surprisingly, CTL did not tolerate the presence of the PABA linker in probe **3**, a compound expected to be an excellent substrate of this enzyme. Although compound **3** is an efficient substrate of CTB, low specificity and high toxicity prevented its use for live cell fluorescence microscopy studies. Probe **4** is an easily synthesized, highly efficient substrate capable of selectively assessing CTB activity in cell lysates under conditions favoring cysteine cathepsin activity. Fluorescence microscopy cell assays indicate that **4** is cell permeable and can efficiently reveal protease activity in viable cervical and breast cancer cell lines. Consistent with the cell lysate assays, the onset of fluorescence in cells treated with **4** can be blocked using the known CTB inactivator CA-074Me. To our knowledge, **4** is the first reported example of a small molecule fluorogenic substrate selective towards CTB over other lysosomal cysteine cathepsins having utility for detecting CTB activity inside live cells. Current efforts in our laboratory seek to synthesize probes using Cbz-Lys-Lys-PABA as a CTB recognition peptide conjugated to a radioactive reporter or fluorophores excited at longer wavelengths for detailed cancer cell assays and *in vivo* molecular imaging.

EXPERIMENTAL SECTION

General Procedures. Proton (^{1}H) and carbon (^{13}C) NMR spectra were acquired at the Lakehead University Instrumentation Laboratory (LUIL) on a Varian Unity Inova 500 MHz spectrometer in DMSO-d_6 with TMS as the internal standard, where *J* (coupling constant) values are estimated in hertz (Hz). Microanalyses were measured for C, H, N using Elementar Vario EL and were within ± 0.4% of theoretical values indicating that all compounds were >95% pure. Thin layer

chromatography (TLC) and silica gel column chromatography were performed using TLC Silica Gel 60 F_{254} (EMD) and SiliaFlash®P60 (SiliCycle), respectively. All other reagents and solvents were used as purchased without further purification. Human Cathepsin B was purchased from Sino Biological Inc. (China), human Cathepsin L from Calbiochem and human Cathepsin S from Novoprotein with kinetic studies performed in a Biotek Synergy 4 plate reader. HPLC was performed on a Agilent 1200 Infinity LC with compounds separated on a Agilent Eclipse XDB-C18 column (4.6 mm, 150 mm, 5 μm) and detected using 1200 series variable wavelength detector (G1314B). All cell culture reagents were purchased from Fisher Scientific. The stock solutions of all peptides were made with DMSO but did not exceed 1% v/v in the media used for the cells assays. Fluorescence was visualized and digitally captured using an inverted fluorescent microscope (Zeiss Axiovert 200 or the Olympus TL4, filter set with excitation maxima 365 nm and emission wavelength > 420 nm) and digital camera attachment (QImaging QICAM Q21310). Compounds **5**,[36] **6/8**,[44] **10**,[45] **11**[46] and **15**[47] were prepared according to literature procedures.

Cbz-Phe-Lys-*N*-ε-BOC-AMC (7): To a solution of **5** (212 mg, 0.52 mmol) in THF – H_20 (3.5:1.5 v/v, 5 mL) and $NaHCO_3$ (44 mg, 0.52 mmol) was added **6** (206 mg, 0.52 mmol) in THF (3.5 mL) and stirred at room temperature for 16 h. The reaction mixture was then concentrated *in vacuo* and extracted with ethyl acetate (15 mL x 3). The combined organic phase was washed with brine (25 mL) and dried (Na_2SO_4). After filtration, the solvent was removed from filtrate and the crude residue was purified by silica gel column chromatography using dichloromethane – methanol (9:1 v/v) as eluent to obtain the pure title compound **7**. White solid, 288 mg (81%); ^{1}H NMR (DMSO-d_6) δ 1.28-1.45 (m, 4H), 1.35 (s, 9H), 1.60-1.70 (m, 1H), 1.70-1.81 (m, 1H), 2.41 (s, 3H), 2.75 (dd, J = 10.9, 13.7 Hz, 1H), 2.83-2.95 (m, 2H), 3.05 (dd, J = 3.6, 15.3 Hz, 1H), 4.31-4.39 (m, 1H), 4.39-4.46 (m, 1H), 4.96 (s, 2H), 6.28 (d, J = 1.3 Hz, 1H), 6.79 (t, J = 5.6 Hz, 1H), 7.16-7.36

(m, 10H), 7.47-7.54 (m, 2H), 7.74 (d, *J* = 8.8 Hz, 1H), 7.79 (d, *J* = 1.9 Hz, 1H), 8.33 (d, *J* = 7.6 Hz, 1H), 10.52 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 18.5, 23.3, 28.7, 29.8, 32.2, 37.9, 54.2, 56.5, 65.7, 77.8, 106.2, 112.8, 115.6, 115.7, 126.4, 126.7, 127.3, 127.9, 128.2, 128.5, 128.8, 129.7, 137.5, 138.5, 142.7, 153.5, 154.1, 156.0, 156.4, 160.5, 171.9, 172.3. Elemental analysis calculated (%) for $C_{38}H_{44}N_4O_8$: C, 66.65; H, 6.48; N, 8.18. Found: C, 66.40; H, 6.74; N, 8.10.

Cbz-Phe-Lys-AMC . TFA (1): To a solution of trifluoroacetic acid – DCM (1:1 v/v, 2 mL) at ice-bath temperature was added **7** (200 mg, 0.29 mmol) and stirred for 15 min. After reaction diethyl ether was added to the reaction mixture to precipitate out the solids. Then the solids were centrifuged out and successively washed with diethyl ether (5 mL x 2) and ethyl acetate (5 mL x 2), and dried under high vacuum in the dark to get the pure title compound **1**. White solid, 204 mg (quant); ^{1}H NMR (DMSO-d_6) δ 1.23-1.80 (m, 6H), 2.33 (s, 3H), 2.61-2.78 (m, 3H), 2.94-3.04 (m, 1H), 4.23-4.32 (m, 1H), 4.32-4.43 (m, 1H), 4.89 (s, 2H), 6.21 (d, *J* = 1.2 Hz, 1H), 7.08-7.31 (m, 10H), 7.45 (dd, *J* = 2.1, 8.6 Hz, 1H), 7.48 (d, *J* = 8.6 Hz, 1H), 7.66 (d, *J* = 8.6 Hz, 1H), 7.70-7.86 (m, 4H), 8.38 (d, *J* = 7.6 Hz, 1H), 10.54 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 18.5, 22.9, 27.2, 31.8, 37.8, 45.0, 54.1, 56.5, 65.7, 106.2, 112.8, 115.6, 115.8, 117.7 (*J* = 300 Hz), 126.4, 126.7, 127.9, 128.2, 128.5, 128.8, 129.7, 137.5, 138.5, 142.6, 153.6, 154.1, 156.4, 158.9 (*J* = 31 Hz), 160.5, 171.8, 172.3. Elemental analysis calculated (%) for $C_{35}H_{37}F_3N_4O_8$: C, 60.17; H, 5.34; N, 8.02. Found: C, 59.82; H, 5.52; N, 7.95.

Cbz-Lys-*N*-ε-BOC-Lys-*N*-ε-BOC-AMC (9): To a solution of **8** (592 mg, 1.24 mmol) in dry DMF (5 mL) **5** (500 mg, 1.24 mmol) and triethylamine (173 µL, 1.24 mmol) were added at 0 °C followed by stirring for 30 min. The solution was allowed to slowly reach room temperature and the reaction stirred for another 16 h. The reaction mixture was then evaporated *in vacuo* and the residue was dissolved in ethyl acetate (20 mL) and successively washed with 1 M citric acid (10 mL

x 2), brine (15 mL) and dried (Na_2SO_4). After filtration, the solvent was removed from filtrate and the crude residue was purified by silica gel column chromatography using ethyl acetate – hexanes (4:1 v/v) as an eluent to obtain the pure title compound **9**. White solid, 935 mg (98%); ^{1}H NMR (DMSO-d_6) δ 1.29-1.44 (m, 6H), 1.37 (s, 18H), 1.45-1.79 (m, 6H), 2.40 (s, 3H), 2.79-2.95 (m, 4H), 3.96-4.08 (m, 1H), 4.33-4.42 (m, 1H), 5.04 (s, 2H), 5.53- 5.61 (m, 1H), 6.28 (s, 1H), 6.77 (t, *J* = 5.0 Hz, 2H), 7.26-7.45 (m, 5H), 7.49 (dd, *J* = 2.0, 8.5 Hz, 1H), 7.72 (d, *J* = 8.5 Hz, 1H), 7.78 (d, *J* = 2.0 Hz, 1H), 8.14 (d, *J* = 7.6 Hz, 1H), 10.45 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 18.4, 23.2, 23.3, 24.9, 25.8, 28.7, 28.7, 29.7, 29.7, 32.1, 33.8, 54.0, 55.1, 65.9, 77.8, 77.8, 106.1, 112.8, 115.5, 115.7, 126.4, 128.2, 128.2, 128.8, 137.5, 142.6, 153.5, 154.1, 156.0, 156.5, 157.1, 160.5, 172.0, 172.7. Elemental analysis calculated (%) for $C_{40}H_{55}N_5O_{10}$: C, 62.73; H, 7.24; N, 9.14. Found: C, 63.12; H, 7.10; N, 8.92.

Cbz-Lys-Lys-AMC.2TFA (2): To a solution of trifluoroacetic acid – DCM (1:1 v/v, 1 mL) at ice-bath temperature was added **9** (100 mg, 0.13 mmol) and stirred for 15 min. After reaction, diethyl ether was added to the mixture to precipitate out the solids. Then the suspension was centrifuged and successively washed with diethyl ether (5 mL x 2) and ethyl acetate (5 mL x 2), and dried under high vacuum in the dark to get the pure title compound **2**. White solid, 60 mg (58%). ^{1}H NMR (DMSO-d_6) δ 1.28-1.83 (m, 12H), 2.41 (s, 3H), 2.69-2.84 (m, 4H), 3.98-4.11 (m, 1H), 4.34-4.47 (m, 1H), 5.04 (s, 2H), 6.28 (d, *J* = 1.2 Hz, 1H), 7.27-7.56 (m, 7H), 7.67-7.94 (m, 8H), 8.23-8.34 (m, 1H), 10.59 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 18.5, 22.9, 22.9, 27.1, 27.2, 31.7, 31.7, 39.1, 45.0, 53.9, 54.8, 65.9, 106.2, 114.1, 115.6, 115.7, 117.7 (*J* = 300 Hz), 126.5, 128.2, 128.3, 128.8, 137.5, 142.6, 153.6, 154.1, 156.5, 158.7 (*J* = 31 Hz), 160.5, 171.9, 172.7. Elemental analysis calculated (%) for $C_{34}H_{41}F_6N_5O_{10}.H_2O$: C, 50.31; H, 5.34; N, 8.63. Found: C, 50.58; H, 5.35; N, 8.79

Cbz-Phe-Lys-*N*-ε-BOC-PAB-OH (12): A solution of **11** (0.83 g, 1.57 mmol), PABA (232 mg, 1.89 mmol) in THF (15 mL) was prepared followed addition of EEDQ (467 mg, 1.89 mmol). After stirring for 16 hours at room temperature, the solvent was removed under reduced pressure and the resulting residue taken into diethyl ether (25 mL) to form a suspension. The solid was filtered out and washed with diethyl ether (25 mL x 2) and dried under vacuum to get the pure title compound **12** as a pale yellow solid, 0.93 g (94%). ^{1}H NMR (DMSO-d_6) δ 1.20-1.50 (m, 4H), 1.36 (s, 9H), 1.50-1.60 (m, 1H), 1.60-1.80 (m, 1H), 2.74 (dd, *J* = 11, 13.5 Hz, 1H), 2.80-2.95 (m, 2H), 3.03 (dd, *J* = 5.5, 13.5 Hz, 1H), 4.30-4.37 (m, 1H), 4.37-4.50 (m, 1H), 4.44 (d, *J* = 18 Hz, 2H), 4.96 (s, 2H), 5.12 (t, *J* = 5.5 Hz, 1H), 6.79 (t, *J* = 5.5 Hz, 1H), 7.15-7.35 (m, 12H), 7.51 (d, *J* = 8.5 Hz, 1H), 7.56 (d, *J* = 8.0 Hz, 2H), 8.21 (d, *J* = 8.0 Hz, 1H), 10.01 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 23.2, 28.7, 29.7, 32.5, 37.9, 40.5, 53.9, 56.5, 63.1, 65.7, 77.8, 119.5, 126.7, 127.4, 127.9, 128.1, 128.5, 128.8, 128.7, 137.5, 137.9, 138.0, 138.5, 156.0, 156.3, 170.8, 172.0. Elemental analysis calculated (%) for $C_{35}H_{44}N_4O_7$: C, 66.44; H, 7.01; N, 8.85. Found: C, 66.32; H, 7.23; N, 8.84.

Cbz-Phe-Lys-*N*-ε-BOC-PABC-AMC (14): AMC (0.25 g, 1.43 mmol) was taken in a 25 mL flask and a solution of 15% phosgene in toluene 10 mL was added. The reaction mixture was refluxed at 120 °C for 16 h under argon. After reaction, the mixture was cooled to room temperature and argon was bubbled into the solution for 10 minutes to remove unreacted phosgene gas. Then the solvents were removed under reduced pressure to dryness yielding 7-isocyanato-4-methylchromen-2-one (**13**) as a white powdered solid (*ca*. 0.29 g) which was used immediately without further purification. A solution of isocyanate **13** (*ca*. 0.29 g, 1.43 mmol) and **12** (0.6 g, 0.95 mmol) in dry THF (5 mL) was stirred at 80 °C under argon for 2 h. After reaction, solvent was removed under reduced pressure to give the crude product **14** which contained unreacted isocyanate **13** and alcohol **12**. To remove the isocyanate **13**, the crude product was dissolved in dichloromethane (25 mL) and

washed successively with 10% aqueous HCl solution (15 mL x 3), water (15 mL), saturated aqueous $NaHCO_3$ solution (15 mL), water (15 mL) and brine (15 mL), and dried ($MgSO_4$). After filtration of $MgSO_4$, the solvents were removed from the filtrate and the solid was taken in ethyl acetate (20 mL) which dissolved unreacted alcohol derivative **12** leaving solid product that was filtered out from the slurry. Repeating the procedure two more times with fresh ethyl acetate afforded analytically pure title compound **14** as a white solid, 0.3 g (38%). The combined filtrates were evaporated under reduced pressure to recover the alcohol derivative **12** as a pale yellow solid, 0.25 g (42%). Compound **14**: ^{1}H NMR (DMSO-d_6) δ 1.20-1.50 (m, 4H), 1.35 (s, 9H), 1.60-1.70 (m, 1H), 1.70-1.80 (m, 1H), 2.51 (s, 3H), 2.74 (dd, *J* = 9.5, 13.5 Hz, 1H), 2.90 (d, *J* = 6 Hz, 2H), 3.05 (d, *J* = 13.5 Hz, 1H), 4.34 (d, *J* = 6.5 Hz, 1H), 4.41 (d, *J* = 6.5 Hz, 1H), 4.96 (s, 2H), 5.14 (s, 2H), 6.24 (s, 1H), 6.78 (s, 1H), 7.15-7.35 (m, 10H), 7.40 (d, *J* = 8.5 Hz, 2H), 7.51 (d, *J* = 8.5 Hz, 1H), 7.56 (s, 1H), 7.64 (d, *J* = 8.5 Hz, 3H), 7.69 (d, *J* = 8.5 Hz, 1H), 8.23 (d, *J* = 7.5 Hz, 1H), 10.14 (s, 1H), 10.27 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 18.4, 23.2, 28.7, 29.8, 32.4, 37.9, 45.0, 54.0, 54.0, 56.5, 65.7, 66.5, 77.8, 104.9, 104.9, 112.4, 114.7, 114.8, 119.6, 126.5, 126.7, 127.9, 128.1, 128.5, 128.7, 129.6, 129.7, 131.4, 137.5, 138.5, 139.4, 143.2, 153.6, 153.7, 154.3, 156.0, 156.3, 156.3, 160.5, 171.1, 172.0. MS $(M + Na)^+$ 856. Elemental analysis calculated (%) for $C_{46}H_{51}N_5O_{10}.1/2H_2O$: C, 65.54; H, 6.22; N, 8.30. Found: C, 65.19; H, 6.27; N, 8.14

Cbz-Phe-Lys-PABC-AMC.TFA (3.TFA): To a solution of trifluoroacetic acid – DCM (1:1 v/v, 2 mL) at ice-bath temperature was added **14** (200 mg, 0.24 mmol) and stirred for 15 min. After reaction, diethyl ether was added to the reaction mixture forming a precipitate. The solids were centrifuged out of the suspension and successively washed with diethyl ether (5 mL x 2) and ethyl acetate (5 mL x 2), and dried under high vacuum in the dark to get the pure title compound **3.TFA**. as a white solid, 204 mg (quant). ^{1}H NMR (DMSO-d_6) δ 1.30-1.84 (m, 6H), 2.39 (s, 3H), 2.73-2.83

(m, 3H), 3.05 (d, *J* = 9.9 Hz, 1H), 4.30-4.37 (m, 1H), 4.40-4.47 (m, 1H), 4.95 (s, 2H), 5.15 (s, 2H), 6.25 (s, 1H), 7.17-7.33 (m, 10H), 7.41 (d, *J* = 8.5 Hz, 3H), 7.53 (d, *J* = 8.9 Hz, 1H), 7.57 (bs, 1H), 7.66 (d, *J* = 8.9 Hz, 2H), 7.70 (d, *J* = 8.0 Hz, 1H), 7.78 (bs, 3H), 8.34 (d, *J* = 8.0 Hz, 1H), 10.21 (s, 1H), 10.29 (s, 1H). ^{13}C NMR (DMSO-d_6) 18.0, 22.4, 26.8, 31.6, 37.4, 38.7, 53.4, 56.1, 65.3, 66.0, 104.5, 111.9, 114.3, 114.4, 119.2, 126.0, 126.3, 127.5, 127.7, 128.1, 128.3, 129.2, 129.3, 131.1, 137.0, 138.1, 138.9, 142.8, 153.2, 153.3, 153.9, 155.9, 160.1, 170.6, 171.8. Elemental analysis calculated (%) for $C_{43}H_{44}F_3N_5O_{10}.1/2H_2O$: C, 60.27; H, 5.29; N, 8.17. Found: C, 60.08; H, 5.20; N, 7.97

Cbz-Phe-Lys-PABC-AMC . HCl (3.HCl): A solution of **14** (100 mg, 0.12 mmol) in methanolic-HCl (0.5M, 5 mL) was stirred at room temperature for 16 h. After reaction, solvents were removed *in vacuo* to dryness and the residue was taken in diethyl ether and stirred for 10 min. The suspension was centrifuged and the supernatant discarded, and the process was repeated twice to get the pure title compound **3.HCl**. White solid, 80 mg (87%). ^{1}H NMR (DMSO-d_6) δ 1.31-1.52 (m, 2H), 1.52-1.86 (m, 4H), 2.38 (s, 3H), 2.65-2.84 (m, 3H), 2.99-3.12 (m, 1H), 4.28-4.40 (m, 1H), 4.40-4.51 (m, 1H), 4.95 (s, 2H), 5.14 (s, 2H), 6.23 (s, 1H), 7.16-7.36 (m, 10H), 7.37-7.46 (m, 3H), 7.51-7.60 (m, 2H), 7.66-7.73 (m, 3H), 8.03 (bs, 3H), 8.43 (d, *J* = 8.3 Hz, 1H), 10.31 (s, 1H), 10.37 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 18.5, 22.8, 27.0, 31.9, 37.8, 38.8, 53.9, 56.6, 65.7, 66.5, 105.0, 112.4, 114.7, 114.8, 119.6, 126.5, 126.7, 127.9, 128.1, 128.5, 128.8, 129.6, 129.7, 131.4, 138.6, 139.5, 143.2, 153.7, 154.3, 156.4, 160.5, 171.1, 172.2. Elemental analysis calculated (%) for $C_{41}H_{44}ClN_5O_8.2H_2O$: C, 61.07; H, 6.00; N, 8.69. Found: C, 60.85; H, 5.79; N, 8.69

Cbz-Lys-*N*-ε-BOC-Lys-*N*-ε-BOC-PAB-OH (16): To a solution of **15** (3.71 g, 6.10 mmol) and PABA (0.9 g, 7.32 mmol) in THF (50 mL) was added EEDQ (1.81 g, 7.32 mmol) and stirred at room temperature under argon for 16 h. After reaction, solvent was removed under reduced pressure

and the residue was taken into diethyl ether (50 mL) to form a suspension. The solid was filtered out and washed with diethyl ether (50 mL x 2) and dried under vacuum to get the pure title compound **16** as a white solid, 2.83 g (65%). ^{1}H NMR (DMSO-d_6) δ 1.14-1.75 (m, 12H), 1.36 (s, 9H), 1.37 (s, 9H), 2.88 (bs, 4H), 3.95-4.05 (m, 1H), 4.31-4.40 (m, 1H), 4.44 (d, *J* = 5.6 Hz, 2H), 5.04 (s, 2H), 5.12 (t, *J* = 5.6 Hz, 1H), 6.77 (t, *J* = 5.6 Hz, 2H), 7.24 (d, *J* = 8.0 Hz, 2H), 7.28-7.40 (m, 4H), 7.43 (d, *J* = 8.0 Hz, 1H), 7.55 (d, *J* = 8.0 Hz, 2H), 8.05 (d, *J* = 8.0 Hz, 1H), 9.96 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 23.2, 23.3, 28.7, 29.7, 29.7, 32.1, 32.4, 53.8, 55.2, 63.1, 65.9, 77.8, 119.4, 127.3, 128.2, 128.2, 128.8, 137.5, 137.9, 138.0, 156.0, 156.0, 156.5, 170.9, 172.5. Elemental analysis calculated (%) for $C_{37}H_{55}N_5O_9.1/2H_2O$: C, 61.48; H, 7.81; N, 9.69. Found: C, 61.50; H, 7.84; N, 9.79

Cbz-Lys-*N*-ε-BOC-Lys-*N*-ε-BOC-PABC-AMC (17): A solution of isocyanate **13** (*ca*. 2.29 g, 11.43 mmol) and **16** (0.9 g, 1.26 mmol) in dry THF (80 mL) was stirred at 80 °C under argon for 2 h. After reaction, solvent was removed under reduced pressure and the residue was successively suspended in diethyl ether (100 mL), stirred for 10 min and filtered, and then repeated with ethyl acetate (50 mL x 2), stirred 10 min and filtered to get the pure title compound **17** as a pale yellow solid, 0.96 g (83%). ^{1}H NMR (DMSO-d_6) δ 1.21-1.45 (m, 8H), 1.34 (s, 9H), 1.37 (s, 9H), 1.45-1.77 (m, 4H), 2.39 (s, 3H), 2.81-2.94 (m, 4H), 3.98-4.04 (m, 1H), 4.34-4.41 (m, 1H), 5.01 (s, 2H), 5.16 (s, 2H), 6.23 (d, *J* = 1.2 Hz, 1H), 6.70-6.83 (m, 2H), 7.28-7.44 (m, 9H), 7.56 (d, *J* = 1.9 Hz, 1H), 7.63 (d, *J* = 8.4 Hz, 2H), 7.69 (d, *J* = 8.8 Hz, 1H), 8.04 (d, *J* = 7.8 Hz, 1H), 10.07 (s, 1H), 10.27 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 14.5, 18.5, 21.2, 23.2, 23.3, 28.7, 28.7, 29.7, 29.7, 32.1, 32.3, 53.8, 55.2, 60.2, 65.9, 66.5, 77.8, 104.9, 112.4, 114.7, 114.8, 119.6, 126.4, 128.2, 128.2, 128.8, 129.6, 131.4, 137.5, 139.4, 143.2, 153.6, 153.7, 154.3, 156.0, 156.0, 156.5, 160.5, 171.2, 172.6. Elemental

analysis calculated (%) for $C_{48}H_{62}N_6O_{12}.1/2H_2O$: C, 62.39; H, 6.87; N, 9.09. Found: C, 62.57; H, 6.96; N, 8.91

Cbz-Lys-Lys-PABC-AMC.2TFA (4): To a solution of trifluoroacetic acid – DCM (1:1 v/v, 2 mL) at ice-bath temperature was added **17** (200 mg, 0.22 mmol) and stirred for 15 min. After reaction, diethyl ether was added to the reaction mixture to precipitate out the solids followed by centrifugation. The supernatant was discarded and the solids obtained were dissolved in water (2 mL) washed with ethyl acetate (5 mL x 2), and the aqueous phase was freeze dried in dark to get the pure title compound **4** as a white solid, 48 mg (47%). ^{1}H NMR (DMSO-d_6) δ 1.23-1.80 (m, 12H), 2.40 (s, 3H), 2.70-2.82 (m, 4H), 4.00-4.08 (m, 1H), 4.36-4.44 (m, 1H), 5.03 (s, 2H), 5.15 (s, 2H), 6.25 (d, *J* = 1.2 Hz, 1H), 7.28-7.48 (m, 9H), 7.57 (d, *J* = 2 Hz, 1H), 7.64 (d, *J* = 8.5 Hz, 2H), 7.67-7.81 (m, 7H), 8.17 (d, *J* = 7.9 Hz, 1H), 10.15 (s, 1H), 10.28 (s, 1H). ^{13}C NMR (DMSO-d_6) δ 18.5, 22.9, 22.9, 27.1, 27.2, 31.7, 32.0, 39.1, 45.0, 53.7, 54.9, 65.9, 66.4, 104.9, 112.4, 114.7, 114.8, 117.7 (*J* = 300 Hz), 119.6, 126.5, 128.2, 128.3, 128.8, 129.6, 131.5, 137.5, 139.3, 143.2, 153.7, 153.7, 154.3, 156.5, 158.7 (*J* = 31 Hz), 160.5, 171.1, 172.5. Elemental analysis calculated (%) for $C_{42}H_{48}F_6N_6O_{12}.1/2H_2O$: C, 53.00; H, 5.19; N, 8.83. Found: C, 52.62; H, 5.34; N, 8.84

HPLC Assays to Evaluate Probe Stability. Compounds **3** and **4** (120 μM) were dissolved in assay buffer (30 mM acetate-NaOH, pH 5.5, 3.0 mM EDTA, 2.0 mM DTT, 10% DMSO) and incubated at 37°C. At 30 minutes, 1 hour and 2 hours, an aliquot was removed and 90 μL injected into an HPLC fitted with a C18 column (Agilent Eclipse XDB). The compounds were separated using a flow rate of 1 mL/min and detected using UV absorbance at 254 nm. Probe **3** had a retention time of 5.9 minutes when eluted with mobile phase A [55% H_2O (0.1% TFA) and 45% ACN (0.1% TFA)] while probe **4** had a retention time of 3.6 minutes when eluted with mobile

phase B [65% H_2O (0.1% TFA) and 35% ACN (0.1% TFA)]. Pure AMC was used as a standard having a retention time of 2.3 minutes using mobile phase A and 2.9 minutes using mobile phase B.

Enzyme Kinetic Studies. To obtain the K_M, k_{cat} and k_{cat}/K_M parameters for each compound as a substrate of CTB, CTL and CTS, concentrations of fluorogenic peptide were varied between ~ 0.5 K_M – 5 K_M while maintaining a constant concentration of activated enzyme. The reaction mixture (150 µl final volume) consisted of 30 mM acetate-NaOH, pH 5.5, 3.0 mM EDTA, 2.0 mM DTT, 10% DMSO, and 0.33 to 3.3 nM human cathepsin B, L or S in 96 well black plates. The samples were pre-warmed at 37°C for 30 min, and the reaction was initiated upon addition of the enzyme. The activity was monitored spectrophotometrically for the release of 7-amino-4-methyl-coumarin (AMC; excitation 380 nm; emission 460 nm). The amount of AMC released from the reaction was determined by a standard curve in assay buffer. The assay to quantify active enzyme by titration with E64 was adapted from Barrett *et al*[48]. Briefly, the final concentration (150 µl reaction volume) consisted of human cathepsin enzyme (~10 nM), assay buffer (30 mM acetate-NaOH, pH 5.5, 3.0 mM EDTA, 1.0 mM DTT) with E64 (ranging from 1.5 to 7.0 nM) and 30 µM Cbz-Arg-Arg-AMC or Cbz-Phe-Arg-AMC at 37 °C. The residual enzymatic activity was monitored spectrophotometrically for the release of 7-amino-4-methyl-coumarin and plotted versus the concentration of E64 to graphically determine the amount of active enzyme.

Preparation of Cell Lysates from HeLa Cervical Cancer Cells. Preparation of the cell lysates and substrate turnover assays were adapted from Giusti *et al*.[49] Briefly, cells were grown to ~ 80% confluency and washed with PBS, treated with lyse buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA) and centrifuged (12,000 rpm in a microcentrifuge) in a 1.5 mL tube. To evaluate CTB activity, 100 µL of the lysis solution was added to a black 96 well plate and diluted with 100 µL activation buffer (100 mM acetate-NaOH, pH 5.5, 5 mM DTT, 5 mM

EDTA) followed by 15 minute incubation at 37 ºC. A 100 μL aliquot of the activated lysate was then withdrawn and added to a 50 μL solution containing 300 μM probe, in 30% DMSO and 70% buffer (100 mM acetate buffer, 5 mM DTT, 5 mM EDTA, pH 5.5). The enzymatic reaction proceeded at 37 °C for 1 hour after which 50 μL was withdrawn and added to 100 μL of sodium choloroacetate (200 mM) to stop the reaction. The fluorescence at 460 nm was measured and normalized to total protein concentration (Bradford Assay) and expressed as a percentage of AMC released from experiments using Cbz-Arg-Arg-AMC. As a control, spontaneous AMC hydrolysis was determined in assay buffer without lysate while hydrolysis by other protease classes was assessed by pre-treating lysates with the cysteine cathepsin inactivator sodium chloroacetate (200 mM in assay buffer). Levels of free AMC were similar in both control reactions and therefore spontaneous hydrolysis was used as a blank and subtracted from the total RFU in each experiment. To determine the CTB specificity of each probe, lysates were prepared from cells treated with CA-074Me (10 μM) and E64d (10 μM) dissolved in serum free DMEM and incubated for 12 hours.

Fluorescence Microscopy using Living Cancer Cells. Approximately 100,000 cells (HeLa or MD-MBA-221) were seeded in a six well plate and cultured in DMEM with 10% fetal bovine serum and 1% antibiotics-antimycotics incubated at 37 °C in a 5% CO_2. The cells were washed with serum free DMEM and a 2 mL portion of serum free media containing 40 μM of probe was added and incubated for 2 hours at 37 °C in 5% CO_2. Prior to florescence imaging, the cells were washed twice with PBS and the media replaced with Phenol Red free DMEM. For the cathepsin B and cysteine cathepsin inhibition experiments, cells were treated with CA074Me (10 μM) and E64d (10 μM) dissolved in serum free DMEM for 12 hours prior to probe addition.

MTT Assays. Approximately 10,000 cells (HeLa or H2N) were seeded in a 96 well plate and cultured in DMEM with 10% fetal bovine serum and 1% antibiotics-antimycotics incubated at 37 °C

in 5% CO_2 overnight. The cells were washed with serum free DMEM and a 200 μL portion of serum free media containing Cbz-Arg-Arg-AMC, probe **3** or **4** (at 1, 10 or 40 μM) was added. To each well, 20 μL of the MTT reagent (5 mg/mL in PBS buffer and passed through a 0.2 μM syringe filter) was added and the plate incubated for 4 hours at 37 °C in a 5% CO_2. The media was then carefully removed and 200 μL of DMSO was added to each well followed by shaking for 5 minutes. The % viability was calculated as a ratio of absorbance at 540 nm of untreated and treated cells. All solutions and procedures involving MTT were kept in the dark as much as possible.

ASSOCIATED CONTENT

Supporting Information

Copies of the proton (^{1}H) and carbon (^{13}C) NMR spectra for all novel compounds synthesized are available in the supporting information. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Email: phenixc@tbh.net. Phone: (+1) 807-766-3355. Fax: (+1) 807-344-1948.

Author Contributions

ΨMAC synthesized and characterized all compounds, ΨIAM performed CTB and developed CTL assays, ǂSB did all cancer cell assays, ǂCCM did the CTL assays, ǂBV performed NMR experiments, HPLC and helped develop CTB assays, ǂ,Ψ,◊IZ help guide the cell assays and ǂ,Ψ,◊,*CPP conceived the research, help guide all experiments and wrote the paper. All listed authors edited the paper.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

Cathepsin B (CTB), Cathepsin L (CTL), Cathepsin S (CTS), [L-3-trans-(Propylcarbamoyl)oxirane-2-carbonyl]-L-isoleucyl-L-proline methyl ester (CA-074Me), (2S,3S)-trans-Epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E64d), *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ), 7-amino-4-methyl-coumarin (AMC), *p*-aminobenzyl alcohol (PABA), Dulbecco's Modified Eagle Medium (DMEM), 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT).

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