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Substrate optimization for monitoring cathepsin C activity in live cells

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Abstract—A series of peptidic fluorogenic substrates were synthesized to develop a flow cytometry assay (FACS) to monitor the proteolytic activity of cathepsin C in live cells. Of the 16 substrates tested, $(NH_2$-aminobutyric-homophenylalanine$)_2$-rhodamine demonstrated the best reactivity and selectivity profile in the FACS assay using the B721 human B-lymphoblastoid cell line. The resulting FACS assay was validated through correlation of the IC_{50} values with a competitive radiolabeling assay against a series of small molecule inhibitors of cathepsin C.

1. Introduction

Cathepsin C (Cat C), also known as dipeptidyl peptidase I (DPPI), is a widely expressed lysosomal cysteine protease of the papain-fold. It functions as a homotetramer with each subunit containing an N-terminal exclusion domain, a heavy (H) chain harboring the catalytic Cys234 and a C-terminal light (L) chain. The exclusion domain partially blocks the prime side of active cleft making it only accessible to the N-terminus of a peptide substrate.[1] This unique structural arrangement makes Cat C an efficient processing enzyme specialized in removal of dipeptide from N-terminus of a protein precursor.

Cat C plays an important function in the activation of hematopoietic serine proteases found in the granules of cytotoxic lymphocytes, such as granzyme A and B,[2] and inflammatory cells, such as chymase, cathepsin G, and netrophil elastase.[3,4] Consequently, loss of function mutations in humans results in a decrease in host defense and susceptibility to microbe infection, leading to severe pre-pubertal peridontitis, Papillon–Lefevre syndrome and Haim–Munk syndrome.[5,6] On the other hand, Cat C deficiency in mice renders resistance to developing sepsis,[7] arthritis,[4,8] and abdominal aortic aneurysms,[9] indicating that Cat C may represent a new opportunity for clinical intervention of inflammatory diseases.

To facilitate the in vivo characterization of Cat C activity and the development of a Cat C inhibitor with cellular efficacy, a sensitive and selective cell based assay is crucial. A number of assay formats have been reported to measure the intracellular activity of Cat C and include enzymatic assays of cell lysates,[3] radiolabeled activity-based probe in intact cells[10] and fluorescently quenched activity based probe in intact cells.[11] However, these methods either require cell lysate preparation and sample separation, or involve covalent inactivation of Cat C and thereby lack the catalytic amplification of the signal. In this communication, we report the synthesis and characterization of a series of rhodamine peptide substrates designed to monitor Cat C activity in intact cells. We identified one rhodamine substrate, **3a**, that is amenable to flow cytometry (FACS), allowing sensitive and selective monitoring of Cat C proteolytic activity in live cells.

2. Substrate design and chemistry

Rhodamine is an attractive fluorphore for cell based enzymatic assays due to its high extinction coefficient, long excitation and emission wavelengths, high

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fluorescence quantum yield, photostability, and membrane permeability.[12] Rhodamine based peptide substrates have been reported to measure cellular activity of proteases of the papain fold.[13] The synthesis of the potential Cat C rhodamine substrates (Table 1) is outlined in Schemes 1a and 1b.

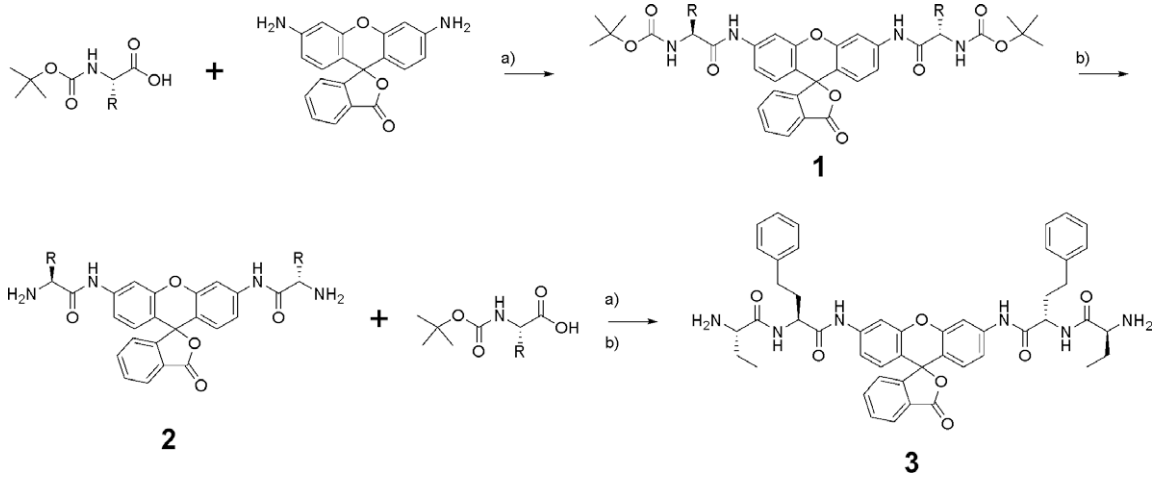
Table 1. Characterization of rhodamine (Rd) substrates in FACS-based assay

#	Structure	*S/N*	Relative signal
3a	$(H_2N\text{-Abu-Hph})_2\text{-Rd}$	7.9	1
3b	$(H_2N\text{-Leu-Hph})_2\text{-Rd}$	1.7	0.06
3c	$(H_2N\text{-Nva-Hph})_2\text{-Rd}$	4.1	0.44
3d	$(H_2N\text{-Pro-Hph})_2\text{-Rd}$	12.2	0.14
3e	$(H_2N\text{-Fpr-Hph})_2\text{-Rd}$	4.3	0.02
3f	$(H_2N\text{-Tze-Hph})_2\text{-Rd}$	3.3	0.05
3g	$(H_2N\text{-Thp-Hph})_2\text{-Rd}$	6.0	0.00
3h	$(H_2N\text{-Chn-Hph})_2\text{-Rd}$	16.9	0.02
3i	$(H_2N\text{-Leu-Leu})_2\text{-Rd}$	1.7	0.08
3j	$(H_2N\text{-Nva-Bip})_2\text{-Rd}$	6.9	0.00
5a	$H_2N\text{-Abu-Hph-Rd-Mor}$	1.0	0.22
5b	$H_2N\text{-Abu-Bip-Rd-Mor}$	3.2	0.03
5c	$H_2N\text{-Fpr-Bip-Rd-Mor}$	2.9	0.01
5d	$H_2N\text{-Leu-Mef-Rd-Mor}$	1.0	0.03
5e	$H_2N\text{-Nva-Mef-Rd-Mor}$	2.2	0.01
5f	$H_2N\text{-Fpr-Mef-Rd-Mor}$	1.3	0.00

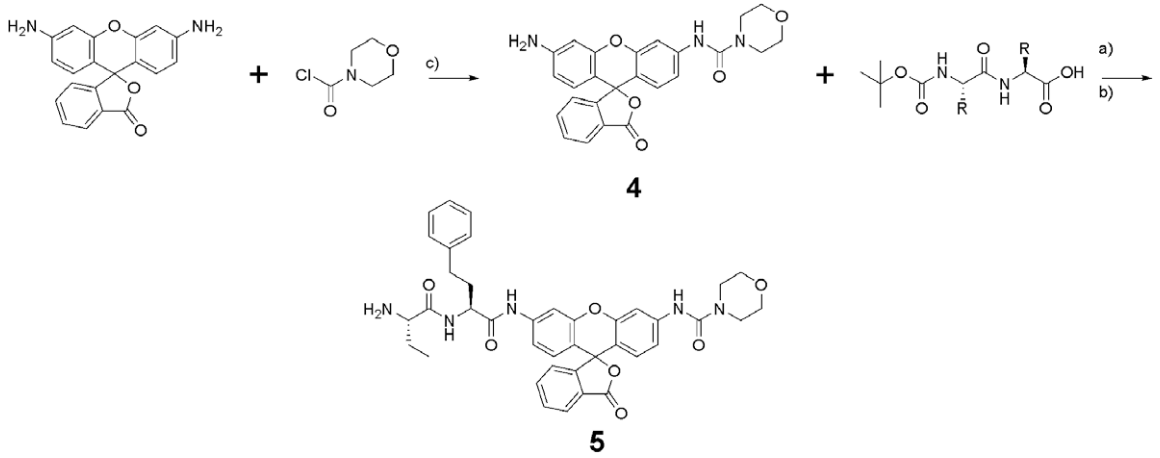
To generate the symmetrical compounds an excess of amino acid (6 equiv) was coupled to rhodamine. To generate a mono-peptidic substrate rhodamine was coupled to morpholinecarbomyl chloride as previously described. Ether symmetric of non-symmetric rhodamine derivatives **2** or **4** was deprotected then coupled to a second amino acid, or dipeptide in the case of **4**. Deprotection of the terminal boc protecting group afforded the desired dipeptidic substrates.

3. Activity of rhodamines as intracellular substrates

To evaluate these substrates, we identified **6** as a tool inhibitor compound that was potent and selective for Cat C with at least 75-fold selectivity over other cathepsin enzymes as well as known to have intracellular activity (Table 2).[14] A known analogue of inhibitor **6** possessing a phenolic group and I^{125} radiolabel (compound **9**), readily enters B721 human B-lymphoblastoid cells and covalently conjugates the lysosomal Cat C en-

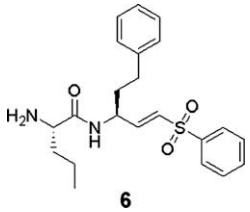
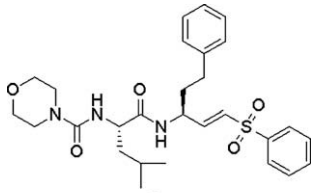
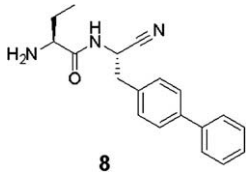


Scheme 1a. Synthesis of symmetric dipeptidic rhodamine substrates ($(H_2N\text{-Xaa-Xaa})_2\text{-Rd}$).



Scheme 1b. Synthesis of non-symmetric mono-peptidic rhodamine substrates ($H_2N\text{-Xaa-Xaa-Rd-Mor}$).

Table 2. Correlation between FACS-based and competitive irreversible radiolabeled inhibitor assays

	Cat C K_i (nM)	Cat C IC_{50} (nM)		Cat K K_i (nM)	Cat B K_i (nM)	Cat S K_i (nM)	Cat H K_i (nM)
		FACS	I^{125}				
 **6**	0.2	70	100	120	1100	5.5	140
 **7**	1800	2600	4000	1.2	64	<0.1	1400
 **8**	1.5	6100	10,000	18,000	$>10^5$	$>10^5$	3700

zyme (Fig. 1). Unlabeled **6** blocks the conjugation of cellular Cat C to the radiolabeled analogue with an IC_{50} of 69 nM. The reactivity and selectivity of the sixteen rhodamine substrates were then analyzed in B721 cells by flow cytometry in the presence of compound **6**. Substrate **3a** generated the highest cellular fluorescent signal and **5a**, the monoamide analogue of **3a**, produced intermediate signal. Other substrates with significant cellular turnover include **3c** and **3d**, both sharing Hph at P1. Cleavage of **3a**, **3c**, and **3d** is dependent on Cat C activity as the signal is sensitive to treatment with compound **6**. Based on the relative velocity and signal-to-noise ratio, substrate **3a**, (NH_2-aminobutyric-homophenylalanine)$_2$-rhodamine ((H_2N-Abu-Hph)$_2$-Rd), was chosen for further validation.

4. Assessment of cathepsin C inhibitors intracellular activity

To validate the (H_2N-Abu-Hph)$_2$-Rd substrate, the cellular potency of three Cat C inhibitors was tested in both the flow cytometry assay with substrate **3a** and the competitive radiolabeled assay with compound **9** (Fig. 1). A good correlation was observed between these two assays (Table 2). Compound **7** is a potent cathepsin S (Cat S) and cathepsin K (Cat K) inhibitor with good intracellular permeability stability and potency as an inhibitor of these proteases. The correlation between the isolated biochemical inhibition, FACS, and competitive radiolabeling support that Cat C activity is solely responsible for the FACS signal. The known selective Cat C inhibitor **8** was also characterized and displayed a good correlation between the FACS and competitive radiolabeling assays. Both activities were approximately 4000- to 7000-fold less potent than the isolated biochemical inhibition. The poor stability of this compound in cellular media and lack of cell permeability could explain the difference in the observed activities. These three compounds demonstrate the utility of the FACS-based activity assay in identifying potent cellular Cat C inhibitors.

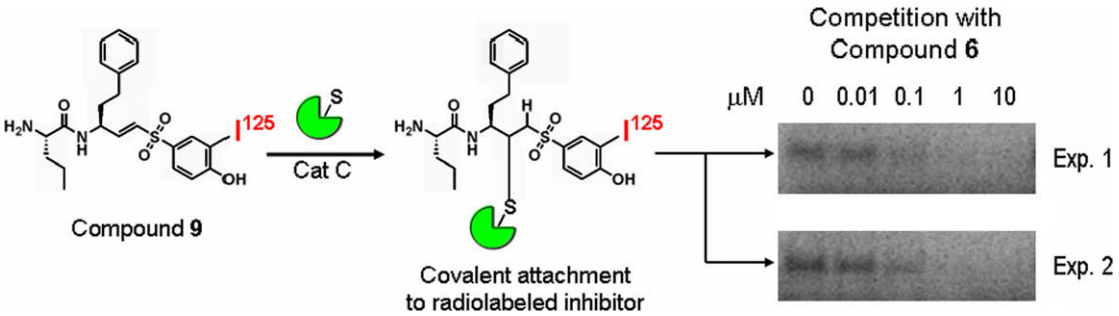


Figure 1. Competitive labeling of intracellular cathepsin C enzyme.

5. Conclusions

In conclusion, we identified **3a** as a selective reagent for monitoring Cat C activity in live cells. The cleavage of **3a** is dependent on Cat C activity and readily detected by FACS. This assay will aid in the investigation of cellular Cat C activity in physiological and pathological conditions. It also has sufficient throughput necessary to support the development of Cat C inhibitors for therapeutic and biomarker development.

6. Experimental

6.1. General materials and methods

All reagents were purchased from Aldrich, Novabiochem, and standard commercial sources. All ^{1}H NMR were recorded at 400 MHz. All ^{13}C NMR were recorded at 100 MHz. Flash chromatography was performed on an Isco using pre-packed silica gel cartridges shielded from light with foil.

6.2. *N*,*N*′-Boc-Hph-rhodamine110 (1a)

To an amber vial were charged a Boc-Hpe-OH (744 mg, 2.66 mmol, 6 equiv), HATU (163 mg, 0.44 mmol, 6 equiv) and DMF (6 mL). After stirring for 5 min DIEA (1.4 mL, 8 mmol, 18 equiv) was added. To the solution was added rhodamine110 and the reaction was allowed to stir for 72 h. A standard aqueous EtOAc workup and purification via silica gel chromatography afforded the desired product.

6.3. *N*,*N*′-NH_2-Abu-Hph-rhodamine110 (3a)

To an amber vial were charged *N*,*N*′-Boc-Hph-rhodamine110 (**1a**) (45 mg, 0.053 mmol, 1 equiv), dichloroethane/TFA 4/1 (v/v) and water. The reaction was allowed to stir for 2 h at room temperature and then all volatile reagents were removed under reduced pressure to afford *N*,*N*′-NH_2-Hph-rhodamine110 as a TFA salt. Boc-Abu-OH (67 mg, 0.317 mmol, 6 equiv), HATU (120 mg, 0.317 mmol, 6 equiv) and DMF (mL) were all charged to an amber vial. After stirring for 5 min DIEA (165 μL, 0.951 mmol, 18 equiv) was added. To the solution was added *N*,*N*′-NH_2-Hph-rhodamine110 and the reaction was allowed to stir for 4 h. A standard aqueous EtOAc workup and purification via silica gel chromatography afforded *N*,*N*′-Boc-Abu-Hph-rhodamine110. This material was TFA deprotected as described above and the desired product was purified from the reaction mixture via HPLC. ^{1}H NMR (400 MHz, MeOH-d_4) δ 8.01 (d, J = 8.2 Hz, 1H), 7.67–7.75(m, 4H), 7.12–7.26 (m, 12H), 6.68 (d, J = 8.4 Hz, 2H), 4.53–4.56 (m 2H), 3.96 (t, J = 6.4 Hz, 2H), 2.65–2.83 (m, 4H), 1.86–2.20 (m, 8H), 1.06 (t, J = 7.2 Hz, 6H), ^{13}C NMR (100 MHz, MeOH-d_4) δ 172.31, 171.35, 170.44, 154.36, 152.87, 142.12, 152.87, 142.12, 141.97, 136.87, 129.57, 129.45, 127.55, 127.24, 126.00, 125.13, 116.83, 115.42, 108.69, 84.20, 55.69, 35.10, 33.12, 26.07, 9.45.

6.4. *N*,*N*′-NH_2-Leu-Hph-rhodamine110 (3b)

Compound **3b** was prepared as described for the synthesis of **3a**. HPLC. ^{1}H NMR (400 MHz, MeOH$_{-d4}$) δ 8.04 (d, J = 8.1, 1H), 7.70–7.80 (m, 4H), 7.15–7.28 (m, 12H), 6.72 (d, J = 8.1 Hz, 2H), 4.57 (dd, J = 0.8, 1.4 Hz, 2H), 4.01 (dd, J = 2.1, 1.4 Hz, 2H), 2.66–2.86 (m, 4H), 2.03–2.22 (m, 4H), 1.68–1.85 (m, 6H), 1.02 (t, J = 1.6 Hz, 11H). ^{13}C NMR (100 MHz, MeOH-d_4) δ 172.31, 171.35, 170.63, 154.41, 152.91, 142.13 136.88, 131.39, 129.56, 129.46, 127.58, 127.26, 126.02, 125.15, 84.22, 55.68, 54.25, 35.13, 34.89, 33.21, 19.16, 14.07.

6.5. *N*,*N*′-NH_2-Nva-Hph-rhodamine110 (3c)

Compound **3c** was prepared as described for the synthesis of **3a** ^{1}H NMR (400 MHz, MeOH-d_4) δ 8.06 (d, J = 8.0 Hz, 1H), 7.70–7.80 (m, 4H), 7.11–7.27 (m, 12H), 6.72 (d, J = 2.2 Hz, 2H), 4.57 (dd, J = 0.8, 8.0 Hz, 2H), 4.01 (dd, J = 2.1, 1.4 Hz, 2H), 2.66–2.86 (m, 4H), 1.68–2.24 (m, 8H), 1.41 (m, 4H), 1.02 (t, J = 1.6 Hz, 6H). ^{13}C NMR (100 MHz, MeOH-d_4) δ 174.36, 171.04, 167.89, 163.14, 160.11, 151.25, 150.92, 141.11, 140.01, 136.88, 131.41, 129.49, 127.27, 116.44, 116.12, 114.91, 106.22, 84.24, 55.68, 51.57, 40.01, 38.21, 33.24, 29.11, 25.38, 21.11, 20.21.

6.6. *N*,*N*′-NH_2-Pro-Hph-rhodamine110 (3d)

Compound **3d** was prepared as described for the synthesis of **3a** ^{1}H NMR (400 MHz, MeOH-d_4) δ 8.04 (d, J = 8.2 Hz, 1H), 7.70–7.83 (m, 4H), 7.14–7.29 (m, 12H), 6.74 (d, J = 3 Hz, 2H), 4.53 (dd, J = 8.2, 1.3 Hz, 2H), 4.38 (dd, J = 2.1, 1.7 Hz, 2H), 3.33–3.45 (m, 4H), 2.66–2.85 (m, 4H), 2.45–2.54 (m, 2H), 2.01–2.21 (m, 10H). ^{13}C NMR (100 MHz, MeOH-d_4) 172.38, 171.24, 169.98, 154.39, 152.91, 142.05, 136.84, 131.40, 129.59, 129.47, 127.62, 127.29, 126.02, 125.12, 116.81, 116.79, 115.53, 108.65, 84.06, 60.97, 55.86, 47.51, 35.05, 33.24, 31.12, 25.0.

6.7. *N*,*N*′-NH_2-Fpr-Hph-rhodamine110 (3e)

Compound **3e** was prepared as described for the synthesis of **3a** ^{1}H NMR (400 MHz, MeOH-d_4) δ 8.04 (d, J = 8.0 Hz, 1H), 7.70–7.83 (m, 4H), 7.14–7.29, 6.74 (d, J = 2.1 Hz, 2H), 5.48 (t, J = 0.9 Hz, 1H), 5.35 (t, J = 1.0 Hz, 1H), 4.53–4.59 (m, 4H), 3.77 (q, J = 4.6, 0.4 Hz, 2H), 3.53 (q, J = 8.9, 0.9 Hz, 2H), 2.56–2.89 (m, 9H), 2.04–2.24 (m, 5H). ^{13}C NMR (100 MHz, MeOH-d_4) δ 172.16, 171.29, 169.37, 154.45, 152.91, 142.09, 141.96, 136.87, 131.41, 129.62, 129.50, 127.31, 126.02, 125.18, 116.92, 115.58, 108.78, 93.70, 91.94, 84.10, 59.91, 55.91, 53.49, 53.25, 38.19, 37.97, 35.16, 33.22.

6.8. *N*,*N*′-NH_2-Tze -Hph-rhodamine110 (3f)

Compound **3f** was prepared as described for the synthesis of **3a** ^{1}H NMR (400 MHz, MeOH-d_4) δ 8.04 (d, J = 8.0 Hz, 1H), 7.70–7.80 (m, 4H), 7.15–7.28 (m, 12H), 6.72 (d, J = 8.0 Hz, 2H), 4.57 (dd, J = 0.8, 1.4 Hz, 2H), 4.01 (dd, J = 2.1, 1.4 Hz, 2H), 2.66–2.86 (m, 4H), 2.03–2.22 (m, 4H), 1.68–1.85 (m, 6H), 1.02 (t,

J = 1.6 Hz, 11H). ^{13}C NMR (100 MHz, MeOH-d_4) 172.18, 171.27, 169.52, 156.13, 154.43, 152.95, 151.52, 142.15, 136.89, 131.45, 129.62, 129.49, 127.29, 126.07, 125.15, 118.74, 117.03, 116.99, 115.66, 108.86, 84.08, 55.55, 54.20, 35.14, 33.31, 33.16.

6.9. *N*,*N*′-NH$_2$-Thp-Hph-rhodamine110 (3g)

Compound **3g** was prepared as described for the synthesis of **3a** ^{1}H NMR (400 MHz, MeOH-d_4) δ 8.04 (d, J = 7.9 Hz, 1H), 7.70–7.83 (m, 4H), 7.14–7.30 (m, 12H), 6.73 (d, J = 2.1 Hz, 2H), 4.55 (dd, J = 2.1, 1.5 Hz, 2H), 3.90–3.96 (m, 4H), 3.74–3.79 (m, 4H), 2.66–2.86 (m, 4H), 2.44–2.58 (m, 5H) 2.13–2.24 (m, 4H), 1.82–1.87 (m, 4H). ^{13}C NMR (100 MHz, MeOH$_{-d4}$) δ 172.73, 171.91, 171.27, 163.42, 163.08, 162.73, 162.35, 162.32, 154.38, 152.93, 142.07, 136.87, 131.43, 129.64, 129.54, 127.32, 126.05, 125.13, 116.81, 115.54, 108.67, 108.65, 84.09, 63.54, 59.15, 56.30, 34.52, 33.55, 32.68, 32.55.

6.10. *N*,*N*′-NH$_2$-Chn-Hph-rhodamine110 (3h)

Compound **3h** was prepared as described for the synthesis of **3a**^{1}H NMR (400 MHz, MeOH-d_4) δ 8.03 (d, J = 7.9 Hz, 1H), 7.69–7.81 (m, 4H), 7.14–7.28 (m, 12H), 4.53 (dd, J = 7.9, 1.5 Hz, 2H), 2.65–2.84 (m, 4H), 2.14–2.26 (m, 9H), 1.79–1.93 (m, 10H), 1.50–1.65 (m, 6H). ^{13}C NMR (100 MHz, MeOH-d_4) δ 173.37, 172.80, 171.31, 163.20, 162.85, 162.50, 162.15, 154.32, 152.90, 142.06, 136.87, 131.42, 129.61, 129.52, 127.27, 126.03, 125.11, 116.86, 115.46, 108.68, 84.16, 61.81, 56.10, 34.55, 33.47, 32.52, 32.34, 25.03, 21.58, 21.53.

6.11. *N*,*N*′-NH$_2$-Leu-Leu-rhodamine110 (3i)

Compound **3i** was prepared as described for the synthesis of **3a** ^{1}H NMR (400 MHz, MeOH-d_4) δ 8.02 (d, J = 7.6, 1H), 7.68–7.80 (m, 4H), 7.01–7.06 (m, 3H), 6.71 (d, J = 7.6 Hz, 2H), 4.58–4.62 (m, 2H), 3.90–3.97 (m, 2H), 1.45–1.80 (m, 12H), 0.87–0.96 (m, 24H), ^{13}C NMR (100 MHz, MeOH-d_4) δ 173.37, 172.65, 170.65, 154.69, 152.36, 140.58, 136.55, 130.25, 128.65, 125.63, 127.63, 125.21, 116.95, 115.32, 108.54, 82.65, 56.96, 54.71, 44.28, 28.63, 27.36, 25.63, 24.15, 23.65.

6.12. *N*,*N*′-NH$_2$-Nva-Bip-rhodamine110 (3j)

Compound **3j** was prepared as described for the synthesis of **3a** ^{1}H NMR (400 MHz, MeOH-d_4) δ 8.01–8.03 (m, 1H), 7.21–7.39 (m, 27 H), 6.90–7.14 (m, 3H),6.58–6.62 (m, 2H), 4.53 (dd, J = 2.2, 1.5 Hz, 2H), 2.65–2.84 (m, 4H), 2.14–2.26 (m, 9H), 1.79–1.93 (m, 10H), 1.50–1.65 (m, 6H). ^{13}C NMR (100 MHz, MeOH-d_4) δ 173.37, 172.80, 171.31, 163.20, 162.85, 162.50, 162.15, 154.32, 152.90, 142.06, 136.87, 131.42, 129.61, 129.52, 127.27, 126.03, 125.11, 116.86, 115.46, 108.68, 84.16, 61.81, 56.10, 34.55, 33.47, 32.52, 32.34, 25.03, 21.58, 21.53.

6.13. N-NH$_2$-Abu-Hph-*N*′-Mor-rhodamine110 (5a)

Boc-Abu-Hph-OH (218 mg, 0.6 mmol, 6 equiv), HATU (228 mg, 0.6 mmol, 6 equiv) and DMF (1 mL) were all charged to an amber vial. After stirring for 5 min DIEA (313 μL, 1.8 mmol, 18 equiv) was added. To the solution was added N-Mor-rhodamine110 (**4**) (44 mg, 0.1 mmol, 1 equiv) and the reaction was allowed to stir for 4 h. A standard aqueous EtOAc workup and purification via silica gel chromatography afforded *N*-Boc-Abu-Hph-*N*′-Mor-rhodamine110. This material was TFA deprotected as described above and the desired product was purified from the reaction mixture via HPLC ^{1}H NMR (400 MHz, MeOH$_{-d4}$) δ 8.02 (d, J = 7.6, 1H), 7.71–7.82 (m, 3H), 7.57 (d, J = 2.4 Hz, 1H) 7.05–7.28 (m, 7H), 6.72 (d, J = 8.8 Hz, 1H), 6.66 (d, J = 8.8 Hz, 1H), 4.56–4.60 (m, 1H), 3.91 (m, 1H), 3.71 (t, J = 4.4 Hz, 4H), 3.52 (t, J = 4.4 Hz, 4H), 2.61–2.37 (m, 2H), 1.79–2.23(m, 5H), 1.07 (t, J = 7.6 Hz, 3H). ^{13}C NMR (100 MHz, MeOH-d_4) δ 173.15, 171.70, 170.94, 165.20, 160.32, 160.11, 159.15, 158.89, 145.32, 140.96, 139.87, 130.98, 127.09, 124.21, 123.07, 120.96, 120.04, 115.06, 113.87, 109.68, 85.32, 81.36, 55.36, 44.36, 43.21, 41.11, 40.39, 36.25, 32.78, 31.01, 30.68, 25.46, 22.98, 20.02.

6.14. N-NH$_2$-Abu-Bip-*N*′-Mor-rhodamine110 (5b)

Compound **5b** was prepared as described for the synthesis of **5a** ^{1}H NMR (400 MHz, MeOH-d_4) δ 7.95–8.04 (m, 4H), 7.63–7.76 (m, 3H), 7.44–7.55 (m, 5H), 7.31–7.38 (m, 4H), 7.24–7.28 (m, 1H), 7.05–7.12 (m, 3H), 6.60–6.68 (m, 2H), 3.81–3.85 (m, 1H), 3.71 (t, J = 4.4 Hz, 4H), 3.52 (t, J = 4.4 Hz, 4H), 3.31–3.35 (m, 2H), 1.89–1.95 (m, 3H), 1.31–1.38 (m, 5H), 1.01–1.05 (m, 3H). ^{13}C NMR (100 MHz, MeOH-d_4) δ 171.80, 171.37, 170.26, 164.91, 157.33, 153.00, 143.89, 141.93, 141.90, 136.97, 131.32, 130.87, 129.89, 129.14, 128.15, 127.75, 12.02, 125.25, 117.38, 116.80, 115.68, 115.68, 114.00, 08.70, 108.60, 67.68, 57.26, 55.81, 55.27, 45.66, 37.02, 31.71, 26.08, 18.72, 7.28, 13.18, 9.41.

6.15. N-NH$_2$-Fpr-Bip-*N*′-Mor-rhodamine110 (5c)

Compound **5c** was prepared as described for the synthesis of **5a** ^{1}H NMR (400 MHz, MeOH-d_4) δ 7.97–7.99 (m, 1H), 7.69–7.74 (m, 3H), 7.22–7.51 (m, 11H), 7.02–7.13 (m, 3H), 6.58–6.61 (m, 2H), 4.98 (m, 1H), 4.55 (m, 1 H) 3.71 (t, J = 4.4 Hz, 4H), 3.52 (t, J = 4.4 Hz, 4H), 3.31–3.35 (m, 2H), 1.80–1.97 (m, 6H), 1.31–1.38 (m, 5H), 1.01–1.05 (m, 3H).^{13}C NMR (100 MHz, MeOH-d_4) δ 171.55, 171.38, 169.13, 159.18, 158.77, 157.32, 153.96, 153.05, 143.89, 141.81, 136.89, 136.74, 130.88, 129.88, 128.13, 127.83, 126.07, 125.28, 120.39, 117.45, 116.49, 115.74, 114.63, 114.01, 108.87, 93.69, 91.93, 67.66, 59.77, 57.48, 55.16, 45.64, 38.91, 37.05.

6.16. N-NH$_2$-Leu-Mef-*N*′-Mor-rhodamine110 (5d)

Compound **5d** was prepared as described for the synthesis of **5a** ^{1}H NMR (400 MHz, MeOH-d_4) δ 7.95–8.01 (m, 3H), 7.53–7.96 (m, 6H), 6.97–7.25 (m, 7H), 6.45–6.81 (m, 7H), 3.81–3.85 (m, 1H), 3.71 (t, J = 4.4 Hz, 4H), 3.52 (t, J = 4.4 Hz, 4H), 3.31–3.35 (m, 2H), 1.89–1.95 (m, 2H), 1.31–1.38 (m, 6H), 1.01–1.05 (m, 3H). ^{13}C NMR (100 MHz, MeOH-d_4) δ 171.90, 171.49, 170.45, 161.18, 158.45, 155.98, 154.89, 153.15, 142.18, 140.67,

139.70, 129.94, 129.52, 128.07, 126.32, 126.00, 123.27, 118.52, 117.01, 115.21, 114.19, 113.00, 112.10, 110.65, 107.54, 107.03, 68.56, 58.21, 50.47, 44.16, 37.29, 35.62, 33.74, 18.62, 14.96.

6.17. N-NH_2-Nva-Mef-*N*′-Mor-rhodamine110 (5e)

Compound **5e** was prepared as described for the synthesis of **5a** ^{1}H NMR (400 MHz, MeOH-d_4) δ 8.00 (d, J = 7.8 Hz, 1H), 7.67–7.76 (m, 3H), 7.53 (s, 1H), 7.11–7.21 (m, 3H), 7.04–7.08 (m, 2H), 6.75–6.81 (m, 2H), 6.61–6.68 (m, 2H), 4.85–4.87 (m, 1H), 3.89–3.95 (m, 1H) 3.71 (t, J = 4.4 Hz, 4H), 3.52 (t, J = 4.4 Hz, 4H), 3.31–3.35 (m, 6H), 1.89–1.95 (m, 4H), 1.31–1.38 (m, 3H), 1.01–1.05 (m, 3H).^{13}C NMR (100 MHz, MeOH-d_4) δ 171.92, 171.39, 170.38, 160.18, 157.35, 154.13, 153.06, 153.02, 143.88, 141.87, 136.76, 131.34, 129.67, 129.17, 127.76, 1236.05, 125.27, 117.48, 117.42, 116.85, 116.85, 115.68, 114.99, 114.00, 108.76, 108.63, 67.68, 57.50, 55.67, 45.66, 38.29, 34.88, 19.13, 14.07.

6.18. N-NH_2-Fpr-Mef-*N*′-Mor-rhodamine110 (5f)

Compound **5f** was prepared as described for the synthesis of **5a** ^{1}H NMR (400 MHz, MeOH-d_4) δ 7.95–8.05 (m, 2H), 7.68–7.76 (m, 3H), 7.51 (s, 1H), 7.0–7.20 (m, 3H), 7.04–7.08 (m, 2H), 6.75–6.81 (m, 2H), 6.61–6.68 (m, 2H), 5.36 (d, J_F = 52 Hz, 1H), 4.76 (t, J = 7.6 Hz, 1H), 4.47 (dd, J = 4, 10.8 Hz, 1H), 3.97 (s, 3H) 3.71 (t, J = 4.4 Hz, 4H), 3.56 (t, J = 4.4 Hz, 4H), 3.35 (s, 4H), 1.31–1.38 (m, 3H), 1.01–1.05 (m, 3H).^{13}C NMR (100 MHz, MeOH-d_4) δ 171.68, 171.36, 169.04, 164.92, 160.16, 159.18, 157.33, 154.05, 153.04, 153.00, 143.88, 141.81, 136.75, 131.38, 129.61, 1289.45, 127.74, 126.06, 125.28, 117.45, 115.72, 114.97, 114.63, 114.66, 114.02, 108.84, 96.68, 91.95 67.66, 59.74, 57.69, 55.65, 55.17,53.42, 45.64, 37.02, 31.7.

6.19. Cathepsin C FACS assay

B721 EBV-immortalized B cells were washed in serum-free and phenol red-free RPMI 1640 media. Cells were resuspended in the same media and 150 μL of 1×10^6 cells/mL was dispensed into a 96-well plate. The cells were then pre-treated with compounds or DMSO to final concentrations of 10, 3.3, 1.1, 0.37, 0.12, and 0 μM. The cell-compound mixture was incubated for 30 min at 37 °C in a 5% CO2 humidified incubator. Following incubation, (H_2N-Abu-Hph)$_2$-Rd substrate **(3a)** and Propidium Iodide were added to the cells to a final concentration of 2 μM and 4 μg/mL, respectively. Samples were immediately read using BD Biosciences FACSCalibur flow cytometer (excitation = 488 nm; emission = 530 nm). Three time points at 15-min intervals were taken to measure the increase of cleaved rhodamine over time. Data were analyzed using FlowJo 5.7.2 software and the EC_{50} for each compound was calculated and plotted using PlateKi software version 4.07.019.

6.20. Competitive radiolabeling assay

B721 EBV-immortalized B cells were washed and resuspended in serum-free and phenol red-free RPMI media. About 500 μL of 1×10^6 cells/mL was plated in a 24-well flat bottom DMSO-resistant plate. Compounds and DMSO were immediately added to a final concentration of 50, 10, 1, 0.1, and 0 μM. The cell-compound mixtures were incubated for 30 min at 37 °C in a 5% CO2 humidified incubator. After incubation, 2 nM of ^{125}I-labeled compound **9** was added to each sample and incubated for another 30 min at 37 °C. Cells were pelleted by centrifugation at 1200 rpm for 10 min, lysed immediately and boiled for 20 min. Samples were resolved on SDS–PAGE gels and the resulting gels were vacuum dried for 2 h. Dried gels were exposed to a phosphor screen (Amersham, cat# 63-0034-89) overnight and bands were detected using Storm 860 phosphorimaging machine and quantified with ImageQuant TL software program (Amersham).

6.21. Enzymatic assays

Recombinant human cathepsin enzymes were used in all enzyme inhibition assays. The standard assay format was 30 μL in a 384-well plate that contained 50 μM fluorogenic peptide substrate (Ac-His-Pro-Val-Lys-7-amino-3-carbamoylmethyl-4-methyl-coumarin for cathepsin S and cathepsin B, Ac-Lys-His-Pro-Lys-7-amino-3-carbamoylmethyl-4-methyl-coumarin for cathepsin K; Ac-His-Lys-Phe-Lys-7-amino-3-carbamoylmethyl-4-methyl-coumarin for cathepsin L; H-Arg-7-amino-4-methyl-coumarin for cathepsin H, NH2-Gly-Phe-7-amino-4-methyl-coumarin for cathepsin C) in 100 mM NaOAc, 1 mM EDTA, 0.01% Brij-35 and 5 mM DTT, pH 5.5 at 37 °C. The recombinant human cathepsin enzyme was preincubated with inhibitor for 20 min prior to addition of substrate. The liberation of the coumarin group was monitored by the increase in fluorescence at an excitation wavelength of 380 nm and an emission wavelength of 450 nm on a Gemini EM fluorometer. The reaction progress curve was fitted to the Morrison equation using PlateKi (BioKin) and the apparent inhibition constant was converted to the inhibition constant (K_i) for competitive inhibitors.

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14. Apparent inhibition constants of compound **6** against recombinant human cathepsin enzymes: 0.2 nM for CatC, 2100 nM against CatB, 250 nM against CatK, 20 nM against CatL, 15 nM against CatS and 137 nM against CatH.