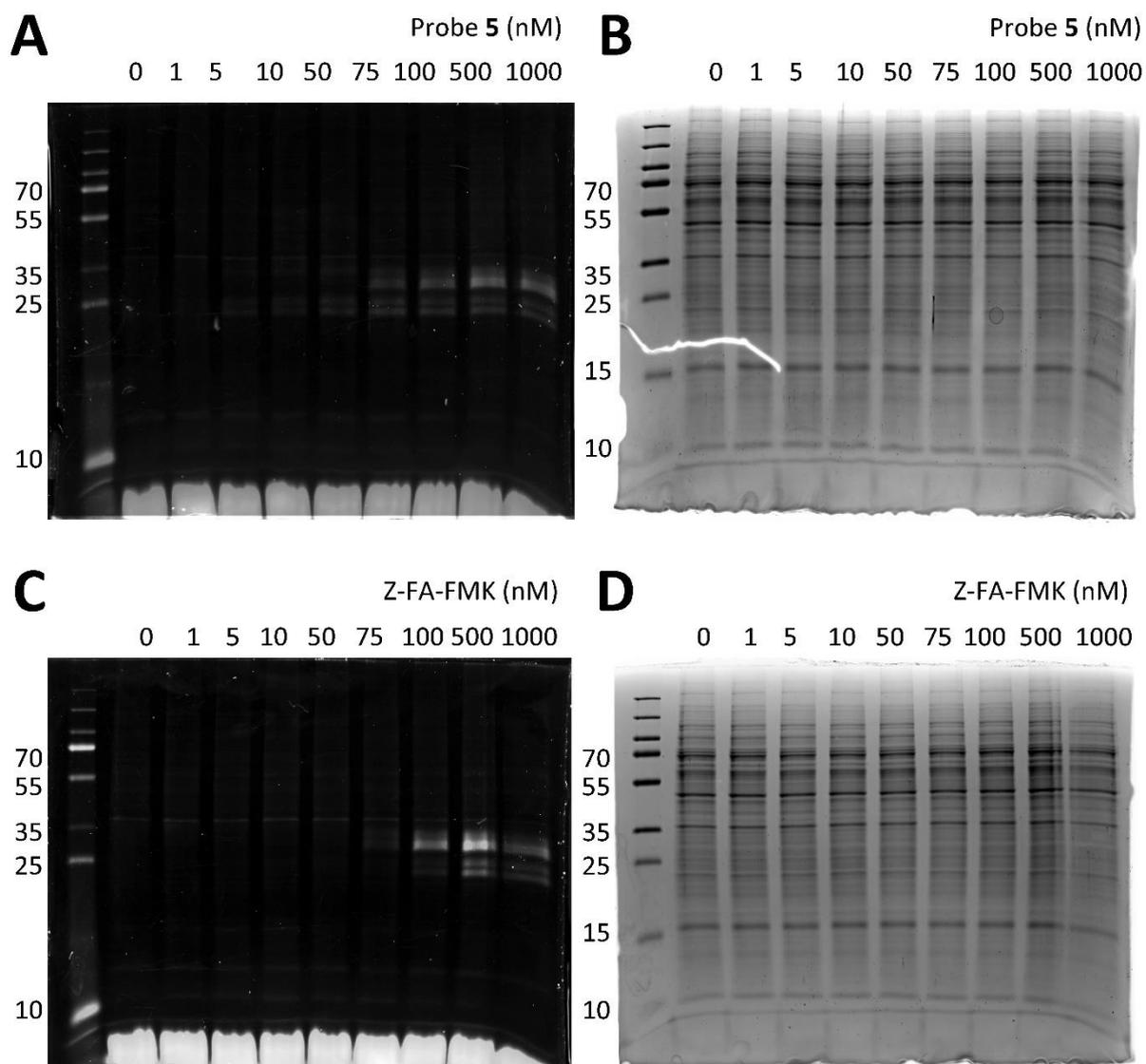
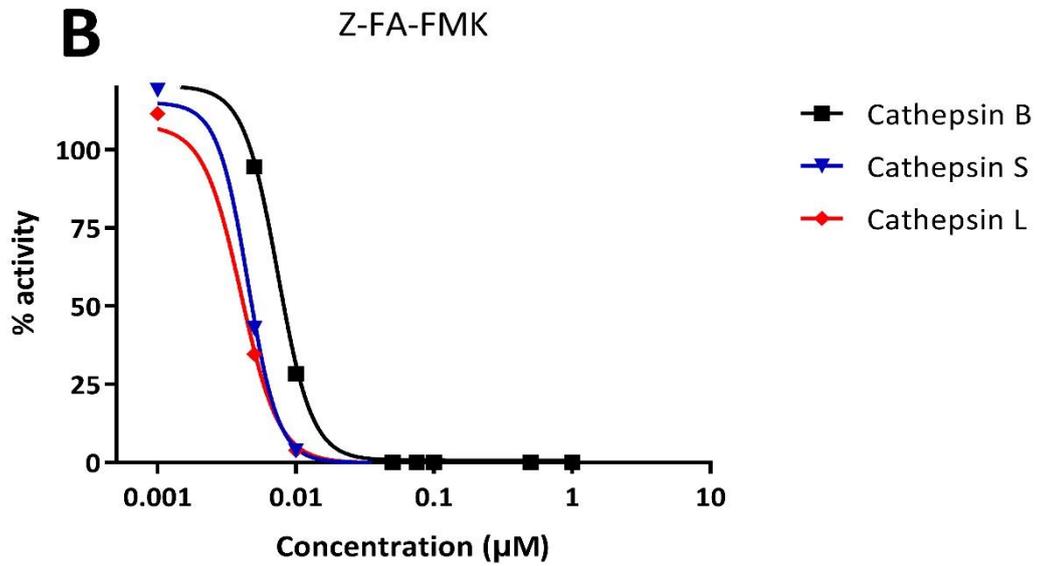
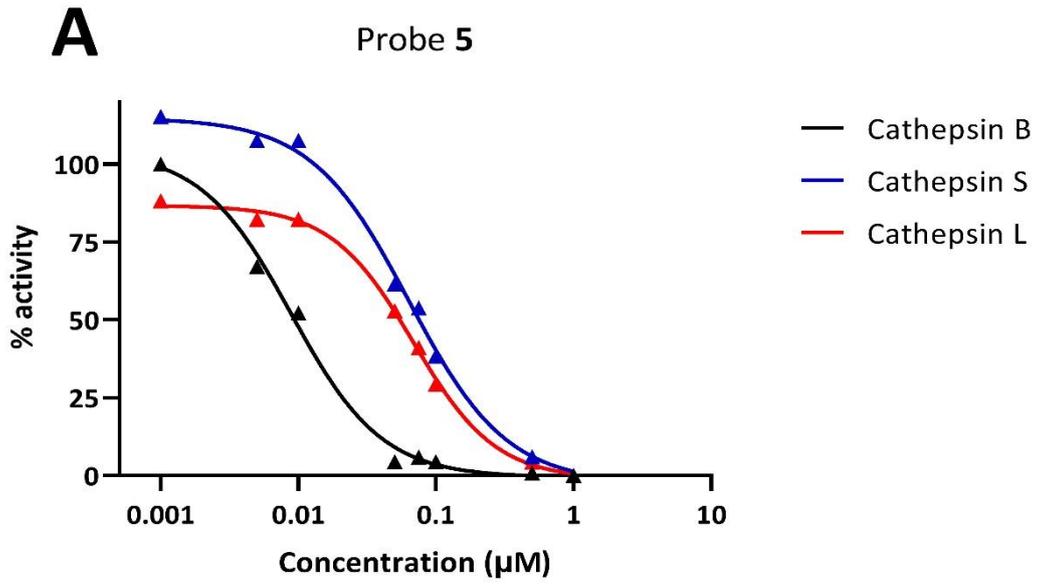


**SUPPLEMENTAL FIGURES**



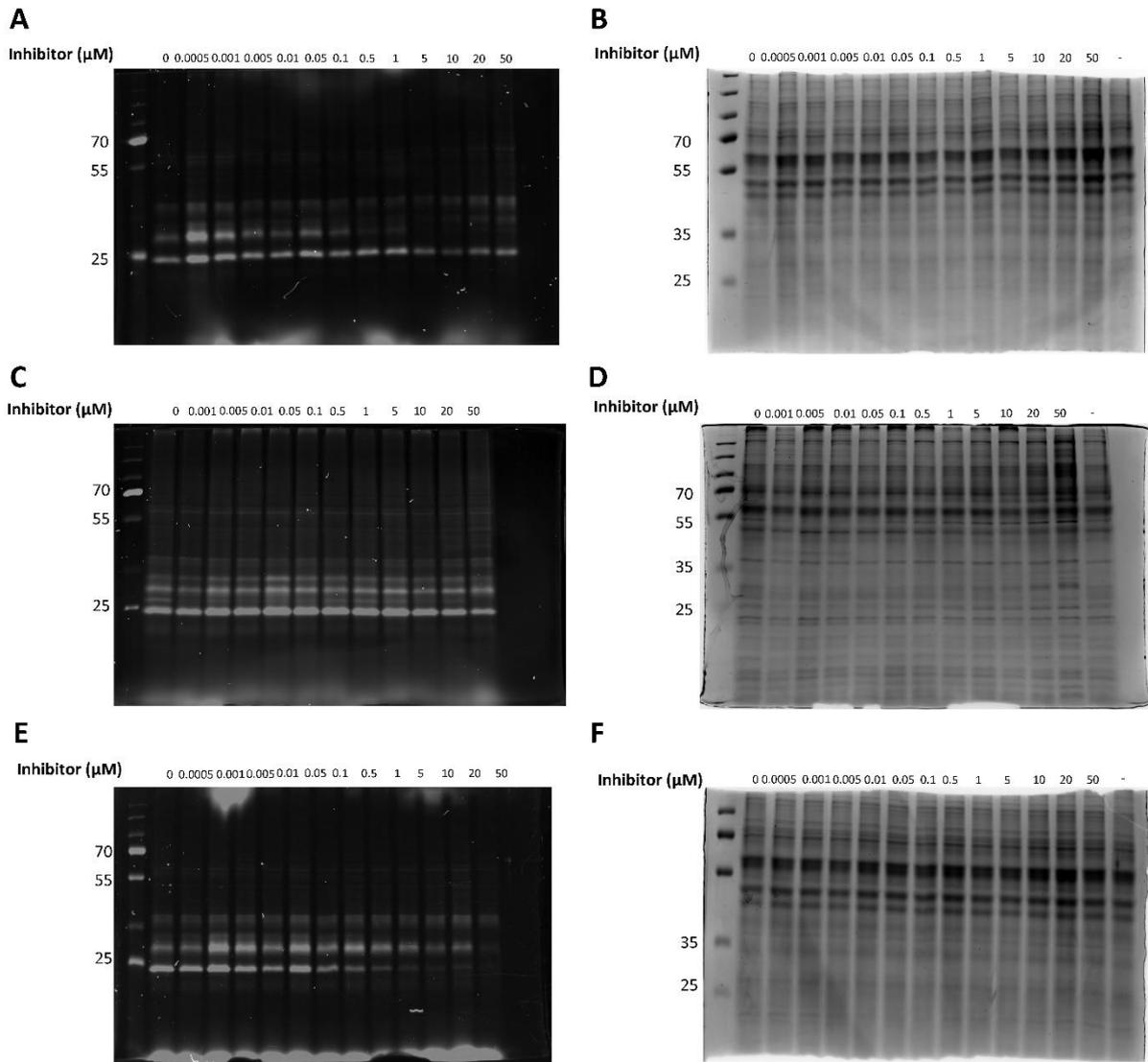
**SI Fig. 1.** (A) Competition experiment in Jurkat lysates between probe 5 and TAMRA-DCG-04 in Jurkat-cell lysates. (C) Competition experiment Jurkat lysates between Z-FA-FMK and TAMRA-DCG-04 in Jurkat-cell lysates. The left gels were fluorescent scan using Cy3 settings, and on the right the same gels stained with coomassie brilliant blue (CBB) are shown (B and D).



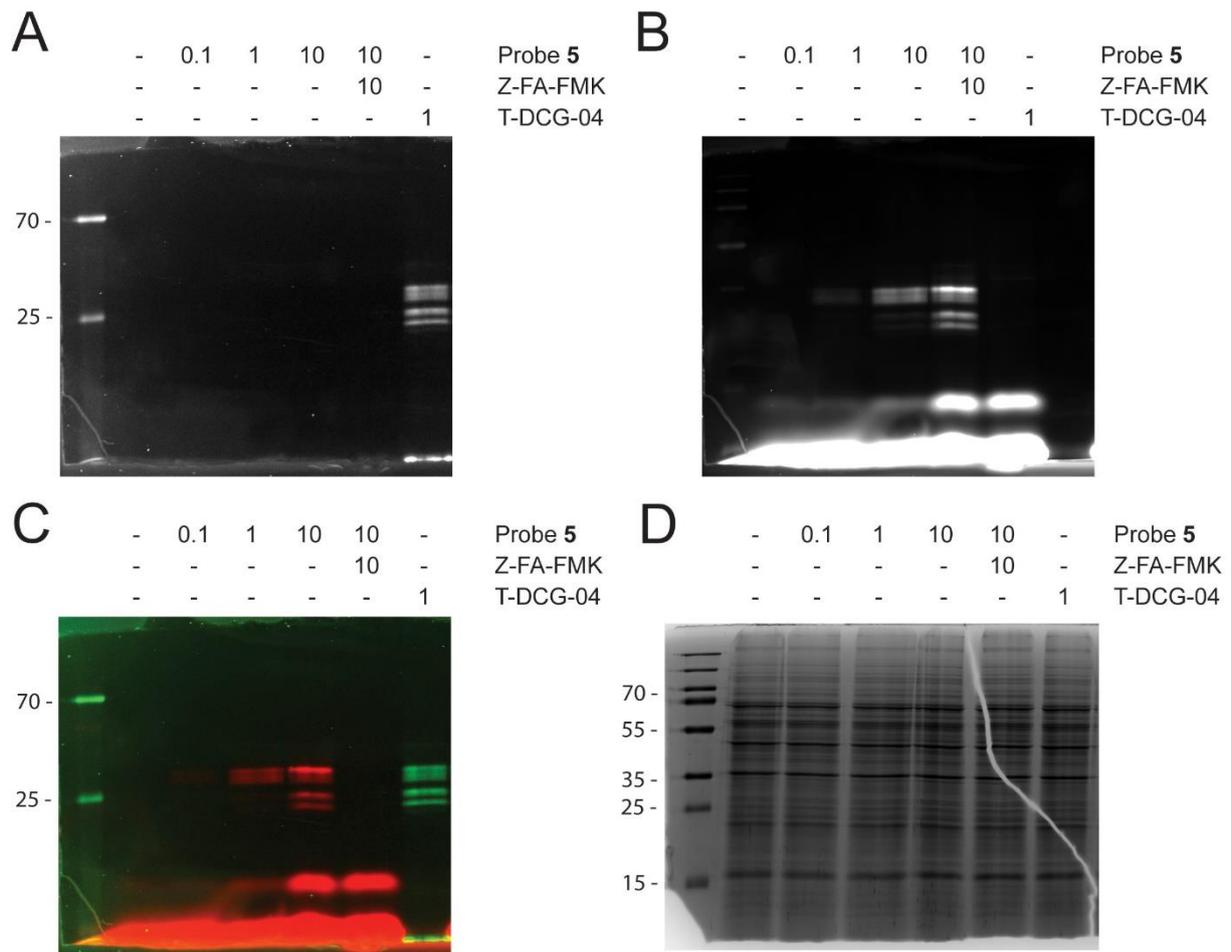
**C**

	IC50	
	Probe 5	Z-FA-FMK
Cathepsin B	8,8	7,4
Cathepsin S	63,4	4,5
Cathepsin L	68,5	4,0

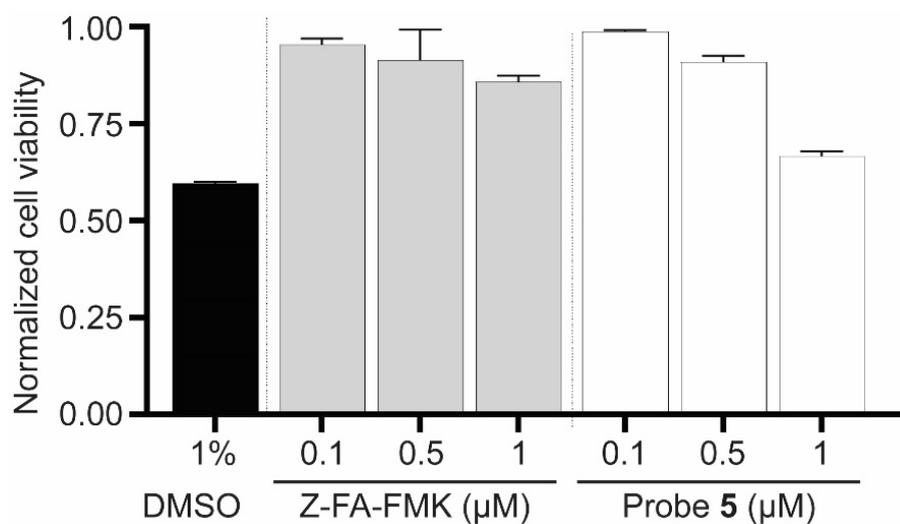
**SI Fig 2. IC50.** Apparent half-maximal inhibitory concentrations (IC50s) were calculated from the quantification of the decrease in labeling intensity of residual cathepsin activity inhibited by probe 5 (A) or Z-FA-FMK (B). IC50 is given in nanomolar (C).



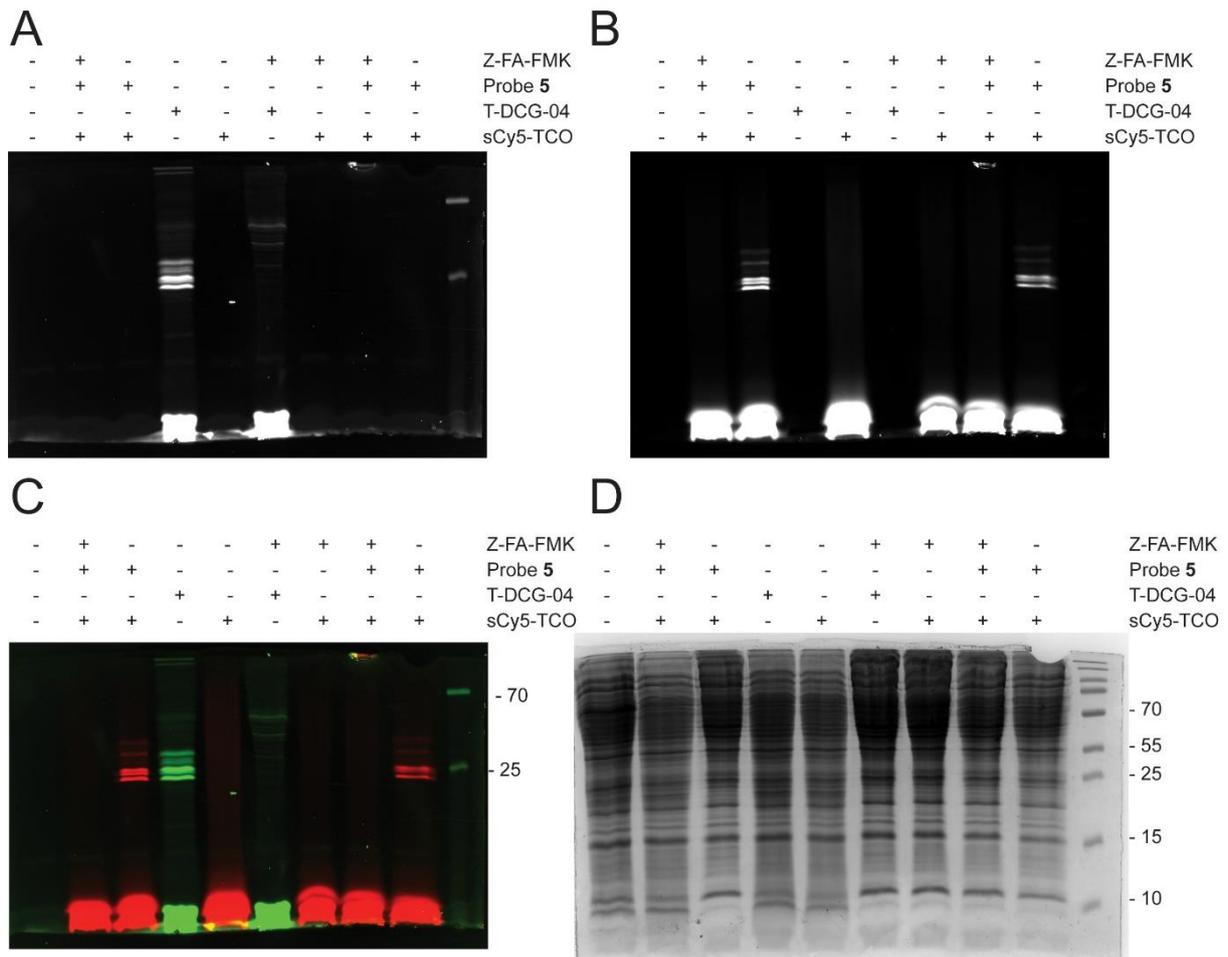
**SI Fig. 3.** Competitive labeling of Cathepsin B, S, and L. RAW 264.7 cells were incubated with the indicated concentration of Cathepsin B (**A-B**), S (**C-D**), and L (**E-F**) inhibitor for 2.5 h. Residual cathepsin activities were labeled with probe 5 (10 $\mu\text{M}$ ) and s-Cy5-TCO (2 $\mu\text{M}$ ).



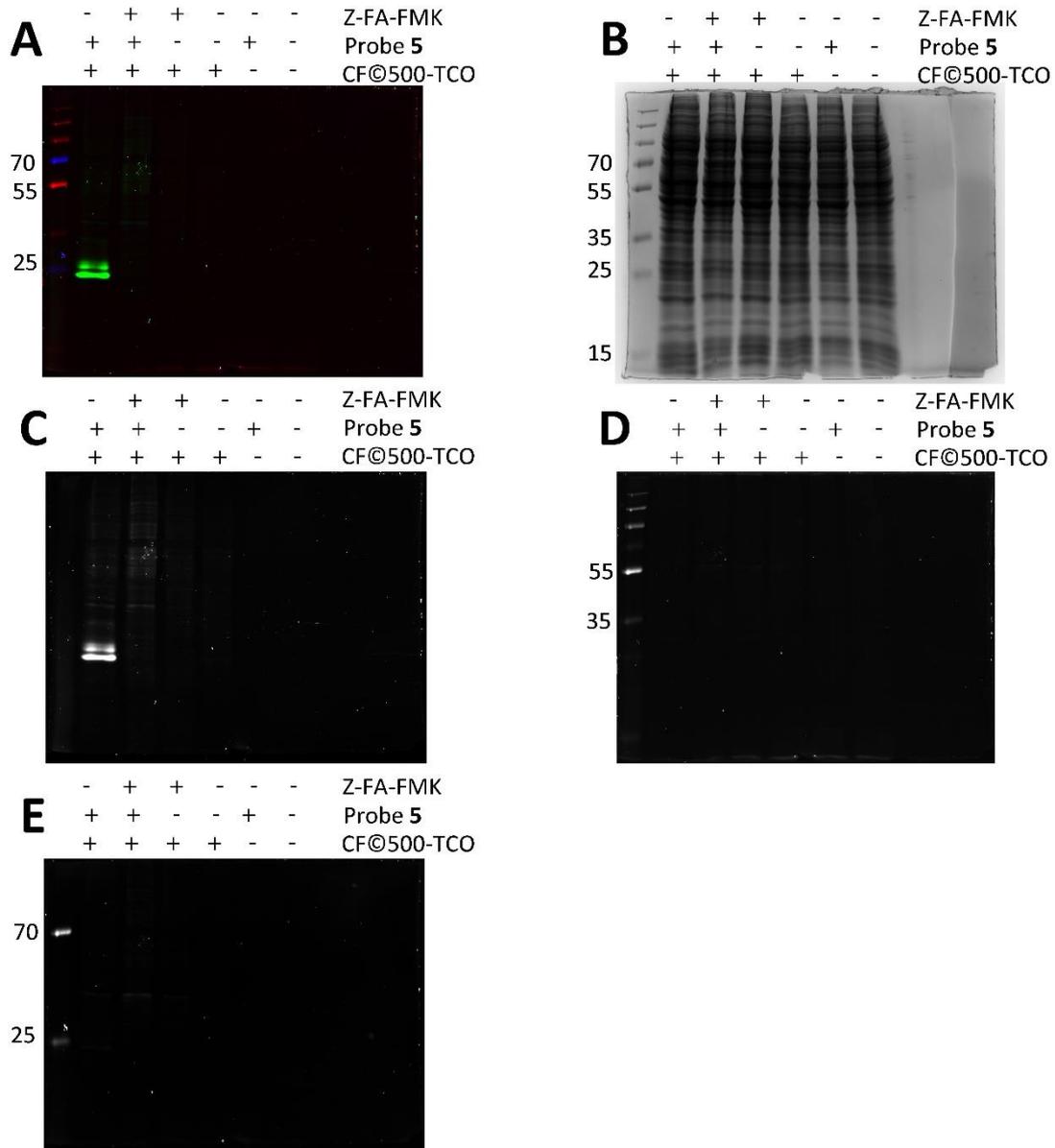
**SI Fig. 4.** Direct labeling in of probe 5 in Jurkat lysates. All concentrations are in  $\mu\text{M}$ . **(A)** Imaging of the Cy3 channel. **(B)** Imaging in the Cy5 channel. **(C)** A composite of the Cy5 (in red) and Cy3 channel (in green). **(D)** The same gel stained with CBB.



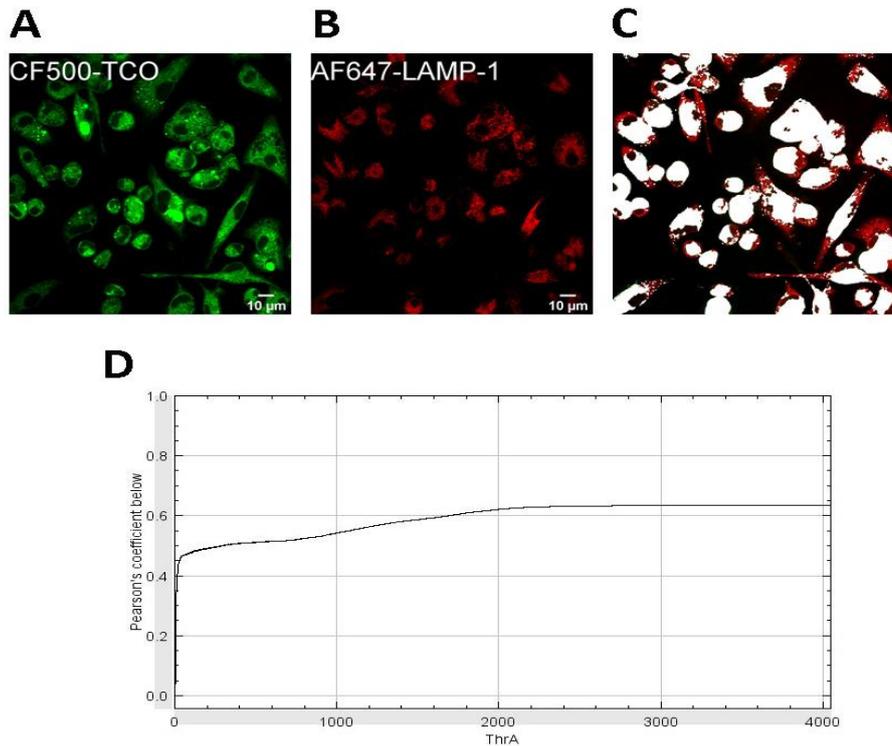
**SI Fig. 5.** Cell viability assay of Jurkat cells incubate with 0,1, 0,5 and 1  $\mu\text{M}$  of Z-FA-FMK or probe 5 for 24 h at 37°C, 5%  $\text{CO}_2$ . The MTT assay revealed that probe 5 is 22% more toxic compared to parent inhibitor Z-FA-FMK. Error bars represent the standard deviation. Data correspond to 1 independent experiments with 3 technical replicates.



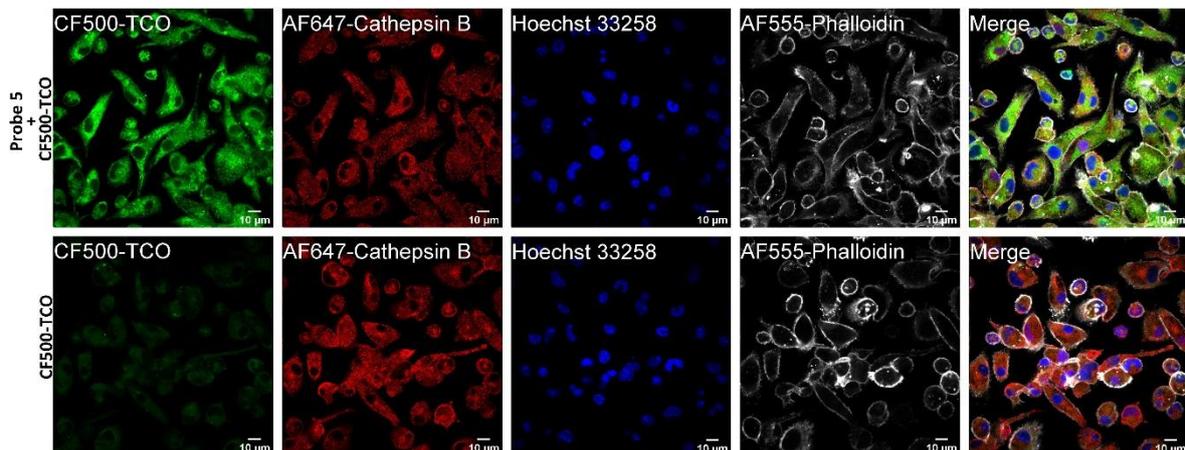
**SI Fig. 6.** Live cell labeling of Jurkat cells with sCy5-TCO as a fluorophore. Concentration of Z-FA-FMK is 5  $\mu$ M, probe 5 is 2  $\mu$ M, TAMRA-DCG-04 is 1  $\mu$ M and sCy5-TCO is 2  $\mu$ M. **(A)** Imaging of the Cy3 channel. **(B)** Imaging in the Cy5 channel. **(C)** A composite of the Cy5 (in red) and Cy3 channel (in green). **(D)** The same gel stained with CBB.



**SI Fig. 7.** Live cell labeling of Jurkat cells with CF@500-TCO as a fluorophore. After probe and fluorophore incubation, the Jurkat cells were lysed and analyzed by SDS-PAGE. The concentration of Z-FA-FMK, probe 5, as well as CF@500-TCO was 2  $\mu$ M. **(A)** Fluorescent scan (AF488, Cy3 and Cy5 channels). **(B)** CBB-stained gel. Separate imaging channels are shown as **(C)** AF488, **(D)** Cy5, and **(E)** Cy3.



**SI Fig 8.** Colocalization analysis of CF@500-TCO and LAMP-1. BMDCs were incubated for 2 h with probe 5 and CF@500-TCO. After uptake, BMDCs were fixed with 2% PFA and processed for immunofluorescence staining with LAMP-1. **(C)** Topological map of the colocalization distribution is given by the overlay of the green **(A)** CF@500-TCO and red **(B)** AF657-LAMP-1 channels. **(C)** The mask of the colocalizing pixels is shown in white. **(D)** Pearson's coefficient was used to measure the dependency of pixels in the green and red-channels. This fluorogram displays the intensity of pixels in the CF@500-TCO channel (ThrA) and the intensity of the corresponding pixels in the AF647-LAMP-1 channel (y-coordinate).

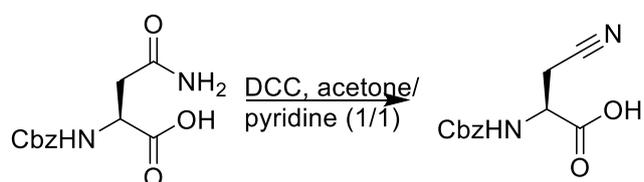


**SI Fig 9.** Imaging of probe 5 and CF@500-TCO ligation by confocal microscopy. BMDCs were incubated for 2 h with probe 5 and CF@500-TCO or CF@500-TCO. After uptake, BMDCs were fixed with 2% PFA and processed for immunofluorescence staining with Cathepsin B (red). The nucleus was stained with Hoechst 33258 (blue) and actin was stained using Phalloidin AF555 (gray). Scale bar is 10 µm (white bar, right corner).

## EXPERIMENTAL METHODS

All commercially available reagents and solvents were used as received unless stated otherwise. Reaction progress was monitored by thin layer chromatography (Sigma, TLC Silica gel 60 F254) via UV visualization ( $\lambda = 254$  nm) and/or ninhydrin stain. All column chromatography purifications were performed using solvents as received and silica gel (Macherey-Nagel, Kieselgel 60 M, 0.04 – 0.63 mm). LC-MS analysis was performed on a Finnigan Surveyor HPLC system (detection at 200-600 nm) with an analytical C18 column (Gemini, 50 x 4.6 mm, 3  $\mu$ m particle size, Phenomenex) coupled to a Finnigan LCQ Advantage MAX ion-trap mass spectrometer (ESI+). Solvent system: A: water (gradient, 10-90%), B: Me-CN (gradient, 90-10%), C: (constant, 10%) 1% TFA (aq). High resolution mass spectrometry (HRMS) spectra were recorded by direct injection (2  $\mu$ L of a 1  $\mu$ M solution in CH<sub>3</sub>CN/H<sub>2</sub>O/tBuOH) on a Thermo Scientific Q Exactive HF Orbitrap mass spectrometer equipped with an electrospray ion source in positive-ion mode (source voltage 3.5 kV, sheath gas flow 10, capillary temperature 275 °C) with resolution  $R = 240.000$  at  $m/z$  400 (mass range  $m/z$  160 – 2000) correlated to an external calibration (Thermo Finnigan). <sup>1</sup>H and <sup>13</sup>C NMR spectra were recorded using a Bruker AV-400 (400/101 MHz). Recorded data was interpreted and analyzed using MestReNova software.

### Boc-Cyanoalanine-OH (1)

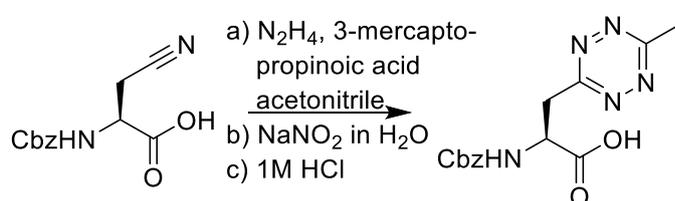


Z-Asn-OH (1.331 g, 5 mmol) was dissolved in 12 mL acetone/pyridine (1/1). DCC (1.135 g, 5.5 mmol) was added, resulting in a suspension. The reaction mixture was stirred for 4 hours at room temperature, after which the reaction mixture was filtered. The filtrate was concentrated and dissolved in 100 mL 0.1 M K<sub>2</sub>CO<sub>3</sub> and the water layer was extracted 3 times with 100 mL DCM. The water layer was acidified using 1M HCL solution till a pH of ~4. The water layer was extracted with 3 times 100 mL DCM, and washed three times with water. The organic layer was dried over MgSO<sub>4</sub>, filtered and concentrated, giving the product as a white powder in a 92% yield (1.14 g, 4.6 mmol)

<sup>1</sup>H NMR (400 MHz, MeOD)  $\delta$  7.40 – 7.28 (m, 5H), 5.14 (s, 2H), 4.55 (dd,  $J = 8.0, 5.3$  Hz, 1H), 3.09 – 2.91 (m, 2H).

<sup>13</sup>C NMR (101 MHz, MeOD)  $\delta$  170.86, 156.87, 136.53, 128.20, 127.78, 127.52, 117.11, 66.64, 50.41, 20.06.

### Z-Methyltetrazinealanine-OH (2)



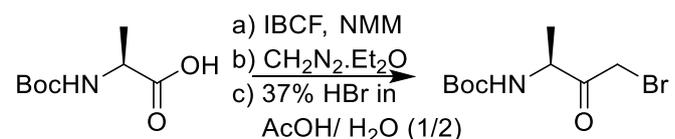
Boc-Cyanoalanine-OH (1) (0.248 mg, 1 mmol), 3-mercaptopropionic acid (87  $\mu$ L, 1 mmol), acetonitrile (522  $\mu$ L, 10 mmol) and hydrazine hydrate (~1 mL, 16 mmol) were added to a microwave tube. The tube was closed and the reaction mixture was stirred overnight at 40 °C. The reaction mixture was cooled down to 0 °C and the seal was carefully punctured. The mixture was added dropwise to a cold 1M NaNO<sub>2</sub> solution. The pH was adjusted to 3-4 by adding 1M HCl in dropwise fashion, ensuring the reaction temperature remained at 0 °C till. During addition of the HCl, gas formation was observed,

and the reaction mixture turned to a bright red. Next, the reaction mixture was extracted using DCM. The organic fractions were combined and washed 3 times with brine. The organic layer was dried over  $\text{MgSO}_4$ , filtered and concentrated. The product was purified by column chromatography and isolated as a pink powder in 13% yield.

$^1\text{H}$  NMR (300 MHz, MeOD)  $\delta$  7.31 – 7.22 (m, 5H), 5.03 (s, 2H), 4.91 (dd,  $J$  = 8.5, 5.4 Hz, 1H), 3.88 (dd,  $J$  = 14.9, 5.5 Hz, 1H), 3.70 (dd,  $J$  = 14.9, 8.6 Hz, 1H), 2.98 (s, 3H).

$^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  172.44, 167.65, 166.86, 156.89, 136.61, 128.06, 127.58, 127.32, 66.28, 52.54, 36.57, 19.69.

### **Boc-Alanine-bromomethyl ketone (3)**

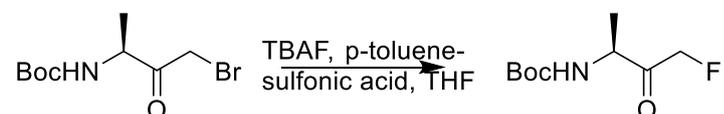


Boc-Ala-OH (0.131 g, 1.65 mmol) was dissolved in 25 ml THF and cooled to  $-10\text{ }^\circ\text{C}$ . To this solution *N*-methylmorpholine (NMM, 0.2 mL, 1.89 mmol) and (0.84 ml, 0.89 g) isobutyl chloroformate (IBCF, 0.26 mL, 2 mmol) were added and the mixture was stirred for 1 hour. A white precipitate was formed upon the addition of IBCF. Diazomethane was generated *in situ* using the procedure described in the Aldrich Technical Bulletin (AL-180)<sup>1</sup>. Ethereal diazomethane (around 10 mmol) was transferred to the stirred solution of the mixed anhydride at  $0\text{ }^\circ\text{C}$ . The reaction mixture was warmed to room temperature over the course of 3 hours. Next, 10 mL of a 1:2 solution of hydrogen bromide (30 wt. % solution in acetic acid) and water was added to the reaction mixture at  $0\text{ }^\circ\text{C}$ . Immediately after the evolution of nitrogen gas stopped, the reaction mixture was diluted with ethyl acetate and transferred to a separatory funnel. The reaction mixture was washed 3 times with water, brine solution, and saturated aqueous  $\text{NaHCO}_3$ . The organic layer was dried over  $\text{MgSO}_4$ , filtered and concentrated. The product was purified by column chromatography (25% ethyl acetate in petroleum ether (40-60)) and isolated as a yellow solid in a 74% yield (0.322 g, 1.2 mmol).

$^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ )  $\delta$  5.58 (d,  $J$  = 7.2 Hz, 1H), 4.29 (p,  $J$  = 7.1 Hz, 1H), 4.01 (d,  $J$  = 2.8 Hz, 2H), 1.24 (s, 9H), 1.19 (d,  $J$  = 7.2 Hz, 4H).

$^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ )  $\delta$  201.69, 155.30, 79.83, 53.41, 32.34, 28.15, 17.06.

### **Boc-Alanine-fluoromethyl ketone (4)**

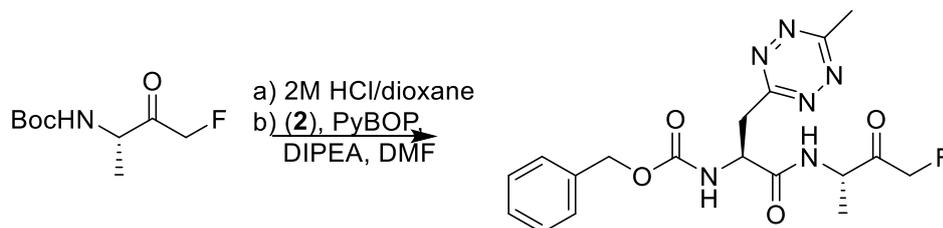


Boc-Alanine-bromomethyl ketone (3) (0.322 g, 1.2 mmol) was dissolved in 3 mL THF and *p*-toluenesulfonic acid (0.630 g, 3.66 mmol) was added. The solution was slowly added to a solution of 1M TBAF in THF solution (7,3 mL). The reaction mixture was refluxed at  $70\text{ }^\circ\text{C}$  for 3.5 hours. After completion of the reaction on TLC, the reaction mixture was concentrated and dissolved in 20 mL ethyl acetate. The organic layer was washed with 3 times brine and dried over  $\text{MgSO}_4$ , filtered and concentrated. The product was purified by column chromatography and isolated as a white solid in 33% yield (81.9 mg, 0.4 mmol).

$^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  5.12 – 4.93 (m, 2H), 4.58 (q,  $J$  = 7.8 Hz, 1H), 1.45 (s, 9H), 1.37 (d,  $J$  = 7.2 Hz, 3H).

$^{13}\text{C}$  NMR (75 MHz,  $\text{D}_2\text{O}$ )  $\delta$  205.49, 155.13, 84.92, 82.47, 52.15, 28.24, 17.57.

### **Carboxybenzyl-Phenylalanine-Alanine-fluoromethyl ketone (5)**



Boc-Alanine-fluoromethyl ketone (**4**) (3.3 mg, 31  $\mu\text{mol}$ ) was dissolved in 2M HCl/dioxane and stirred at room temperature for 2 hours. The reaction was concentrated and dissolved in DMF. Z-Methyltetrahydropyridine-4-yl-OH (**2**) (9.8 mg, 31  $\mu\text{mol}$ ), PyBOP (37 mg, 72  $\mu\text{mol}$ ) and DIPEA (21  $\mu\text{L}$ , 120  $\mu\text{mol}$ ) were added and the reaction mixture was left for 3 hours at room temperature. The reaction mixture was concentrated and dissolved in 10 mL DCM. The organic layer was washed 3 times with saturated aqueous  $\text{NaHCO}_3$  and brine. The product was purified by HPLC and isolated as a pink powder in a 12% yield (1.53 mg, 3.8  $\mu\text{mol}$ ).

$^1\text{H}$  NMR (500 MHz,  $\text{CDCl}_3$ )  $\delta$  7.34 (q,  $J = 7.4, 6.3$  Hz, 6H), 7.26 – 7.21 (m, 1H), 6.17 (d,  $J = 8.6$  Hz, 1H), 5.09 (d,  $J = 5.4$  Hz, 2H), 5.08 – 4.98 (m, 2H), 4.94 – 4.88 (m, 1H), 4.79 (q,  $J = 7.5, 7.0$  Hz, 1H), 3.79 (t,  $J = 5.6$  Hz, 2H), 3.04 (s, 3H), 1.40 – 1.32 (m, 3H), 1.31 (s, 3H).

$^{13}\text{C}$  NMR (126 MHz,  $\text{CDCl}_3$ )  $\delta$  204.57, 170.17, 167.99, 166.70, 128.69, 128.49, 128.21, 84.53, 83.06, 67.64, 52.85, 51.60, 37.00, 30.94, 21.16, 16.44.

$^{19}\text{F}$  NMR (471 MHz,  $\text{CDCl}_3$ )  $\delta$  -75.93.

### **Cell culture**

#### **Jurkat cells**

The human leukemic T cell line, Jurkat cells were grown in RPMI-1640 (Sigma-Aldrich) supplemented with 10% heat inactivated FCS (Fetal Calf Serum), 2mM glutamax<sup>TM</sup> (GIBCO), 50 IU/ml penicilline, 50  $\mu\text{g}/\text{ml}$  streptomycin and 50  $\mu\text{M}$  2-mercaptoethanol (Gibco) at 37 °C and 5%  $\text{CO}_2$ . The cells were maintained in logarithmic phase of growth and passaged every 3 days.

#### **Bone Marrow Dendritic Cells (BMDCs)**

The BMDCs were differentiated and cultured as previously described.<sup>2</sup> Briefly, bone marrow cells were harvested from the femurs and tibias of eight to twelve-week-old male C57BL/6 mice. The ends of the bones were removed and the marrow collected by centrifugation at 300 rcf. spun down. The suspension was collected, passed through a 70  $\mu\text{m}$  filter, and spun down at 1200 rpm for 5 min. The supernatant was discarded, and the cells were treated with Ammonium-Chloride-Potassium Lysis Buffer (ACK) for 5 min, quenched by adding 10 ml of PBS and spun down. The cells were then resuspended in 10 ml IMDM medium containing 10% FCS, 2 mM glutamax<sup>TM</sup> (GIBCO), 50 IU/ml penicillin, 50  $\mu\text{g}/\text{ml}$  streptomycin, 50  $\mu\text{M}$  2-mercaptoethanol and 20 ng/mL recombinant mouse GM-CSF (Immunotools). Fresh medium was added on day 2, loosely adherent cells were split on day 4 and used for experiments on day 8.

#### **RAW 264.7 cells**

The RAW cells were cultured in DMEM high glucose (Gibco<sup>®</sup>) supplemented with glutamine (GlutaMAX<sup>TM</sup>), sodium pyruvate and phenol red, 10% fetal calf serum, and antibiotics (100 units/ml

penicillin, 100 µg/ml streptomycin) at 37°C, and 5%CO<sub>2</sub>. The cells were passaged every 2–3 days. The cells were suspended via a cell-scraper.

### **Cell viability assay**

A MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) assay was performed to ensure the concentration of probe **5** used in the experiments was not cytotoxic. Briefly, Jurkat cells (2x10<sup>5</sup> cells per well) were seeded per triplicate in a 96-well plate (Sarstedt). Then, 0,1, 0,5 and 1µM of probe **5** or Z-FA-FMK were added. As a negative control, Jurkat cells on RPMI 1640 complete medium were used. In addition, DMSO (1%) was used as a positive control for toxicity.<sup>3</sup> Cells were incubated for 24 hours at 37 °C and 5% CO<sub>2</sub>, and 95% humidity.

The medium was removed after spun down the cells three minutes at 300xg at room temperature. Next, 90 µL of RPMI 1640 complete medium and 10 µL of MTT (final concentration of 0.5 mg/ml in PBS) were added to each well and incubated for 4h at 37 °C, 5% CO<sub>2</sub> and 95% humidity. The formed formazan was dissolved with 100 µl of DMSO per well and incubated 2 min with constant shaking (400 rpm). The absorbance of the formazan was measured at 570 nm using a CLARIOstar microplate reader. Results were normalized between untreated cells as 100% and only media as background signal.

### **Lysate preparation**

Jurkat cells (5 x10<sup>7</sup>) were washed twice with cold PBS (Sigma-Aldrich). Subsequently, 50 µl of NaOAc lysis buffer (50 mM NaOAc, 150 mM NaCl, 1 mM DTT, 0,5% NP40) was added. The cells were incubated for 3 hours at 4 °C with constant shaking at 550 rpm. The sample was spun down at 20,238 rcf for 5 minutes at 4 °C. The protein concentration was measured using a Bradford assay (Biorad). The supernatant was frozen and stored at -80 °C for further experiments.

### **Competitive cathepsin labeling in Jurkat T-cell lysates**

Lysate was diluted to a concentration of 1 µg protein/µL using NaOAc lysis buffer and aliquoted to 20 µL per sample. 0.2 µL of the respective probe concentration in DMSO was added to each sample and all samples were incubated for 30 minutes at room temperature. After the first incubation, 0.2 µL of 100 µM DMSO stock of TAMRA-DCG-04 was added to each sample to a final concentration of 1 µM and was incubated for an additional 30 minutes at room temperature. After the last incubation, 6.7 µL of 4x SDS loading buffer was added to each sample and boiled at 95 °C for 5 minutes. After separation by SDS-PAGE, ABP labeling was assessed by a ChemiDoc MP imaging system (Bio-Rad) (Cy3 channel, Green Epi Illumination, 602/50 Filter). Fluorescence intensities were quantified using ImageJ software. Apparent IC<sub>50</sub>s were calculated by Graphpad Prism using a non-linear fit.

### **Direct labeling in Jurkat T-cell lysates**

Lysate was diluted to a concentration of 1 µg protein/µL using NaOAc lysis buffer. To 20 µL of sample, 0.2 µL of different concentrations of probe **5** (0,1 µM, 1 µM, 10 µM final concentrations), Z-FA-FMK (1 µM final concentration) or DCG-04 (1 µM final concentration) were added and were incubated for 30 minutes at room temperature. Afterwards, sulfo-Cy5-*trans*-cyclooctene (sCy5-TCO, 2 µM final concentration) was added to the samples and incubated in the dark for an additional 30 minutes. After the last incubation, 6.7 µL of 4x SDS loading buffer was added to each sample and boiled at 95 °C for 5 minutes. After separation by SDS-PAGE, ABP labeling was assessed by a ChemiDoc MP imaging system (Bio-Rad) (Cy5 channel, Red Epi Illumination, 700/50 Filter and Cy3 channel, Green Epi Illumination, 602/50 Filter).

### **Live cell labeling with sCy5-TCO**

1.5x10<sup>7</sup> Jurkat cells were suspended in 500 µl RPMI and were incubated with either 2 µM Z-FA-FMK or probe **5** for 2 hours at 37 °C and 5% CO<sub>2</sub>. After incubation, the cells were washed three times with PBS. Subsequently, 20 µl of NaOAc lysis buffer (50 mM NaOAc, 150 mM NaCl, 1 mM DTT, 0,5% NP40) was added. The cells were incubated for 3 hours at 4 °C with constant shaking at 550 rpm. The sample was spun down at 20,238 rcf for 5 minutes at 4 °C. After lysis, each sample was incubated with sCy5-TCO (2µM final concentration) for 30 minutes. As a control, the lysate of 1.5x10<sup>7</sup> Jurkat cells without inhibitor was incubated with the TAMRA-DCG-04- (2µM final concentration). After the last incubation, 6.7 µL of 4x SDS loading buffer was added to each sample and boiled at 95 °C for 5 minutes. After separation by SDS-PAGE, ABP labeling was assessed by a ChemiDoc MP imaging system (Bio-Rad) (Cy5 channel, Red Epi Illumination, 700/50 Filter)

#### **Live cell labeling with CF@500-TCO**

1.5x10<sup>7</sup> Jurkat cells in 300 µl RPMI were incubated with either 2 µM Z-FA-FMK or probe **5** for 2 hours at 37 °C and 5% CO<sub>2</sub>. After incubation, the cells were washed three times with PBS at 37 °C. Next, the cells were incubated with CF@500-TCO (2 µM final concentration) for 2 hours at 37 °C and 5% CO<sub>2</sub>. After incubation, the cells were washed three times with PBS at 37 °C. After the last wash, 100 µL of 1x SDS loading buffer was added to the cell pellet and samples were boiled at 95 °C for 5 minutes. After separation by SDS-PAGE, ABP labeling was assessed by a ChemiDoc MP imaging system (Bio-Rad) (AF channel, Blue Epi Illumination, 532/28 Filter)

#### **Competitive labeling of Cathepsin B, S and L.**

RAW 264.7 cells were seeded 5E6 cell/ 500µl per well on 48-well plate in DMEM medium without serum. Cells were incubated at 37°C for 24 hours. Next, the cells were wash with DMEM medium without serum. Then cells were incubated 2.5h with different concentrations of cathepsin B, L or S inhibitors. After incubation the cells were washed 3 times with PBS. Cells were lysate with 20 µl of NaOAc lysis buffer. The cells were incubated for 3 hours at 4 °C with constant shaking at 550 rpm. The sample was spun down at 20,238 rcf for 5 minutes at 4 °C. After lysis, each sample was incubated with Probe **5** (10µM final concentration), and sCy5-TCO (2µM final concentration) for 30 minutes. Then, 2 µL of 4x SDS loading buffer was added to each sample and boiled at 95 °C for 5 minutes. After separate on by SDS-PAGE, ABP labeling was assessed by a ChemiDoc MP imaging system (Bio-Rad) (Cy5 channel, Red Epi Illumination, 700/50 Filter)

#### **Sample staining**

BMDCs were seeded 2000 cell/µl per well on 8-well chamber slides (ibiTreat) in phenol red-free IMDM medium. The BMDCs were incubated with 100 ng/mL of lipopolysaccharide (LPS) overnight. We washed the cells three times with phenol red-free IMDM medium. Then, cells were incubated with 10 ng/mL LPS to increase cathepsin activity together with 2µM of probe **5** for 2 hours at 37 °C and 5% CO<sub>2</sub>. Cells were washed with PBS three times after each incubation step. CF@500-TCO (2 µM) was added and incubated for 2 hours to wells previously incubated with probe **5**. After incubation with Probe **5** + CF@500-TCO or DMSO+CF@500-TCO, the cells were fixed with 2% PFA for 30 minutes at room temperature (RT). We included as controls samples stained with FA-FMK (2µM), Probe **5** (2µM) and CF@500-TCO (2 µM), as well as samples only stained with FA-FMK (2µM) and CF@500-TCO (2 µM). After fixation, cells were washed and glycine (20mM) in PBS was used to quench unreacted aldehyde. Then cells were permeabilized with 0.01% saponin in PBS for 20 minutes at RT. Afterwards the cells were stained for F-actin using 5 µL AlexaFluor™555 (Life Technologies) in 200 µL 1% BSA in PBS/well for 30 minutes in the dark. Lysosomal membrane was stained with LAMP-1 (Biolegend, clone 1D4B, 1:200 dilution, staining in 1% BSA, in PBS) at concentration of 0.5 mg/mL for 1 hour at RT. Lastly, the nuclei were stained with Hoechst 33258 (2µg/ml) for 10 minutes. Samples were imaged directly in

glycerol/DABCO mounting medium. For the staining with Cathepsin B, samples were first blocked 1h with 1%BSA in PBS. Then, incubated with polyclonal primary antibody (proteintech (12216-1-AP), 1:400 dilution in 1%BSA in PBS) for 2h at 4C. Afterward, the samples were washed 3 times with PBS and incubated with secondary antibody AF647-Conjugated Anti-Rabbit IgG (1:400 dilution in 1%BSA in PBS) for 2 h at room temperature.

### **Confocal microscopy**

The samples were imaged with a AR1 HD25 Nikon confocal microscope, using a CFI plan Apo Lambda 100x/Numerical Aperture (N.A)=1.45 oil objective. Hoechst 33258, CF@500-TCO, AlexaFluor™555-Phalloidin, AlexaFluor™ 647-LAMP-1 or Cathepsin-B were excited using the 405, 488, 561 and 647 nm laser lines, respectively, and images were acquired using the resonant scanner and the DU4 detector (495LP (450/50), 560LP (525/50), 640LP (595/50, 700/75)). Poisson noise was removed from the image by the built-in Nikon Denoise.ai software. Z-stacks were acquired from the samples with 0.2µm steps and are all presented as maximum intensity projections. Colocalization was calculated with the plug JACoP in imageJ.

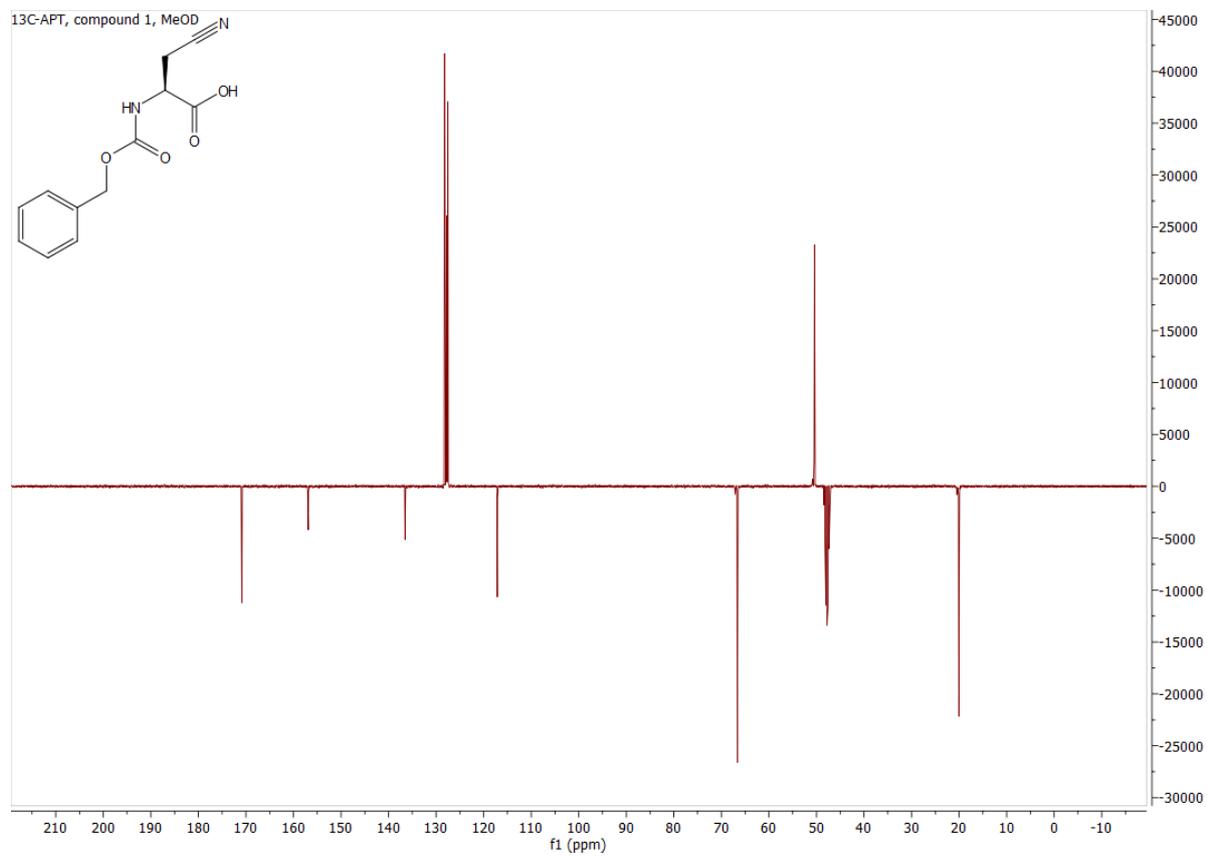
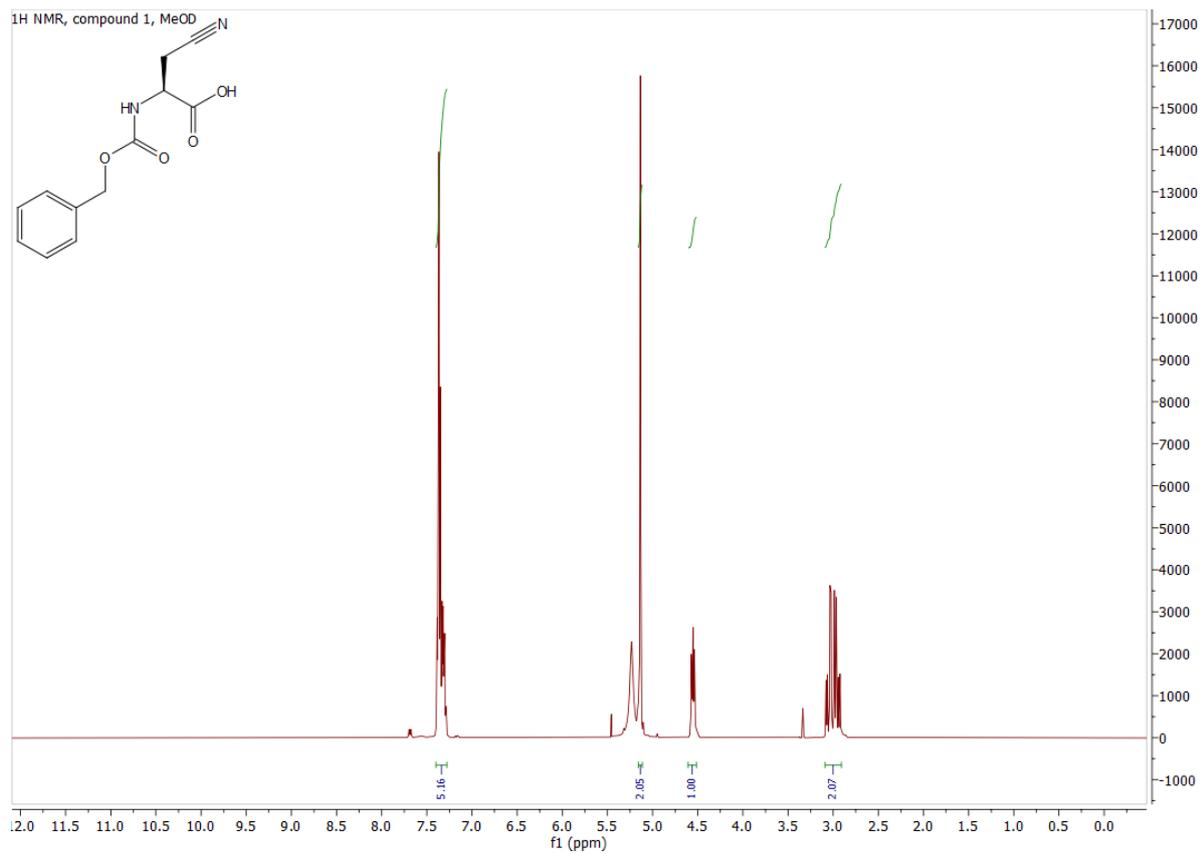
### **Cellular uptake**

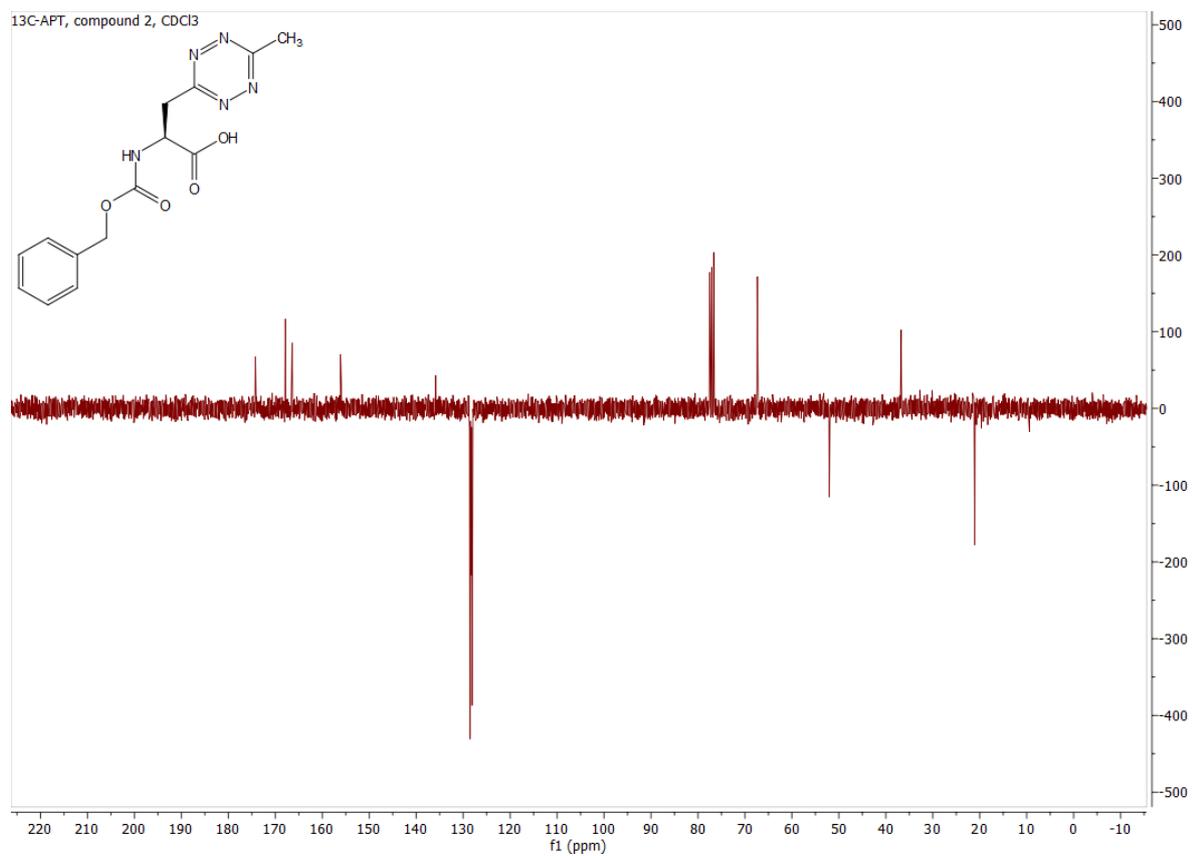
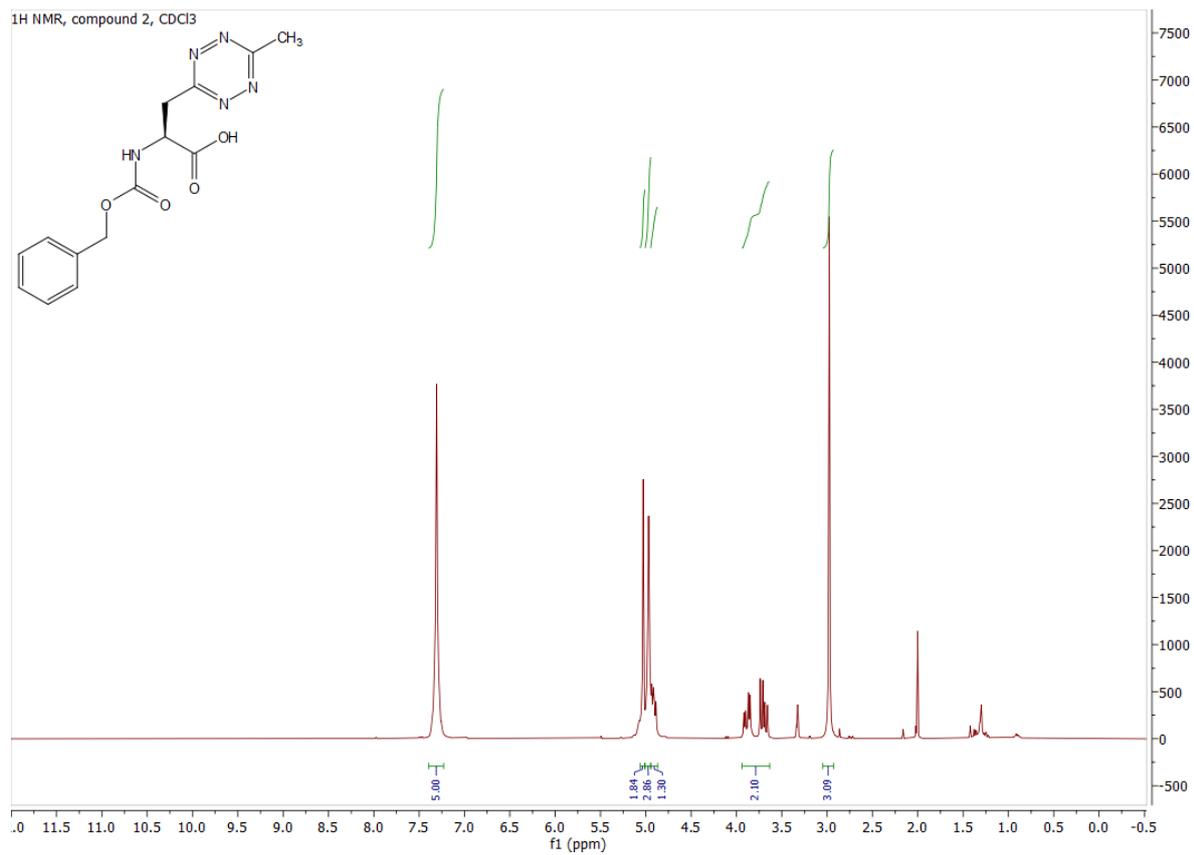
Samples were incubated with FA-FMK, Probe **5**, and CF@500-TCO as previously described in the sample staining section. Before fixation, the cells were incubated with live/dead Zombie-NIR dye for 15 min at RT (1:500, in PBS, biolegend). Then, cells were washed with FACS buffer (PBS, 2µM EDTA, 0.5%BSA) and resuspended in 100µl of FACS buffer. The samples were acquired in a Guava® easyCyte™ 12 HT Flow Cytometer (Luminex). Mean intensity fluorescence (MFI) was calculated for CF@500-TCO in live cells using flowjo.

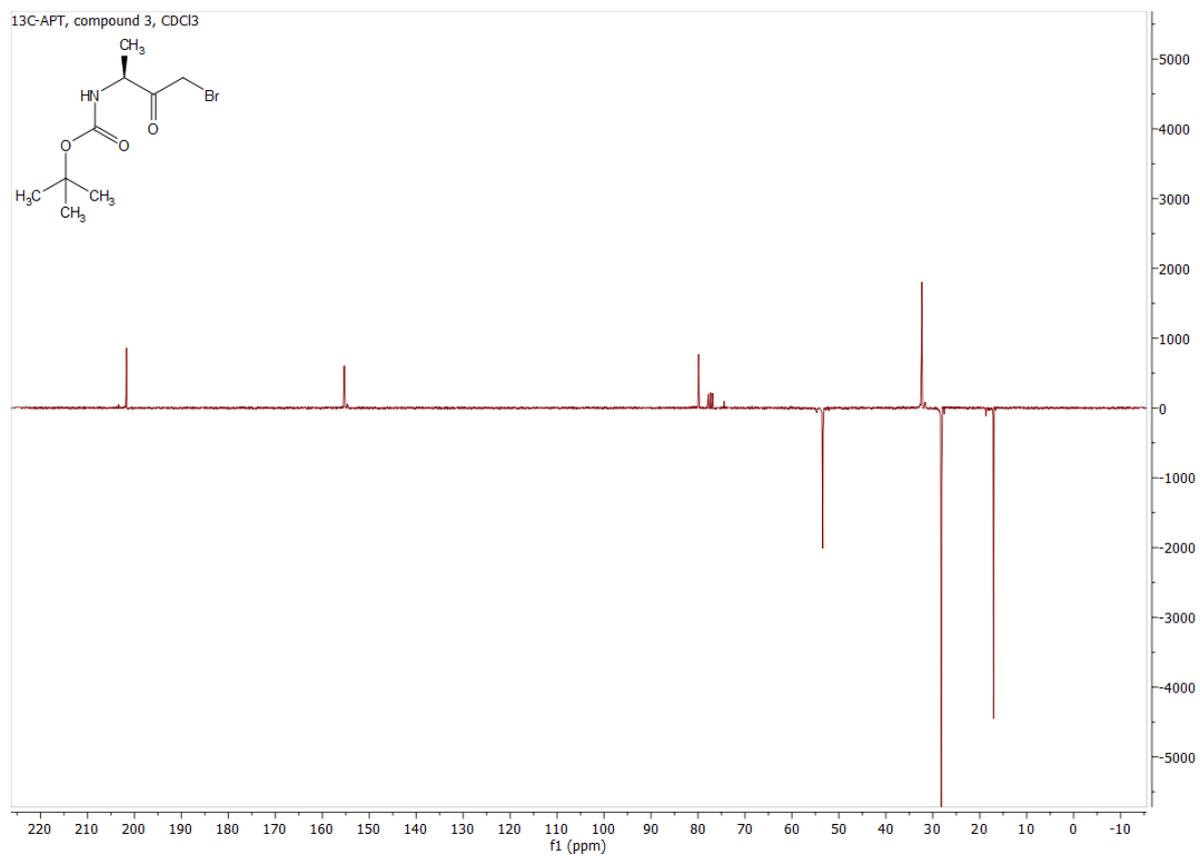
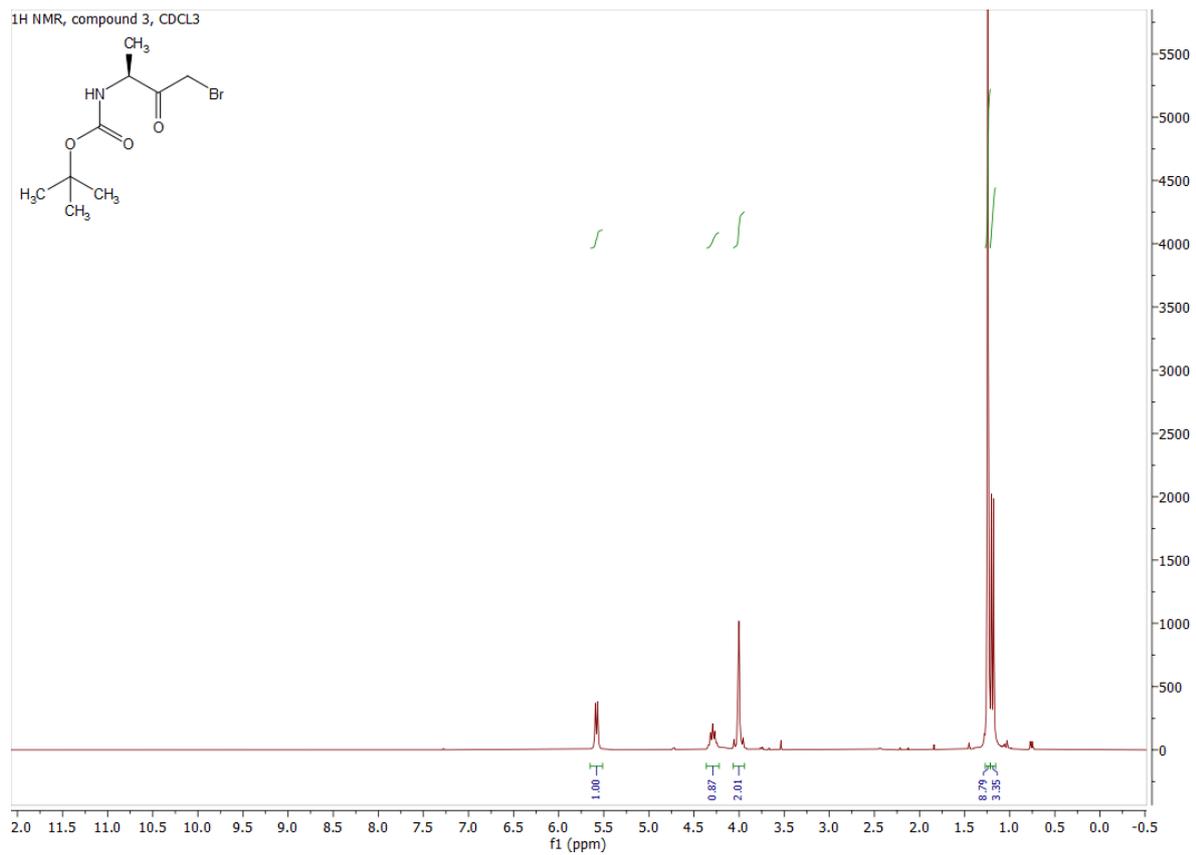
### **Statistical analysis**

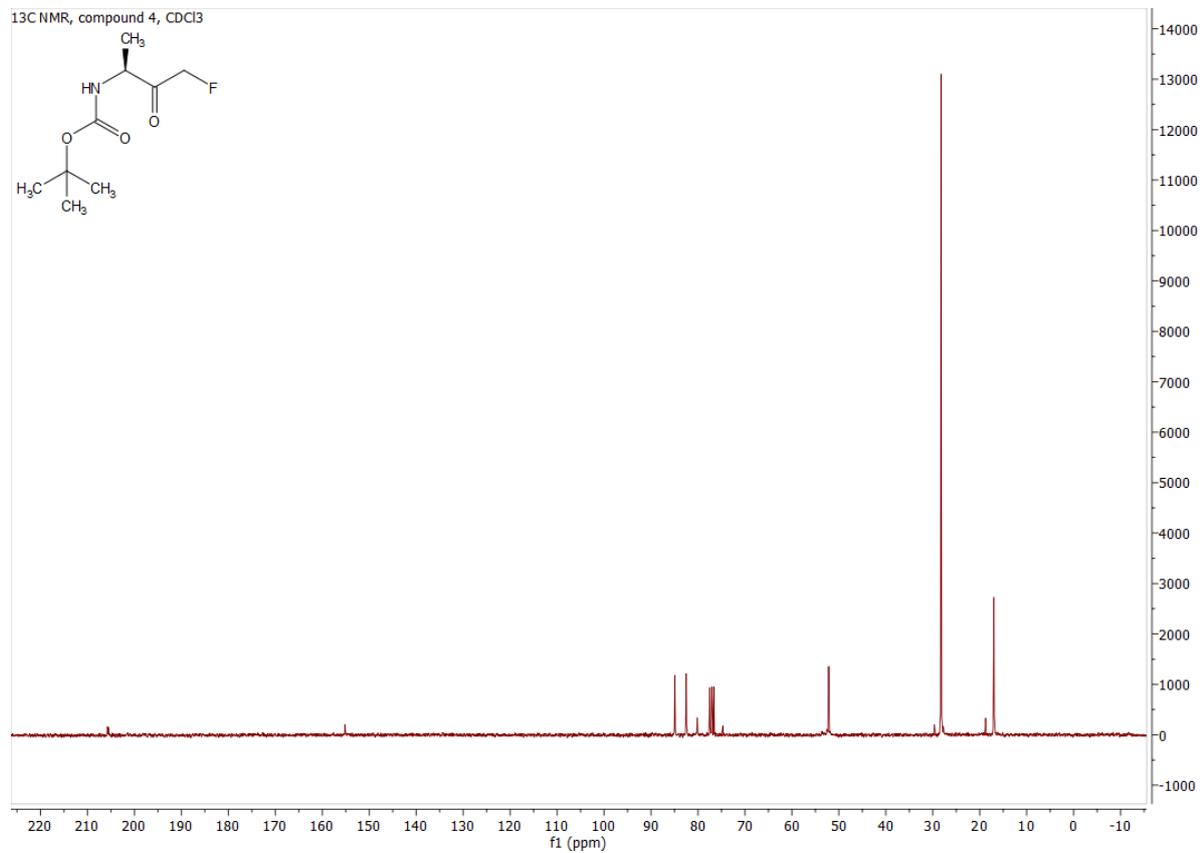
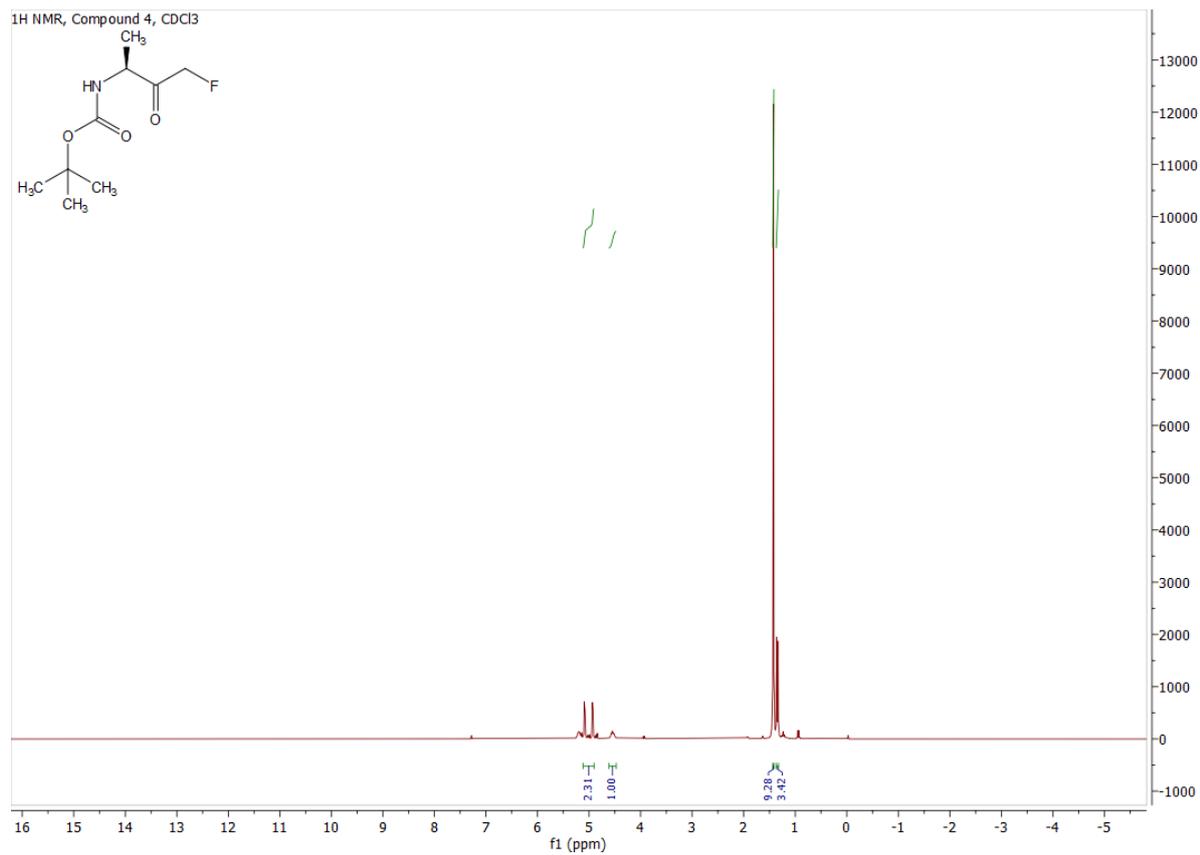
Mean and Standard Deviation (SD) were calculated for each variable studied. All graphics were performed using GraphPad Prism software, version 6.00 for MAC (GraphPad Software, La Jolla, California, USA, [www.graphpad.com](http://www.graphpad.com)).

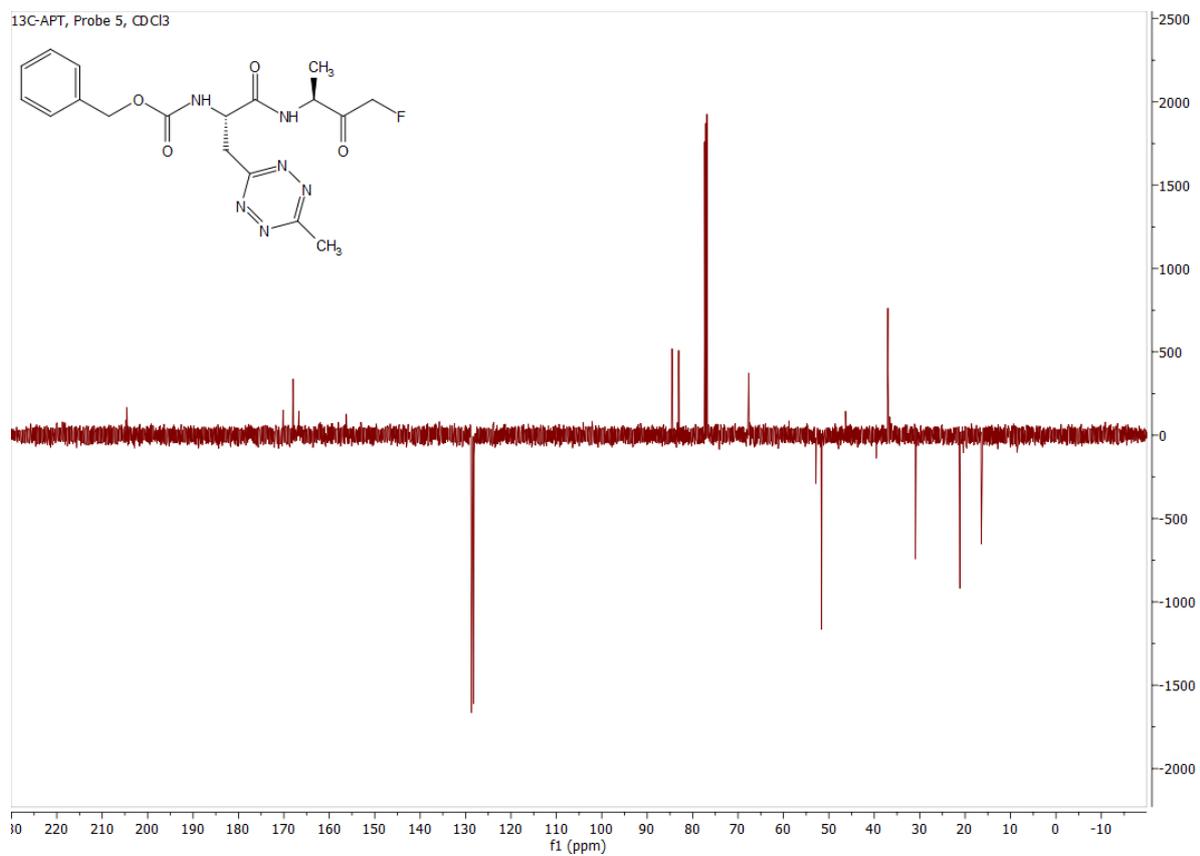
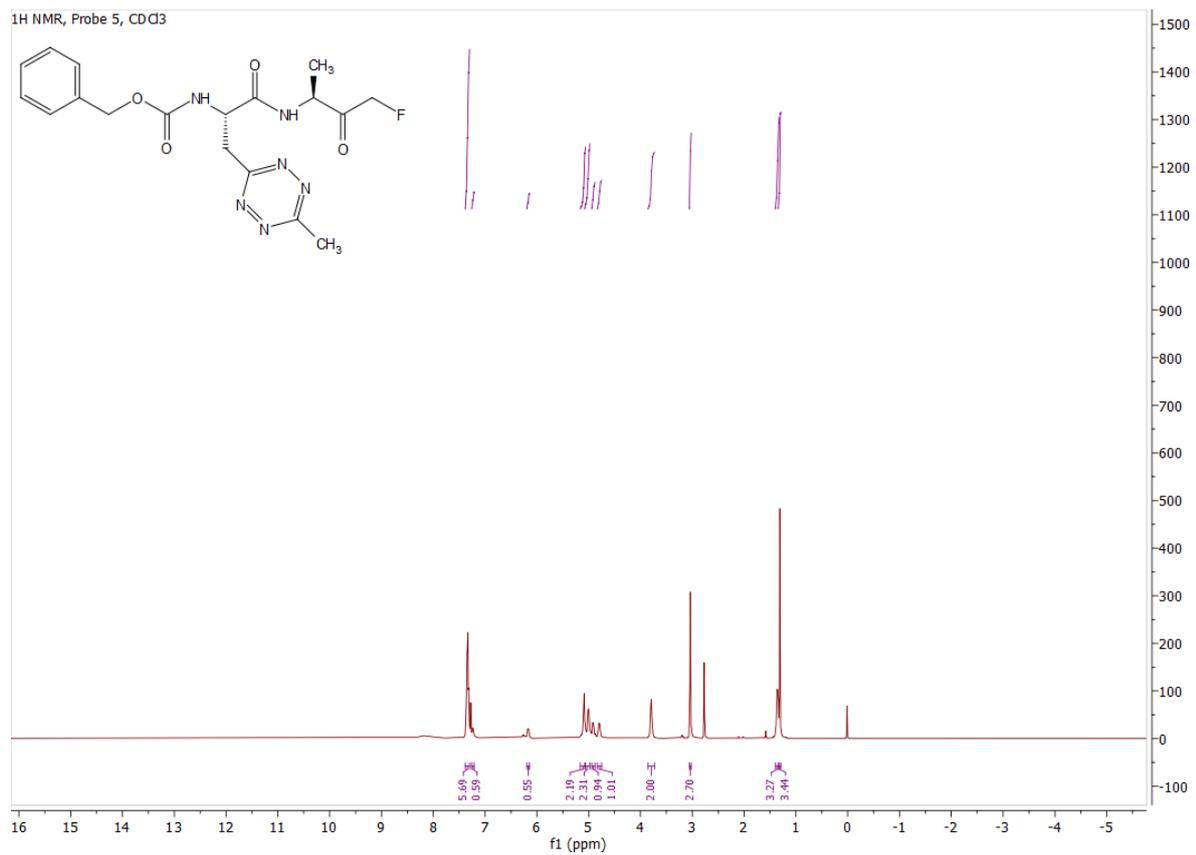
## NMR spectra

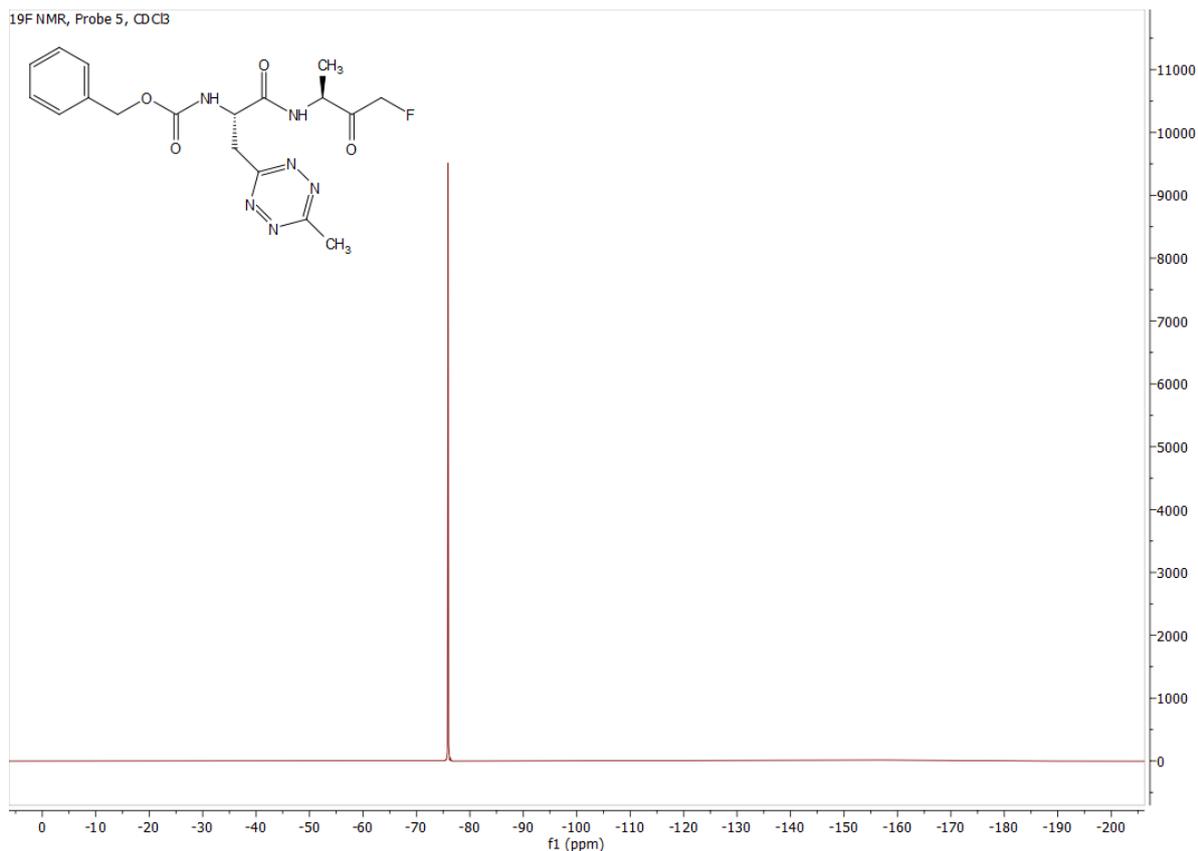












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