

A Multifunctional Protease Inhibitor to Regulate Endo-Lysosomal Function and Receptor Turnover

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Supplementary information

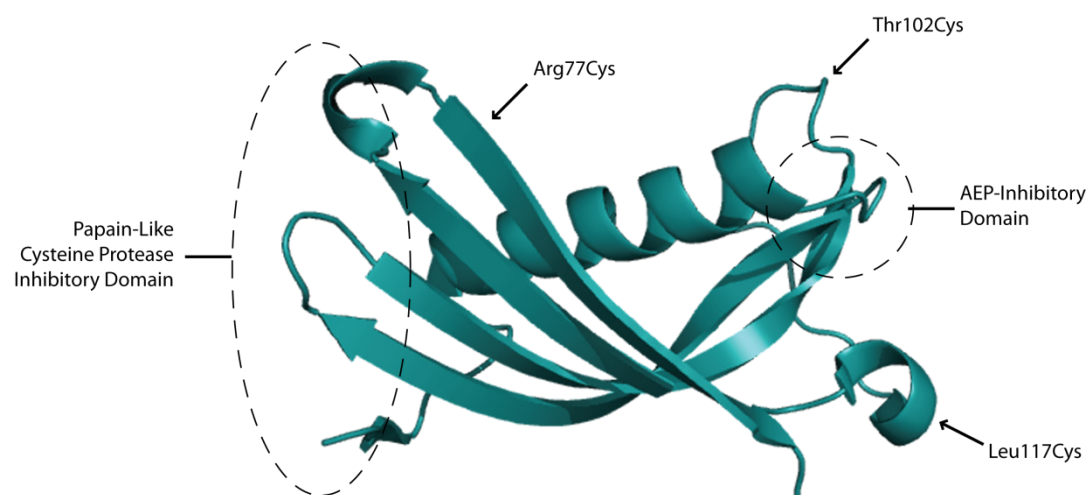


Figure S1a: Localisation of inhibition sites on cystatin C and potential solvent-accessible mutation positions. Three different cystatin mutants were tested. Mutation sites were picked for solvent accessibility, as well as for their location, with residues which are part of the inhibitory motifs (as highlighted by the dashed circles), excluded. We chose three mutations, one on a β -sheet (R77C), one on an α -helix (L117C) and on a non-structured loop region of the protein (T102C).

Parameter	T102C	L117C	R77C
Transient expression levels after purification	11 mg/L	7 mg/L	8 mg/L

Table S1b: Expression levels (after purification of the three mutants)

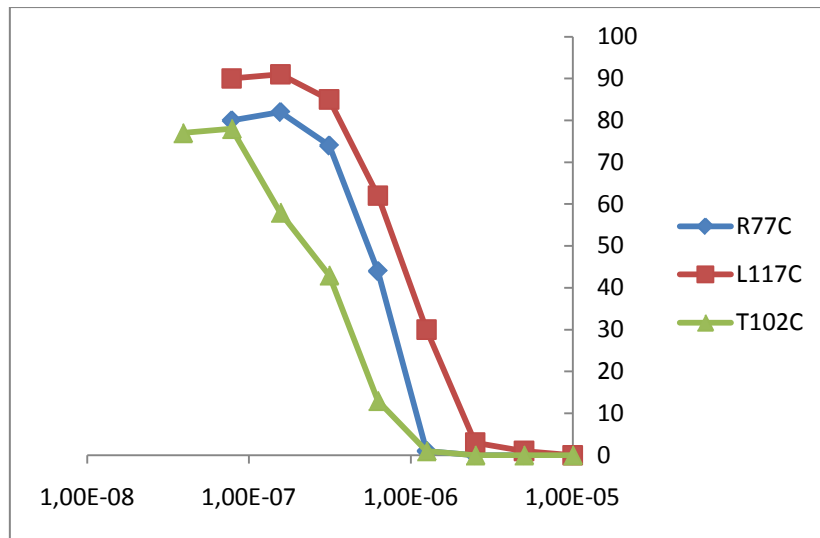
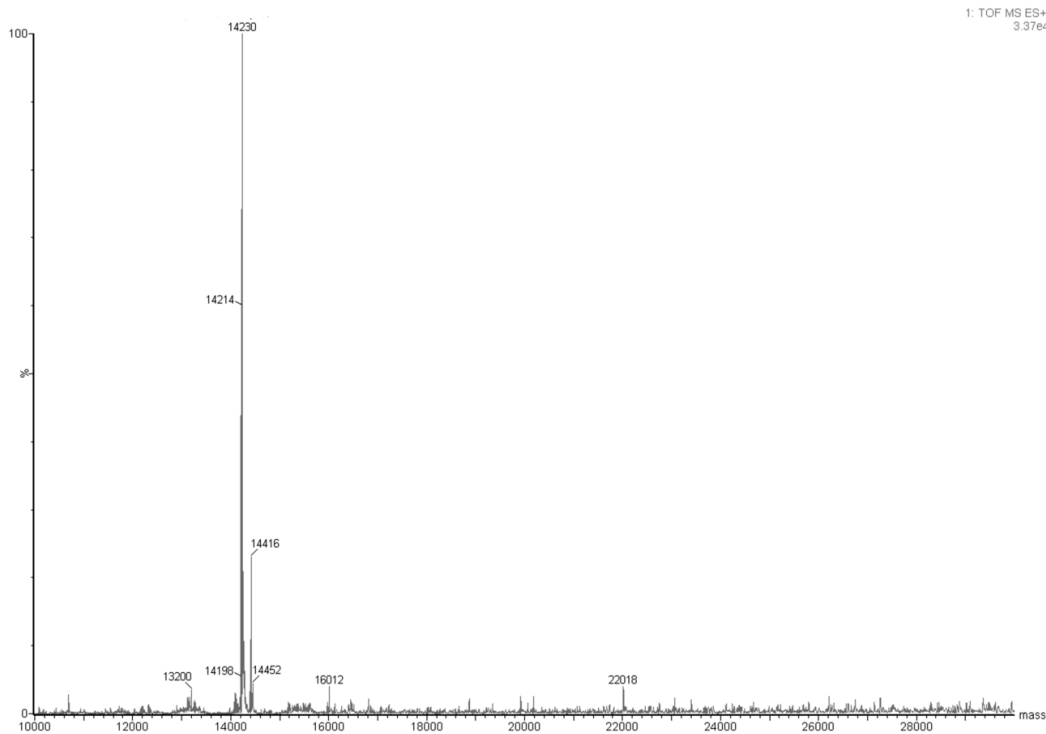


Figure S1c: relative inhibitory rates of the three mutants as determined by serial dilution of the various cystatin C mutants (concentration in mM) in presence of 5 ng of Cathepsin L/well.



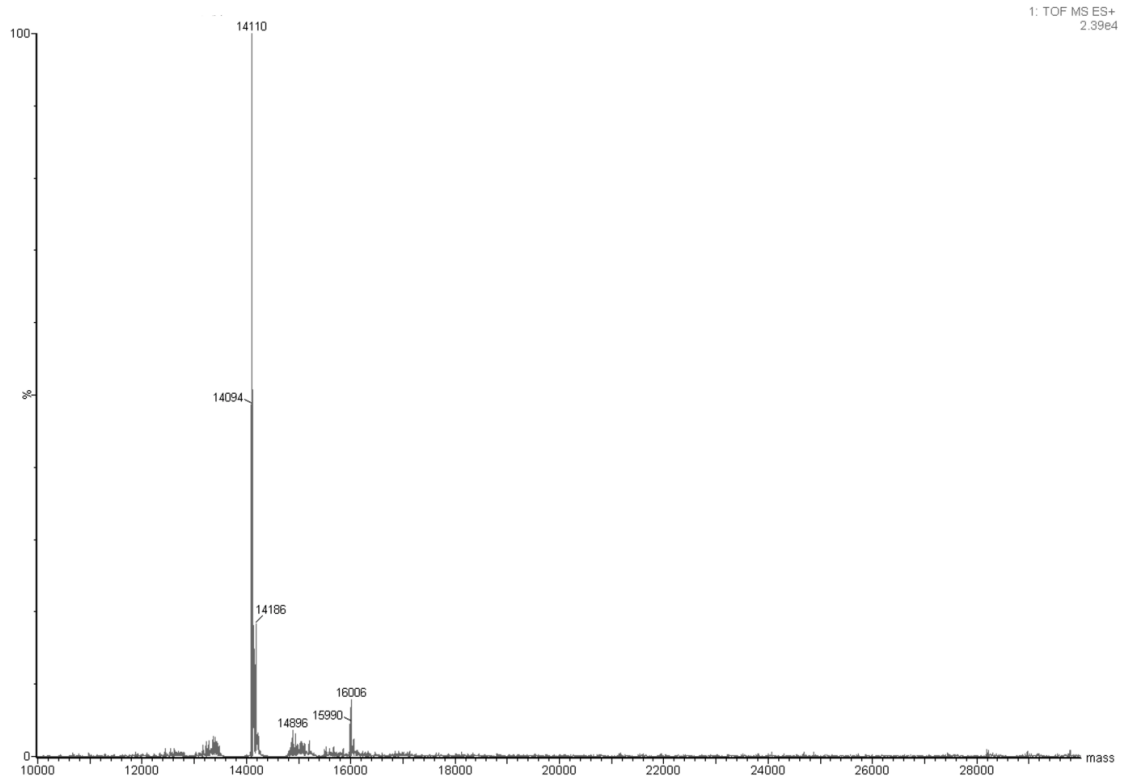


Figure S2: Cystatin C required a reduction step prior to the modification with the MTS-pepstatin reagents, as otherwise no modification was observed. Reduction using 5 mM DTT at room temperature for 10 minutes was sufficient for the disappearance of the oxidised species (mw 14,230) and the appearance of a single product (mw 14,110)

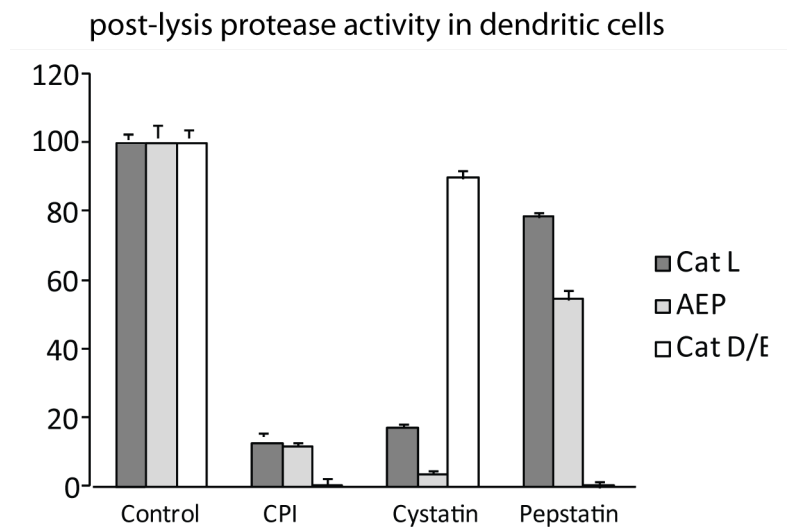


Figure S3: Post-lysis protease activity measured after incubation with various inhibitors in mouse BM-DCs. Cells were incubated with inhibitors, washed and lysed. Protease activity was determined with substrates specific for each of the families of proteases post-lysis. CPI is clearly shown to inhibit representative members of all three families, whereas cystatin C and pepstatin A only inhibit their respective target families. Initial rates of fluorescence emerge with the PBS-control values set to 100% have been plotted.

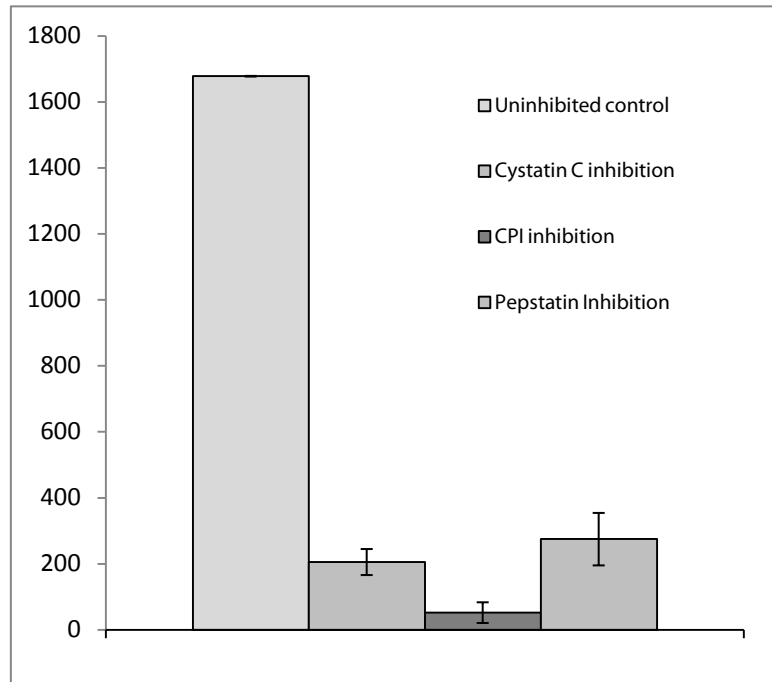


Figure S4: Inhibition of proteolysis of Bodipy-modified casein (enzchek substrate) by cystatin C, pepstatin A and CPI. It can be seen that CPI more strongly inhibits the fluorescence dequenching (resulting from proteolysis) than do cystatin c and pepstatin A alone. Initial rates of fluorescence emergence are plotted (0-2hours)

Experimental information

Purified human Cystatin C was purchased from Genway (11-511-248839). Anti-CD63 was a kind gift from professor J.J. Neefjes, Recombinant cathepsins came from R&D systems. Pepstatin A and fluorescent substrates were purchased from Bachem. N-Boc-L-Cysteine Methanethiosulfonate was purchased from Toronto Research Chemicals (B646250). Bodipy-modified casein was purchased from in Vitrogen.

Cell Culture

DHFR-negative CHO cells were grown in DMEM-based medium containing 10% dialysed FCS, 5 mM Glutamine and 0.1 mM hypoxanthine and 0.01 mM thymidine. HeLa cells were grown in DMEM supplemented with 10% FCS. A20 cells were grown in RPMI supplemented with 10% FCS and 5 mM L-glutamine. PBMN-DCs and macrophages were grown and isolated as described previously [reference].

Cloning and Expression of Cystatin C-T102C-6His mutant

The Cystatin C was amplified as described previously¹ using the following primers: *CysCFor* (caggattacaattggtaccatggccgggcccc) and *CysCRev* (gcctactcgcagcttaatgatgatgatgatggtcctgacag) to introduce a C-terminal 6-Histidine tag. The amplification product was cloned into a pcDNA-DHFR vector used previously² between XhoI and KpnI restriction sites.

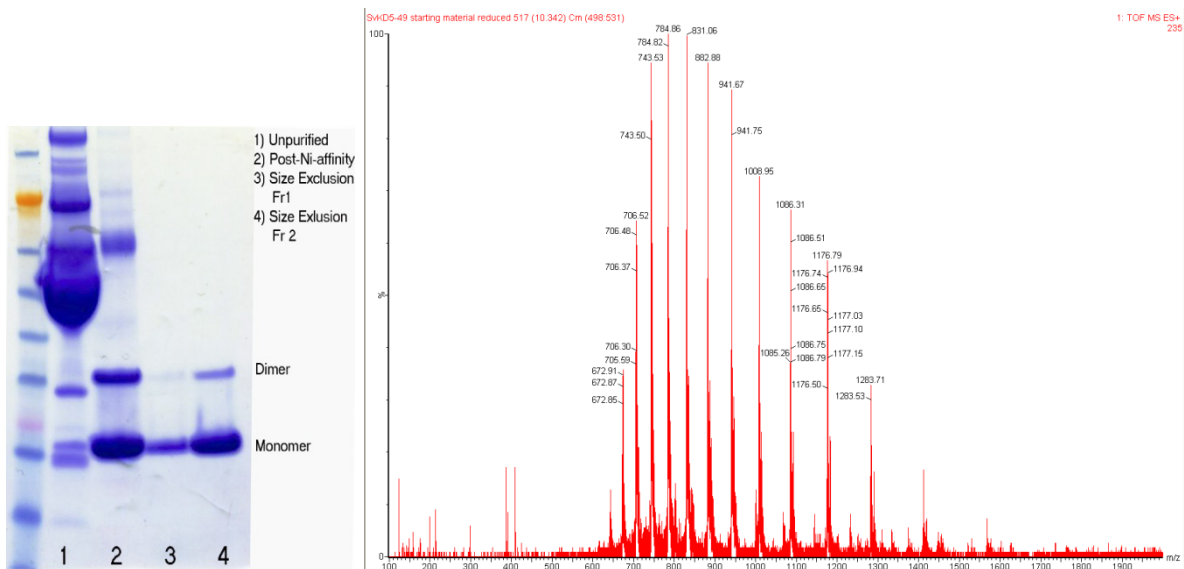
Threonine 102 was determined to be solvent accessible using the NAccess software³ based on the crystal structure of human Cystatin C⁴ and domain-swapped human Cystatin C⁵. It was also a residue away from the inhibitory motifs of both the papain-like Cathepsins and asparagine endopeptidase⁶. The mutations were introduced with primers *CysCT102Cfor* (caggtgtaccaagtgccagcccaacttg) and *CysCT102Crev* (ccaagttgggctggcacttggtacacgtg).

DHFR-negative CHO cells were grown in DMEM-based medium containing 10% dialysed FCS, 5 mM Glutamine and 0.1 mM hypoxanthine and 0.01 mM thymidine. Following transfection using lipofectamine with DHFR-CysC-T102C-6His plasmid, the hypoxanthine and thymidine supplement were removed and the cells were cultured at low density in 15 cm dishes (10^4 cells per dish) in medium containing 20 nM methotrexate (MTX). After 2 days the medium was replaced by medium containing 50 nM MTX. The cells were grown at 5% CO₂ at 37 °C for a further 2 days upon which single colonies had begun to form. These were picked and placed in a 96-well plate (tissue culture treated) in medium containing 100 nM methotrexate.

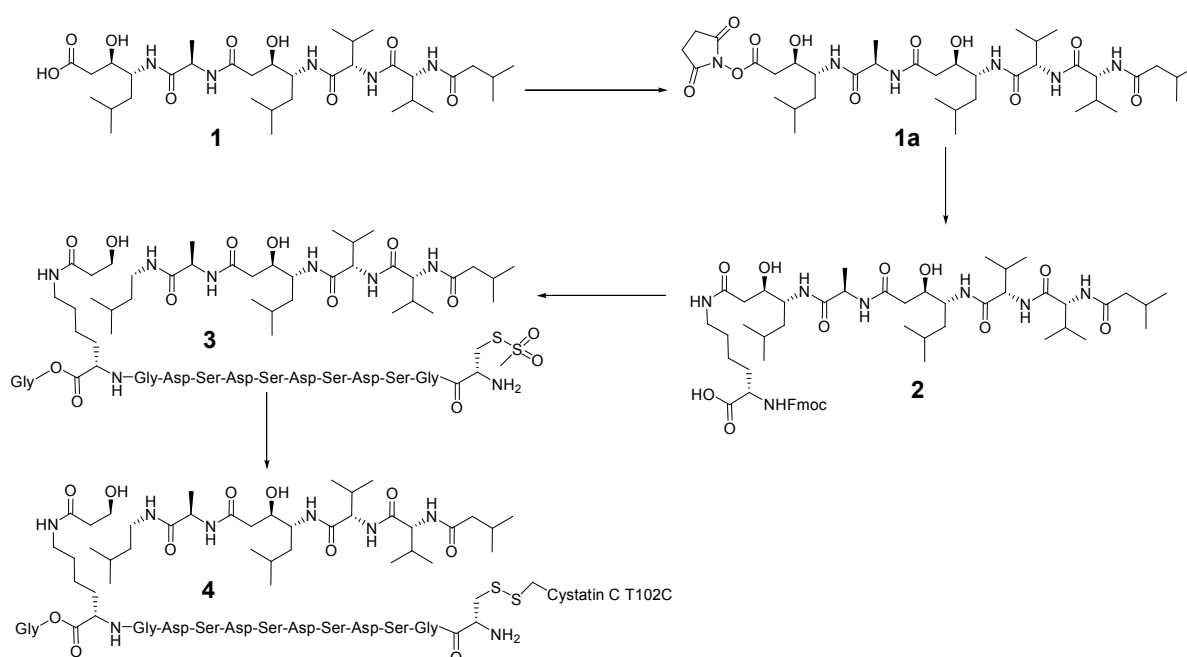
The colonies were assessed for Cystatin C-expression levels using an anti-Cystatin C antibody MAB1196 (R&D Systems, mouse anti-Human CysC; 1:3000 dilutions) and the highest producing clones were harvested and transferred to a 24-well dish. Here they were allowed to grow to 80% confluency prior to the addition of medium containing increasing amounts of MTX (upto 2 mM). At the final MTX-concentration one clone (SvKD2-25-A7) was selected for large-scale production of Cystatin C.

This clone was grown to confluency in 10 225 cm² tissue culture flasks in medium containing 2 mM MTX. The cells were incubated at 37 degrees for 2 weeks prior to harvest of the supernatant. The pH of the supernatant (2 L) was adjusted to 8.0 and NaCl was added to a final concentration of 250 mM. The supernatant was filtered through a 0.22 µm filter prior to passing it over 6 mL of NiNTA agarose (Quiagen) at 4 °C. The resin was washed with 10 column volumes of 50 mM NaPO₄, 300 mM NaCl (pH 8.3) and 5 column volumes of the same buffer containing 5 mM imidazole. The bound protein was eluted by gently shaking the agarose with 2 x 8 mL of 500 mM imidazole containing buffer followed by elution.

The eluent was passed over a Superdex G75 column (GE Healthcare, XK26/60) in batches of 1.5 mL. The fractions containing pure Cystatin C were pooled and concentrated by ultrafiltration (MWCO 6-8000) to yield 21 mL of a 0.8 mg/mL protein solution (as determined by UV absorbance). Mass spectrometry (after reduction):



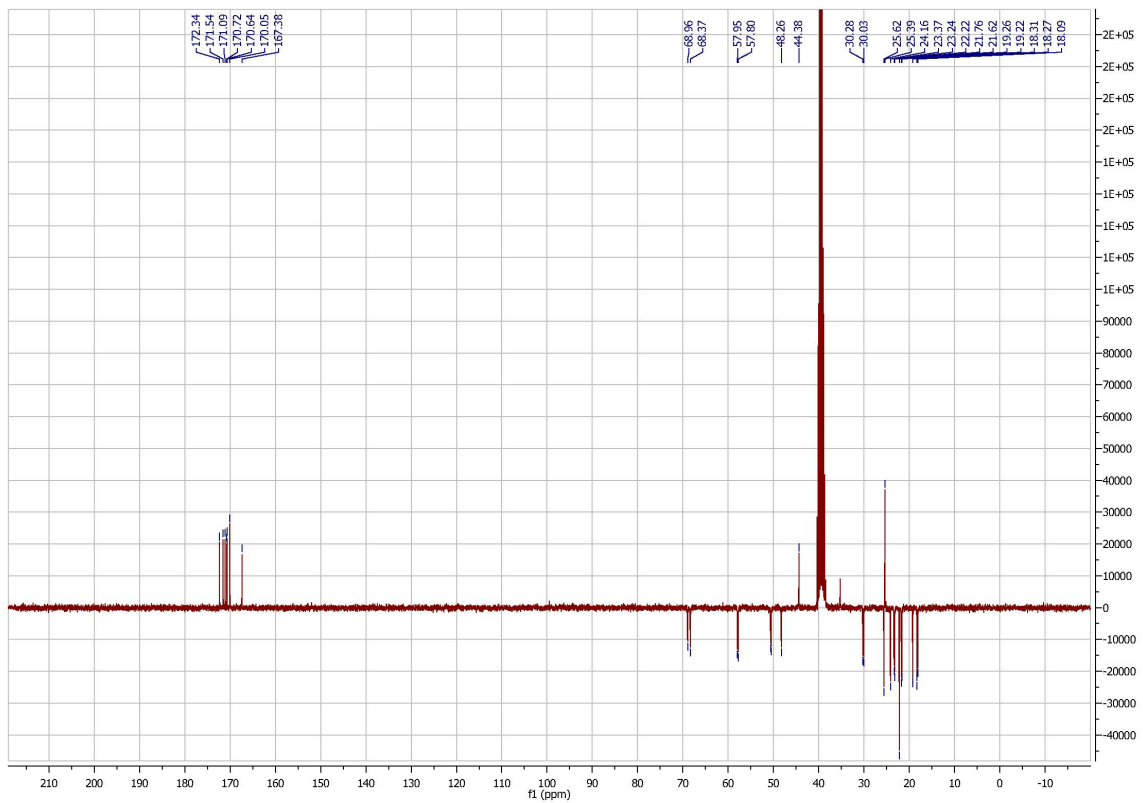
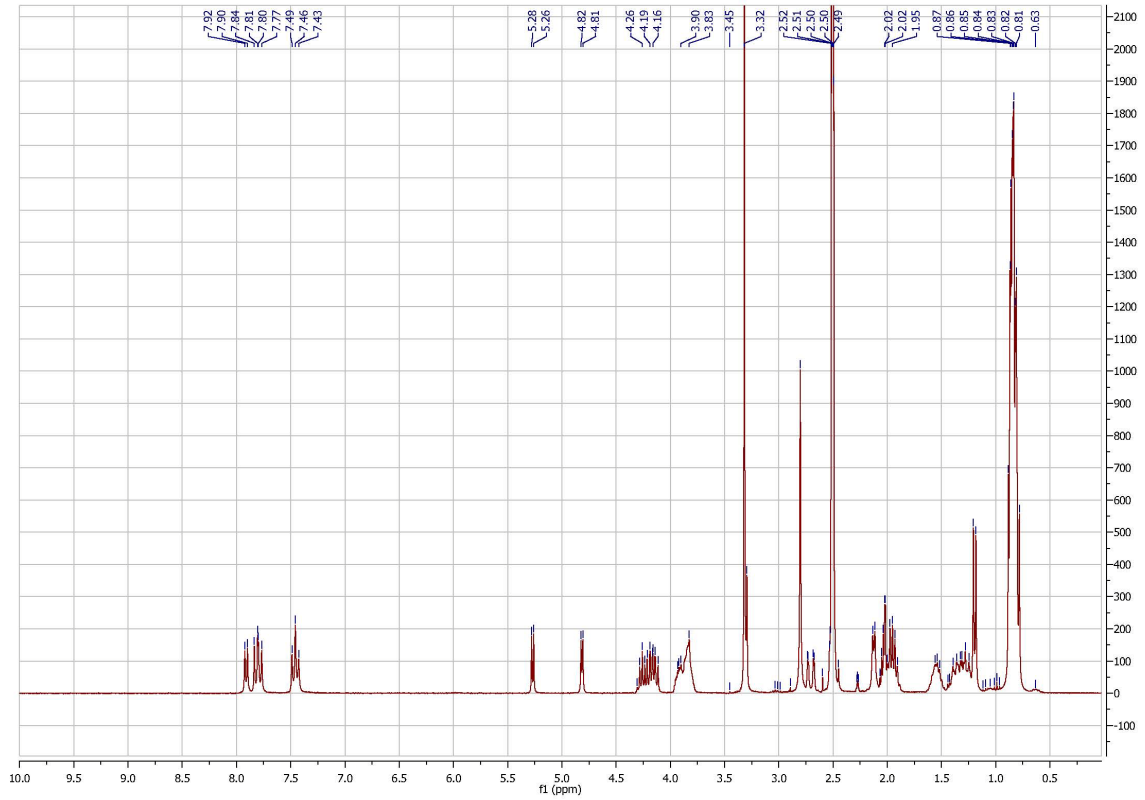
Extended synthetic scheme



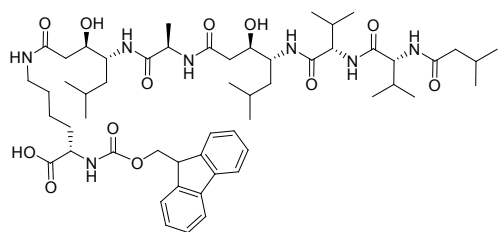
Pepstatin A-*N*-hydroxysuccinimidyl ester (**1a**)

Pepstatin (147 mg, 0.21 mmol) was dissolved in DMF (15 mL). *N*-Hydroxysuccinimide (217 mg, 1.4 mmol) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (323 mg, 1.7 mmol) were added and the mixture was stirred at room temperature for 23 hours. The mixture was concentrated under high vacuum to yield a glass-like solid, which was washed with water (3 x 15 mL) and diethyl ether (3 x 15 mL) to yield a white powder (153 mg, 93% yield) of Pepstatin A-*N*-hydroxysuccinimidyl ester. m/z (ESI+) observed 783.4; calculated: 783.5; ^1H NMR (300 MHz, DMSO) δ 7.91 (d, $J = 7.3$ Hz, 1H, NH), 7.80 (dd, $J = 11.3, 9.0$ Hz, 2H, 2 x NH), 7.46 (t, $J = 9.2$ Hz, 2H, 2 x NH), 5.27 (d, $J = 5.8$ Hz, 1H, OH), 4.82 (d, $J = 5.0$ Hz, 1H, OH), 4.33 – 4.07 (m, 3H), 3.83 – 3.94 (m, 4H), 2.80 (s, 4H, HOSu), 2.71 (dd, $J = 16.5, 2.2$ Hz, 2H), 2.21 – 1.84 (m, 5H), 1.67 – 1.46 (m, 2H), 1.47 – 1.23 (m, 4H), 1.19 (d, $J = 7.1$ Hz, 3H, CH₃), 0.97 – 0.72 (m, 30H, 10 x CH₃).

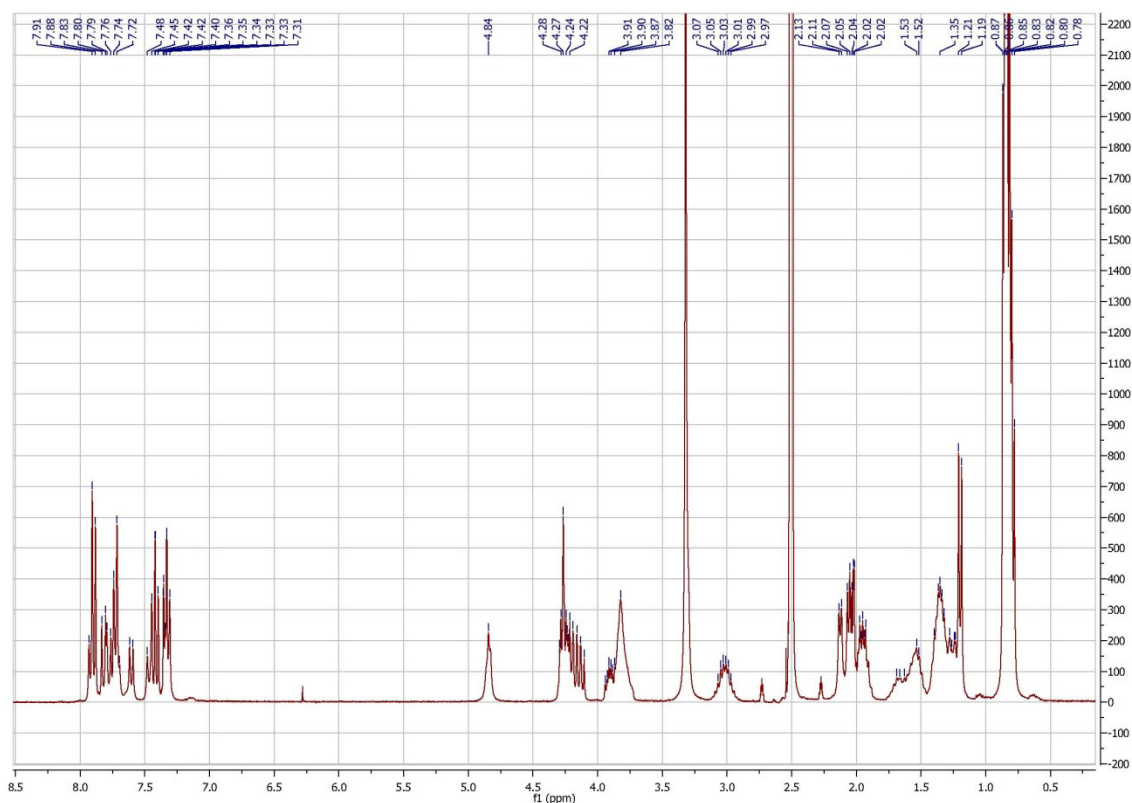
^{13}C NMR (75 MHz, DMSO) δ 172.34, 171.54, 171.09, 170.72, 170.64, 170.05, 167.38, 68.96, 68.37, 57.95, 57.80, 50.64, 50.53, 48.26, 44.38, 40.36, 40.08, 39.80, 39.52, 39.24, 38.96, 38.68, 38.49, 35.27, 30.28, 30.03, 25.62, 25.39, 24.16, 23.37, 23.24, 22.22, 21.76, 21.62, 19.26, 19.22, 18.31, 18.27, 18.09.

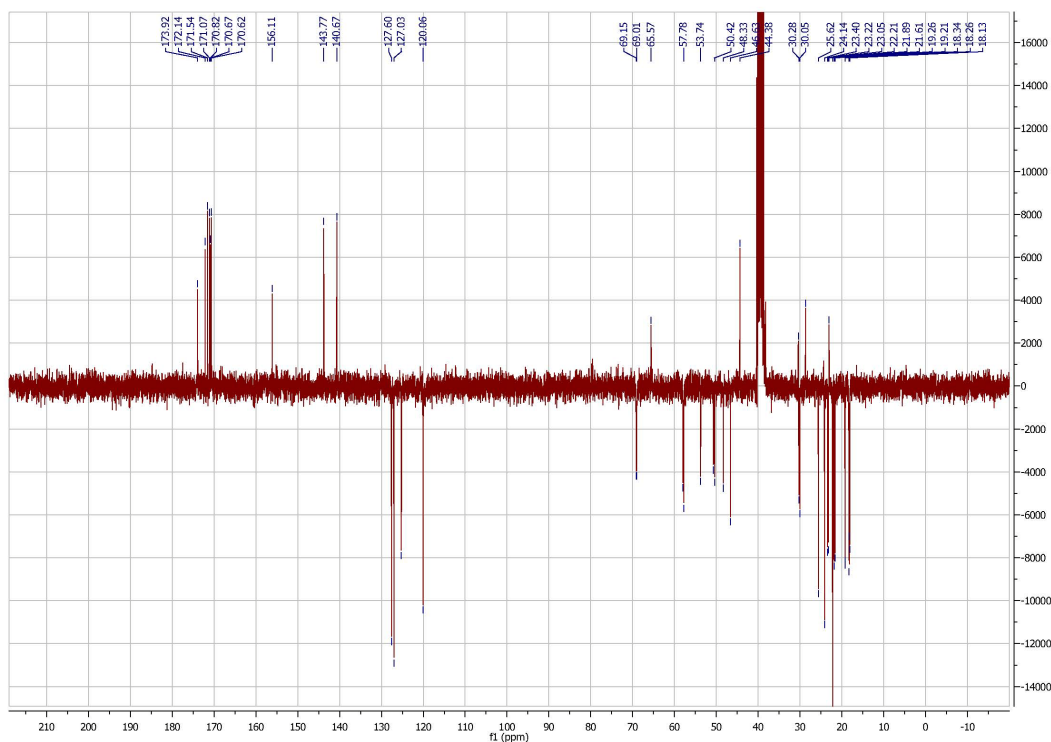


Pepstatin A-lysine (2)



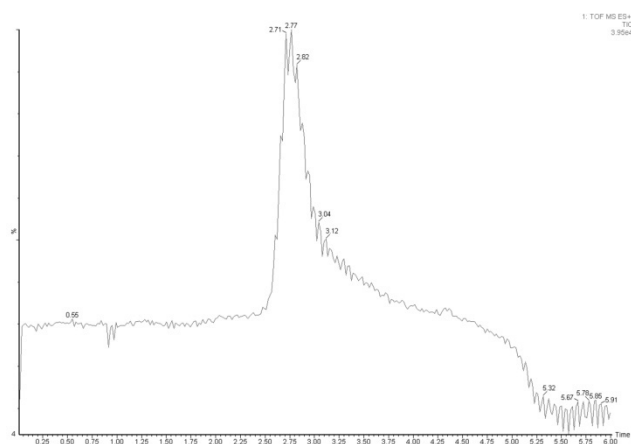
Pepstatin (164 mg, 0.21 mmol) was dissolved in DMF (20 mL). Fmoc-Lysine 77.2 mg, 0.21 mmol) was added and shaken for 2 days at room temperature. The reaction mixture was concentrated under vacuum and washed with 0.1 M HCl-solution (2 x 2 x 50 mL) and water (6 x 50 mL) before being lyophilised to yield 148 mg of a white solid, which could be further purified by HPLC. m/z (ESI+) observed: 1036.6; calculated 1036.6. ^1H NMR (300 MHz, DMSO) δ 7.91 – 7.29 (m, 15H, 7 x NH & Ar-H), 4.84 (s, 2H, 2 x OH), 4.33 – 4.05 (m, 6H), 3.98 – 3.67 (m, 5H), 3.02 (dt, $J = 11.8, 6.5$ Hz, 2H), 2.20 – 1.81 (m, 9H), 1.77 – 1.22 (m, 15H), 1.20 (d, $J = 7.0$ Hz, 3H), 0.95 – 0.66 (m, 30H, 10 x CH_3). ^{13}C NMR (75 MHz, DMSO) δ 173.92, 172.14, 171.54, 171.07, 170.82, 170.67, 170.62, 156.11, 143.81, 143.77, 140.67, 127.60, 127.03, 125.24, 120.06, 69.15, 69.01, 65.57, 57.96, 57.78, 53.74, 50.71, 50.42, 48.33, 46.63, 44.38, 40.35, 40.07, 30.41, 30.28, 30.05, 28.68, 25.62, 24.14, 23.40, 23.22, 23.05, 22.21, 21.89, 21.61, 19.26, 19.21, 18.34, 18.26, 18.13.

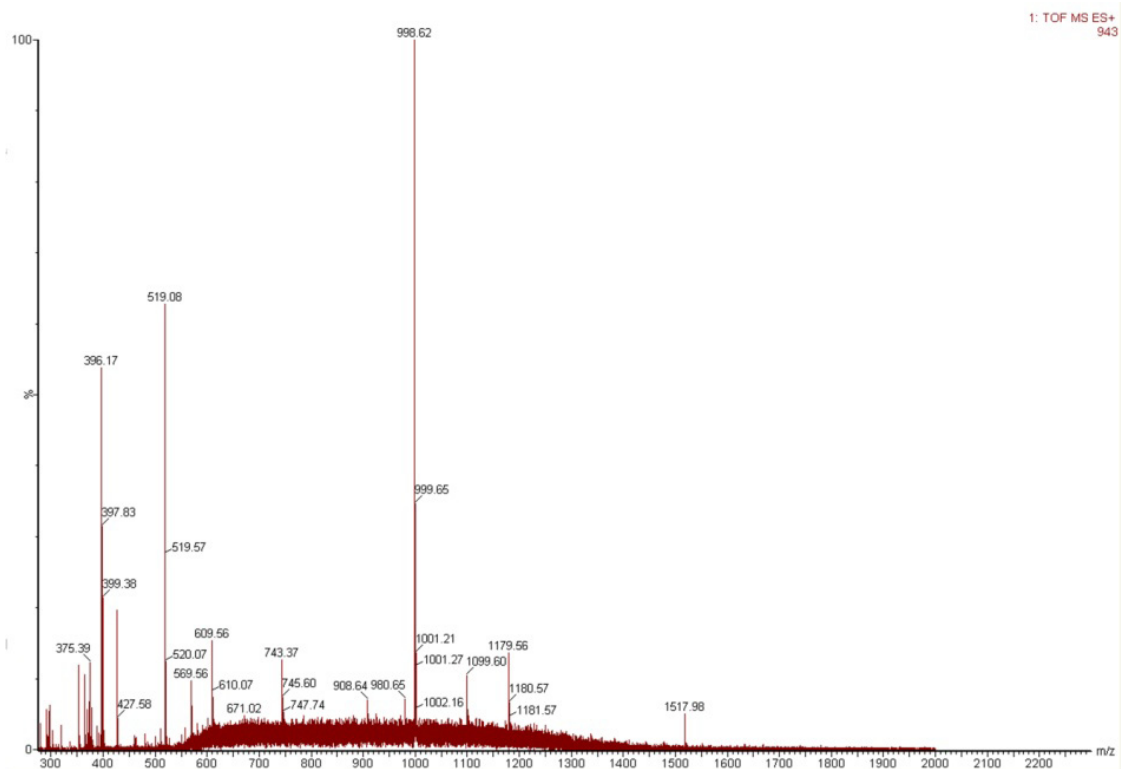




Pepstatin-Lys-Peptide-MTS (3)

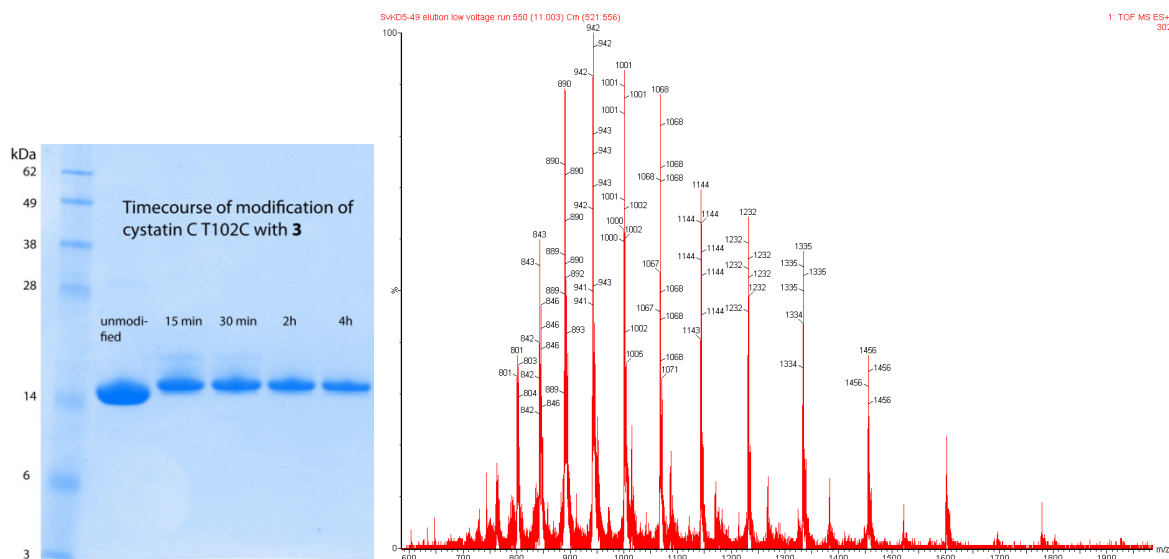
3 was synthesised by standard Fmoc-solid phase peptide chemistry using Pybop/DIPEA as coupling agent and N-methylpyrrolidone as the solvent on a Syro I peptide synthesizer. The initial coupling of Pepstatin-Lysine-Fmoc to the glycine-resin was conducted manually using 2 equivalents of **2**, 4 equivalents of 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide and 4 equivalents of *N*-hydroxybenzotriazole as coupling agents. Boc-Cysteine MTS (TRC Research Chemicals) was introduced manually after the automated synthesis using the 4 molar equivalents of the amino acid, 8 equivalents 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and 8 equivalents of *N*-hydroxybenzotriazole for 2 hours. *m/z* (ESI⁺) observed: 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and 8 equivalents of *N*-hydroxybenzotriazole for 2 hours. *m/z* (ESI⁺) observed: 998.62 [M*2(H₂O)/2]; calculated 998.51

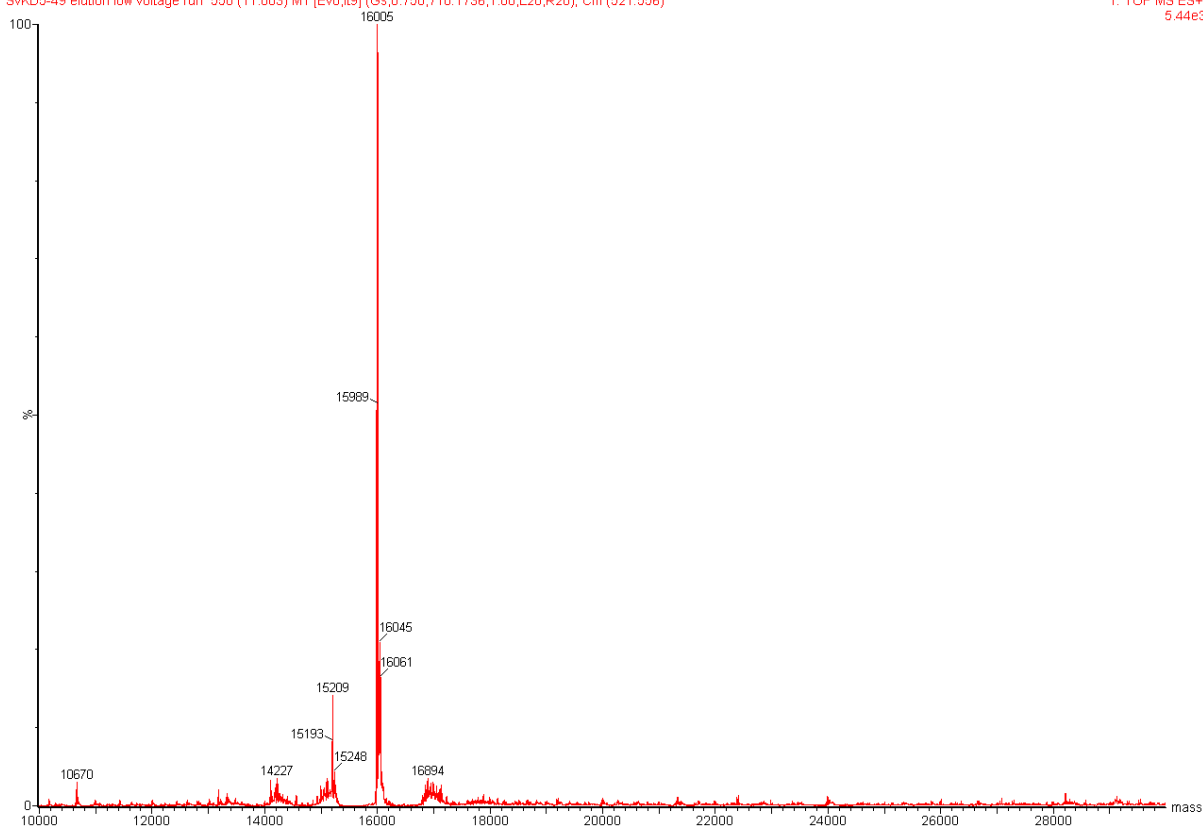




Synthesis of the Cystatin-Pepstatin Conjugate (**4**)

Dithiothreitol (DTT) was added to a solution of Cystatin C-T102C-6His (1 mg/mL in PBS, 2.5 mL) to a final concentration of 5 mM. The mixture was gently shaken at room temperature for 10 minutes and buffer exchanged into phosphate buffered saline (PBS) by Sephadex G-25 resin (GE Healthcare). The reduced protein (50 μ M 3.5 mL) was added to a solution of **3** in DMSO (10 mM, 200 μ L) and the mixture was gently shaken at room temperature for 2 hours, after which SDS-PAGE and LC-MS analysis showed the formation of a single product. The protein was purified after modification using nickel affinity chromatography, followed by dialysis into PBS. (6000-8000 MWCO, 3 x 4L).





Protease inhibition studies

All protease inhibition studies were carried out as described previously⁷⁻⁹ on a FLUOstar Optima Fluorimeter (BMG) with 360 nm excitation and 460 nm emission wavelength filters as described previously.^{1-2,9-10} Recombinant Cathepsin D and Cathepsin S were purchased from R&D Systems. Recombinant Asparagine Endopeptidase was expressed and activated as described previously.²

Inhibition of asparagine endopeptidase

To a AEP (100 ng/well) in assay buffer (50 mM NaOAc, 300 mM NaCl, pH 4.5, 50 μ L) was added 10 μ L of the inhibitors (in PBS). The plate was then incubated at room temperature for 20 minutes. Z-Ala-Ala-Asp-MEC was then added (100 μ M, Bachem, 60 μ L) and 7-amino-4-methylcoumarin release was measured by fluorescence spectroscopy over time. Initial rates were plotted against inhibitor concentration.¹⁰

Inhibition studies of Cathepsin D activity

Cathepsin D (100 ng/well) inhibition studies were performed as previously described⁹. To a solution of cathepsin D in assay buffer (NaOAc, 200 mM, pH 4.5) were added 10 μ L of the inhibitors (in PBS). The plate was then incubated at room temperature for 20 minutes. Mca-Gly-Lys-Pro-Ile-Leu-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH₂ was added (50 μ L; 10 μ M) and 7-amino-4-methylcoumarin release was measured by fluorescence spectroscopy over time. Initial rates were plotted against inhibitor concentration.

Inhibition studies of Cathepsin L

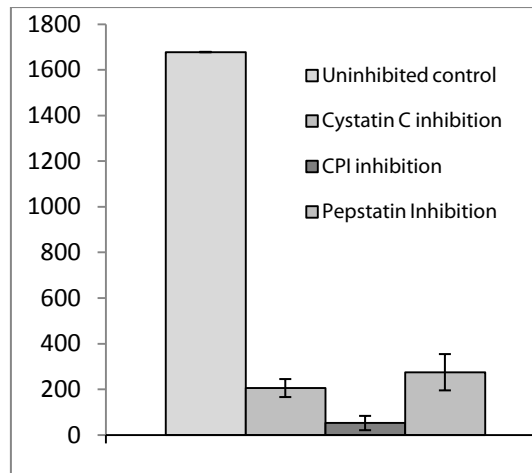
To a solution of activated mouse Cathepsin L (R&D systems, 0.25 ng/ μ L) in assay buffer (25 mM MES, pH 5.5, 30 μ L) were added various serial dilutions of Cystatin C (from 1.4 μ M), Cystatin-Pepstatin and Pepstatin alone. The mixture was gently shaken at room temperature for 5 minutes prior to the addition of Z-Leu-Arg-AMC (40 μ M in assay buffer, made from a 1 mM DMSO Stock solution). 7-amino-4-methylcoumarin release was measured by fluorescence spectroscopy over time. Initial rates were plotted against inhibitor concentration¹¹.

Inhibition of proteases in dendritic cell lysates

Three spleens from C57/B6 mice were homogenised in a glass homogeniser in 16 mL of citrate buffer (200 mM, pH 5.5). The cells were lysed by repeated freeze-thaw cycles (6 cycles). The supernatant was cleared by centrifugation at 18,000 *g* for 30 minutes. The cleared supernatant had a protein concentration of 5 mg/mL. 20 μ g of lysate was used as protease source in the same inhibition assays as for the recombinant proteases.

Validation of Enzcheck substrate using purified macrophage lysosomes

Lysosomes isolated from bone marrow derived macrophages using a Percoll density gradient fractionation (400 ng/ μ L, 10 μ L) were resuspended in assay buffer (100 mM Citrate, 2 mM DTT, pH 4.5, 0.5% v/v triton X-100; 590 μ L). 50 μ L of this diluted lysosomal suspension was plated in triplicates in a 96-well flat-bottomed plate. 10 μ L of either cystatin, pepstatin or CPI (3 μ M final concentration) was added to each set of three wells with PBS as a control. The mixtures were incubated at room temperature for 20 minutes prior to the addition of EnzChek substrate (Invitrogen, catalog number E6638, 20 μ g/mL in assay buffer). Fluorescence emergence (excitation 485nm; emission 530nm) was measured every 5 minutes on a fluorescent plate reader at 37 °C. Initial rates of fluorescence emergence were plotted for each of the inhibitors.



Determination of proteolysis inhibition in live cells using Enzchek substrate

A20 cells (5×10^6 /mL; 100 μ L/well) were plated in a 96-well plate. Inhibitors (28 μ M in PBS + 1% DMSO; 50 μ L) were added to each wells and the cells were incubated at 37°C, 5% CO₂ for 30 minutes.

After this time Enzchek (Invitrogen, catalog number E6638, 20 μ g/mL in DMEM + 10% FCS) was added and the fluorescence emergence (excitation 485nm; emission 530nm) was measured every hour on a fluorescent plate reader at 37 °C. Rates of fluorescence emergence were plotted for each of the inhibitors.

Inhibition of endo-lysosomal protease activity in live Bone-marrow derived dendritic cells

To a suspension of dendritic cells, derived from bone marrow precursors as described previously¹², (10⁷ Cells, 100 μ L) the inhibitors were added (70 μ M; 100 μ L in PBS). The cells were incubated at 37 °C, 5% CO₂, for 3 hours. 1 mL of cold cRPMI with 10% FCS was added to each tube and the cells were collected by centrifugation. The cells were washed 3 times with medium prior to lysis (50 μ L; 50 mM Citrate, 1% Triton X-100, pH 5.0). 5 μ g of this protein mixture was used to determine residual proteolytic activity as described previously¹.

In Vitro digest of proteins with purified lysosomes

Lysosomes isolated from bone-marrow derived macrophages using a Percoll density gradient fractionation (400 ng/ μ L, 3.6 μ L) were resuspended in assay buffer (50 mM Citrate, pH 4.5, 0.5% Triton X-100; 59 μ L). Inhibitor (70 μ M; 12 μ L in PBS +5% DMSO) was added and the mixture incubated for 15 minutes. Protein substrate (1 mg/mL in PBS, 9 μ L) was added to the mixture and the reaction was incubated at 37 °C. At the indicated timepoints 20 μ L of the reaction mixture was removed and boiled with LDS-sample buffer and analysed by SDS-PAGE.

Immunofluorescence (IF) microscopy

IF was performed according to standard protocols. For visualization of fluorescent substrate processing (Enzchek, vide infra), murine A20 B-cell blasts were grown on coverslips and incubated with enzchek (1 µg/mL final concentration) in presence or absence of protease inhibitors as indicated (20 µM) for 5 hours under standard growth conditions. Following treatment, cells were fixed with 3.7% paraformaldehyde in PBS, Triton X-100 permeabilized (0.1% in PBS), and immunostained against CD63, visualized using an anti-mouse secondary antibody-Alexa 647 conjugate on a Leica SP-2 confocal microscope using a 63x magnification objective.

Analysis of EGFR endocytosis and trafficking was performed as previously described with the following modifications. HeLa cells were grown on coverslips and serum starved in the absence or presence of CPI (20 µM; 0.1% FCS in DMEM-PBS 1:1) and stimulated with EGF (100 ng/mL final concentration) for 5 or 90 minutes. Samples were prepared as previously described. All images were collected using a Leica SP-2 confocal microscope equipped with a 63x magnification objective. Post-image processing and data analysis were performed using ImageJ. Image quantification was based on 25-50 cells per condition per experiment and significance was calculated using a standard Student's T-test.

Determining the effect of CPI on EGF-signalling

COS7 cells growing in 12-well tissue culture plates were preincubated for 1 hour with or without cystatin C (0.35mg/ml) or CPI (0.32 mg/ml) and then stimulated with 100ng/ml EGF for the times shown. The cells were scraped from the well in 50µl lysis buffer containing 50mM Tris, 150mM NaCl, 1mM EGTA, 1mM EDTA, 1% NP40 plus protease inhibitors (Roche, Miniprotease tablet). Aliquots were heated in SDS sample buffer and run on a 4-12% MOPS % gel (Invitrogen). After transfer to Hybond ECL membrane EGF receptor was revealed with a rabbit anti-EGF receptor antibody (Santa Cruz) followed by peroxidase conjugated donkey anti-rabbit (Jackson) and a standard ECL protocol (Millipore). The blot was then stripped and reprobed for phospho-Erk (p42/44 MAP kinase; Cell signalling) and as a loading control, Rsk2 (Santa Cruz) with appropriate secondary antibodies. The signals were quantitated using ImageJ analysis software.

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