Characterization of a new series of non-covalent proteasome inhibitors with exquisite potency and selectivity for the 20S β5-subunit

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INTRODUCTION

The mammalian 26S proteasome is a 2500 kDa multi-catalytic complex involved in intracellular protein degradation. We describe the synthesis and properties of a novel series of non-covalent di-peptide inhibitors of the proteasome used on a capped tri-peptide that was first identified by high-throughput screening of a library of approx. 350 000 compounds for inhibitors of the ubiquitin–proteasome system in cells. We show that these compounds are entirely selective for the β5 (chymotrypsin-like) site over the β1 (caspase-like) and β2 (trypsin-like) sites of the 20S core particle of the proteasome, and over a panel of less closely related proteases. Compound optimization, guided by X-ray crystallography of the liganded 20S core particle, confirmed their non-covalent binding mode and provided a structural basis for their enhanced in vitro and cellular potencies. We demonstrate that such compounds show low nanomolar IC50 values for the human 20S β5 site in vitro, and that pharmacological inhibition of this site in cells is sufficient to potently inhibit the degradation of a tetra-ubiquitin–luciferase reporter, activation of NFκB (nuclear factor κB) in response to TNF-α (tumour necrosis factor-α) and the proliferation of cancer cells. Finally, we identified capped di-peptides that show differential selectivity for the β5 site of the constitutively expressed proteasome and immunoproteasome in vitro and in B-cell lymphomas. Collectively, these studies describe the synthesis, activity and binding mode of a new series of non-covalent proteasome inhibitors with unprecedented potency and selectivity for the β5 site, and which can discriminate between the constitutive proteasome and immunoproteasome in vitro and in cells.

Key words: chymotrypsin-like, immunoproteasome, 26S proteasome, proteasome inhibitor, β5-subunit, ubiquitin–proteasome system (UPS).

Abbreviations used: Ac, acetyl; AMC, 7-aminomethylcoumarin; Boc, t-butoxycarbonyl; HBTU, O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate; HEK, human embryonic kidney; LC50, half-maximal lethal concentration; MPD, 2-methyl-2,4-pentanediol; NFκB, nuclear factor κB; IκB, inhibitory protein of NFκB; NFκB-Luc, NFκB–luciferase; PA, proteasomal activator; PDL, poly-o-lysine; RNAi, RNA interference; siRNA, small interfering RNA; Suc, succinyl; TEV, tobacco etch virus; TNF-α, tumour necrosis factor-α; 4XUb-Luc, tetra-ubiquitin–luciferase; UPS, ubiquitin–proteasome system; Z, benzoyloxycarbonyl.

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The structural co-ordinates of the yeast 20S proteasome with the indicated ligand bound reported will appear in the PDB under accession codes: 3MG0 (bortezomib); 3MG4 (compound 1); 3MG6 (compound 6); 3MG7 (compound 8); 3MG8 (compound 16).
Figure 1  Examples of covalent (A) and non-covalent (B) proteasome inhibitors

and is being evaluated for the treatment of other malignancies [21–23]. Bortezomib induces cell death through a variety of transcriptional, translational and post-translational mechanisms, and may be preferentially cytotoxic to cancer cells by enhancing endoplasmic reticulum stress, increasing the expression of pro-apoptotic factors and/or inhibiting pro-survival or DNA-damage repair pathways [4–6,21–23]. More recently, two further closely related di-peptide boronic acids, CEP-18870 and MLN9708, have been described that inhibit cancer cell proliferation in vitro and show anti-tumour activity in solid and haematological preclinical tumour models [24,25].

Bortezomib binds with very high affinity to the β5 site of the proteasome, and to a lesser extent the β1 and β2 sites [15], and behaves as a slowly reversible inhibitor (t1/2 ∼ 110 min for dissociation from the β5 site [25]). Analysis of the crystal structure of the yeast 20S in complex with bortezomib indicates that the high affinity of the drug is partly mediated by the covalent interaction of the boron atom with the nucleophilic oxygen of Thr1Oγ, and by the hydrogen bonding between the two acidic boronate hydroxy groups and the amine groups of Gly47N and Thr1N within each active site [5,9,26]. These properties are likely to contribute to the effectiveness of bortezomib as an anti-cancer agent for the treatment of haematological malignancies, but may limit its distribution and therefore broader utility in solid tumours due to the high tissue abundance of the target (the proteasome has been estimated to comprise 2% of total cellular protein [6]). More recently, the β-lactone salinosporamide A (NPI-0052) [27] and the epoxyketone carfilzomib (PR-171) (Figure 1A) [28], which are analogues of lactacystin and epoxomicin respectively, have demonstrated activity in preclinical models of multiple myeloma and chronic lymphocytic leukaemia, and have entered clinical trials for advanced solid and haematological malignances [23]. These covalent inhibitors have comparable potency to bortezomib, but differ in that they form essentially irreversible adducts with the active site Thr1Oγ residues of the proteasome [4,5,9,10,15,27–30].

Although the reactive ‘warheads’ of covalent proteasome inhibitors can contribute to enzyme potency, they can also lack specificity and be excessively reactive and unstable [10,11,15], properties which may limit their efficacy in vivo. Non-covalent inhibitors that are readily reversible in their interactions with the catalytic 20S β-subunits provide an alternative mechanism for proteasome inhibition. Such inhibitors may offer therapeutic
advantages by enabling more widespread tissue distribution through their more rapid binding and dissociation kinetics. TMC-95A is a complex natural cyclic tri-peptide (Figure 1B) that competitively and non-covalently inhibits the chymotryptsin-like activity of the proteasome with nanomolar potency, and also inhibits the caspase-like and trypsin-like activities at higher concentrations [4,5,9,10,31]. The high affinity of TMC-95A is due to its rigid heterocyclic ring system, which provides specific hydrogen-bonding interactions within each 20S active site without covalent modification of the catalytic threonine residues [4,5,9,32]. In addition, synthetic analogues of TMC-95A have been described, including endocyclic biphenyl-ether macrocycles and non-constrained linear tri-peptides that bind non-covalently with altered affinities for the trypsin- and caspase-like sites relative to the chymotryptsin-like site [5,9,33,34]. TMC-95A shows cytotoxicity in the low micromolar range in cancer cells, although the mechanism-based effects of TMC-95A or its derivatives have not been demonstrated [4]. Other non-covalent proteasome inhibitors include a series of 5-methoxy-1-indane di-peptide benzamides (e.g. CVT-659) [35,36] and 2-aminobenzylstatine derivatives (Figure 1B) [37] that selectively inhibit the chymotryptsin-like site with submicromolar potencies, although these compounds also have poor cellular activity. However, homology modelling of a 2-aminobenzylstatine compound in the chymotryptsin-like binding pocket of the proteasome has led to the design a non-covalent tri-methoxy-L-phenylalanine-containing tri-peptide with maintained selectivity and greatly increased cell potency (Figure 1B) [38]. This compound is the most potent non-covalent proteasome inhibitor described to date, inhibiting the chymotryptsin-like activity in MDA-MB-435 cells and their proliferation with IC50 values of 20 nM and 60 nM respectively [38].

In an effort to identify cell-active inhibitors of the UPS (ubiquitin–proteasome system) with novel chemical scaffolds, we have screened a library of approx. 350000 compounds using a cell-based assay that monitors accumulation of a 4xUb-Luc (tetra-ubiquitin–luciferase) reporter in response to proteasome inhibition [15,39]. Using this approach, we report the identification of a C- and N-terminally capped tri-peptide derived from the unnatural amino acid S-homo-phenylalanine that potently and selectively inhibits the chymotryptsin-like activity of the mammalian and yeast 20S proteasomes. Further optimization, guided by X-ray crystallography of compounds in complex with purified yeast 20S, has yielded a series of non-covalent di-peptide inhibitors of the proteasome with unprecedented in vitro and cellular potencies. The synthesis, binding mode and cellular activity of these compounds are described in the present study.

**EXPERIMENTAL**

**Cell culture**

Cells were from the A.T.C.C. (Manassas, VA, U.S.A.), with the exception of the diffuse large B-cell lymphoma lines which were obtained from the following sources: Karpas-1106P, Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany); WSU-DLCL2, Asterand (Detroit, MI, U.S.A.); and OCI-Ly10, provided by Dr Louis M. Staudt (National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.). Cells were cultured at 37°C in a humidified air/6% CO2 atmosphere in medium supplemented with 10% fetal bovine serum, except for the medium for Karpas-1106P and OCI-Ly10 cells which contained 20% fetal bovine serum, and 100 units/ml penicillin/100 µg/ml streptomycin (all from Invitrogen), as specified: Calu6 cells, minimum essential medium; H460, WSU-DLCL2 and Karpas-1106P cells, RPMI 1640 medium; HCT116 and HT29 cells, McCoy’s 5a medium; and OCl-Ly10, Iscove’s modified Dulbecco’s medium. Clonally-derived stable MDA-MB-231 cells expressing four tandem copies of ubiquitin fused to firefly luciferase (4xUb-Luc) and HEK (human embryonic kidney)-293 cells expressing NFκB-Luc [NFκB (nuclear factorκB)–luciferase] were generated and maintained as described previously [15].

**Reporter assays**

Cells were seeded at 10000 cells per well in white BioCoat™ PDL (poly-d-lysine)-coated 384-well plates (BD Biosciences) at 16–24 h prior to compound treatment. For the 4xUb-Luc assays, MDA-MB-231 cells were incubated with compound for 8 h. For NFκB-Luc assays, HEK-293 cells were pre-treated for 1 h with proteasome inhibitor and then stimulated with 10 ng/ml recombinant human TNF-α (tumour necrosis factor-α) (R&D Systems) for a further 3 h in the continued presence of the compound. Firefly luciferase activity was measured using BF-Glo™ reagents according to the manufacturer’s instructions (Promega) in a LEADseeker™ plate reader (GE Healthcare Life Sciences). Inhibition of NFκB-Luc activity was calculated relative to a no-compound (DMSO) control, whereas 4xUb-Luc reporter accumulation was expressed as a fold increase in luciferase activity over the DMSO control.

**Cell viability assay**

Calu6, HT29, MDA-MB-231 cells (each at 2000 cells/well), H460 cells (1000 cells/well) and HCT116 cells (1500 cells/well) were plated in black clear-bottomed BioCoat™ PDL-coated 384-well plates (BD Biosciences). Cells were incubated with compound for 72 h, after which the medium was removed to leave 25 µl per well. An equal volume of ATPlight™ reagent (PerkinElmer) was then added and luminescence was measured using a LEADseeker™ instrument.

**siRNA (small interfering RNA) transfection and assay**

MDA-MB-231 4xUb-Luc cells were transfected in a 384-well format with 10 nM siRNAs (siGENOME SMARTpool, Dharmacon) using DharmaFECT 1 (DH1) reagent (Dharmacon) as follows. For the preparation of the RNAi (RNA interference) transfection mixture for each time point, 40 µl of OptiMEM I (Invitrogen) was dispensed into duplicate wells of a 384-well plate each containing 9 µl/dish of 0.5 µM siRNA in siRNA buffer (Dharmacon). OptiMEM (50 µl) containing 0.53 µl of DH2 transfection reagent (Dharmacon) was then added to each well and the plates were incubated at room temperature (22°C) for 20 min. The transfection mixture (14 µl) from duplicate wells was then transferred into six replicate wells of two separate BioCoat™ PDL-coated 384-well cell plates (BD Biosciences), one for 4xUb-Luc assay and one for ATPlight assay (white and black clear-bottomed respectively). Reverse transfection was performed by the addition of 50 µl of MDA-MB-231 4xUb-Luc cells to each well to give 1200 cells/well. At 48, 72 or 96 h after transfection, one set of duplicate plates was assayed for 4xUb-Luc reporter activity and cell viability. For each time point, six replicate wells of cells transfected with buffer only and the non-targeting GL2 luciferase control duplex were each treated with either DMSO or 1 µM bortezomib for 8 h prior to performing the 4xUb-Luc assay.
Cell-based Proteasome-Glo™ β5 assays

Calu6 cells were plated as for the reporter assays and incubated with compound for 1 h at 37°C. B-cell lymphoma subtypes Karpas-1106P, WSU-DLCL2 and OCI-Ly10 were plated at 20000 cells per well in 384-well plates and treated with compounds identically. The activity of the 26S proteasome was measured in situ after compound removal by monitoring hydrolysis of the β5 (chymotrypsin-like) substrate Suc-LLVY-aminoluciferin (where Suc is succinyl) in the presence of luciferase using the Proteasome-Glo™ assay reagents according to the manufacturer’s instructions (Promega). Luminescence was measured using a LEADseeker™ instrument.

In vitro assays of purified 20S

The peptidase activities of purified human erythrocyte and peripheral blood monocyte 20S proteasomes (Boston Biochem™) were assayed using fluorogenic tri- and tetra-peptide substrates coupled to AMC (7-amino-4-methylcoumarin) [40] (AnaSpec) in the presence of recombinant PA28α (proteasomal activator 28α; Boston Biochem™). The following selective substrates were obtained from AnaSpec, with the exception of Z-LLE-AMC (where Z is benzoyloxy carbonyl), which was from Boston Biochem™, and were each used at a final concentration of 15 μM to assay the constitutive (c) and immunoproteasome (i) sub-sites: β1c, Z-LLE-AMC; β2c, Ac-KQL-AMC (where Ac is acetyl); β5c, Ac-WLA-AMC; β1i, Ac-PAL-AMC; β2i, Ac-KQL-AMC; and β5i, Ac-ANW-AMC. Reactions were performed at 37°C in 384-well black microtiter plates (Corning) using 0.25 mM 20S and 12 nM PA28α in a final volume of 50 μl of buffer containing 20 mM Hepes, pH 7.4, 0.5 mM EDTA and 0.01% BSA. Peptidase activity was measured by monitoring the AMC liberation over time with a Polarstar Galaxy fluorimeter (BMG Labtechnologies) using excitation and emission wavelengths of 340 nm and 460 nm respectively. Percentage inhibition of 20S activity was calculated relative to the controls with DMSO and 10 μM ML599698, an analogue of bortezomib that contains a phenyl group in place of the N-terminal pyrazine cap.

In vitro assays of 20S proteasomes in cell extracts

B-cell lymphoma extracts were prepared by hypotonic lysis and assayed for proteasome activity as described previously [15] using 10 μg of protein extract per reaction in the presence of 12 nM PA28α and the following substrates, each at 100 μM, to monitor the catalytic activity of the c or i 20S sub-sites, as indicated: β1i, Ac-PAL-AMC; β5i, Ac-ANW-AMC; β5c, Ac-WLA-AMC; and β5 Suc-LLVY-AMC.

Kinetic analysis of purified 20S inhibition

Inactivation of purified human erythrocyte 20S was determined by monitoring the hydrolysis of the β5-specific fluorogenic peptide substrate Suc-LLVY-AMC in the presence of different concentrations of inhibitor. Substrate cleavage was continuously monitored as a change in the fluorescence emission at 460 nm (excitation wavelength, 360 nm) and plotted as a function of time. Assays were performed at 37°C in cuvettes in a final volume of 2 ml in a reaction buffer containing 20 mM Hepes, pH 7.5, 0.5 mM EDTA, 0.01% SDS, 0.25 mM 20S and 10 μM Suc-LLVY-AMC, with continuous stirring. The apparent dissociation constant, $K_{i}^{app}$, was determined by non-linear least-fit of the fractional velocity, $v/v_0$, as a function of [I], where $v_0$ is the steady-state residual activity of the enzyme in the presence of inhibitor (I) and $v_0$ is the initial velocity in the absence of inhibitor:

$$\frac{v}{v_0} = \frac{1}{1 + \frac{[I]}{K_{i}^{app}}}$$

The dissociation constant, $K_i$, was calculated from the apparent dissociation constant, $K_{i}^{app}$, using the following expression, where [S] is the substrate concentration and $K_m$ is the substrate binding constant (for 20 μM Suc-LLVY-AMC):

$$K_i = \frac{K_{i}^{app}}{1 + \frac{[S]}{K_m}}$$

Protein turnover assay

Bulk protein turnover was measured in HCT116 cells according to previously published procedures [41], as described in the Supplementary material at http://www.BiochemJ.org/bj/430/bj4300461add.htm.

Synthesis of compound 1, the capped tri-peptide 5-methyl-N-((S)-1-((S)-1-((S)-1-(4-methylbenzylamino)-1-oxo-4-phenylbutan-2-ylamino)-1-oxo-4-phenylbutan-2-yl)pyrazine-2-carboxamide

The trimeric compound 1 was identified by LC-MS as an impurity in an active well identified during the high-throughput screen [42] and its structure was confirmed by full characterization of material prepared according to Scheme 1, as detailed in the Supplementary Experimental section at http://www.BiochemJ.org/bj/430/bj4300461add.htm.

Synthesis of capped di-peptide proteasome inhibitors

The synthesis of di-peptide analogues was carried out using standard peptide-coupling conditions, typically using HBTU (O-benzotriazolyl-N,N,N,N′,N′-tetramethyluronium hexafluorophosphate) to activate the carboxylic acids according to Scheme 2. The P1 and P2 residues (R$_i$ and R$_j$ respectively) were installed as described above for the preparation of compound 1 by coupling the appropriate amine to a Boc (t-butoxycarbonyl)-protected α-amino acid, followed by de-protection to give intermediates II as hydrochloride salts. The three-step elaboration of II to the di-peptide analogues of compound 1 (IV) was carried out using automated solution-phase parallel synthesis techniques in deep-well plates without purification of intermediates, but with rigorous purification and full characterization of final products. A representative procedure for the parallel synthesis of compound arrays of IV can be found in the Supplementary Experimental section.

Purification of yeast 20S

Yeast 20S wild-type and open-gate (an N-terminal tail deletion from the α3 and α7 subunits, designated α3/7ΔN) mutant containing a TEV (tobacco etch virus)-protease-cleavable Protein A-derived tag in the core particle subunit Pre1 (β4) were affinity-purified from the SDL135 and yMS159 strains respectively (gifts from Dr M. Schmidt and Dr D. Finley, Harvard University Medical School, Boston, MA, U.S.A.) [43,44] on IgG-Sepharose 6 Fast Flow (GE Healthcare), according to the procedure of Leggett et al. [45], except that ATP was omitted from the buffers and the enzyme was eluted from the IgG resin by incubation with the His$_6$-TEV protease (Invitrogen) overnight at 4°C. The resultant
Scheme 1 Synthesis of compound 1
(i) ArCH₂-NH₂, HBTU, NMM (N-methylmorpholine), DMF (N,N-dimethylformamide), 25°C, 24 h; (ii) 4 M HCl, dioxane; (iii) N-Boc-homo-phenylalanine, TBTU [O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium-tetrafluoroborate], collidine, DMF, 25°C, 3 h; (iv) 4-methylpyrazinecarboxylic acid, TBTU, collidine, DMF, 25°C, 3 h.

Scheme 2 Synthesis of compound IV
(i) R₁-NH₂, HBTU, NMM (N-methylmorpholine), DMF (N,N-dimethylformamide), 25°C, 24 h; (ii) 4 M HCl, dioxane; (iii) P₃ Boc-amino acid, HBTU, NMM, DMF, 25°C, 24 h; (iv) R₄COOH, HBTU, NMM, DMF, 25°C, 24 h.

yeast 20S was purified further by gel filtration on a 24 ml Superose 6 10/30 column (GE Healthcare) in 10 mM Tris/HCl, pH 7.5, containing 1 mM EDTA, concentrated to 30 mg/ml using an Amicon Centriplus YM-100 centrifugal filter device (Millipore) and stored at −80°C.

X-ray crystallography
Crystals of purified wild-type and the open-gate mutant yeast 20S proteasome were grown in hanging drops at room temperature, as described previously [46], using a drop volume of 1.5 μl of 20S (20 mg/ml in 10 mM Tris/HCl, pH 7.5, and 1 mM EDTA) and 0.5 μl of reservoir solution containing 100 mM Mes, pH 7.0, 40 mM Mg(CH₃COO)₂, 15% MPD (2-methyl-2,4-pentanediol) and 10 mM EDTA. Proteasome–inhibitor complexes were generated by soaking crystals overnight in reservoir buffer containing 1 mM compound, 10% DMSO and 20% MPD followed by an additional 5 h in reservoir buffer containing 1 mM compound, 10% DMSO and 25% MPD before being flash-cooled in liquid nitrogen. Crystal data and refinement statistics are given in Supplementary Table S1 at http://www.BiochemJ.org/bj/430/bj4300461add.htm. Data were collected using the Structural GenomiX (SGX)-CAT beamline at the Advanced Photon Source (APS) synchrotron of the Argonne National Laboratory (U.S. Department of Energy Chicago, IL, U.S.A.) and processed using the programs IMOSFLM [47] and SCALA [48]. Starting co-ordinates for each of the proteasome–inhibitor structures were taken from PDB entry 1G0U [43]. SigmaA-weighted Fₒ – F_c difference electron density for the β5 active site was used to model inhibitor co-ordinates, starting with a conformation generated using the small-molecule topology generator PRODRG [49]. Model building was performed using the program Coot [50] and refinement was carried out using the CCP4i graphical user interface [51] to the REFMAC program [52].

RESULTS

Cell-based screen for inhibitors of the UPS
To identify novel cell-active small-molecule inhibitors of the UPS, a high-throughput screen of the Millennium Pharmaceuticals, Inc., library was performed for compounds that stabilized 4xUb-Luc in MDA-MB-231 cells. In this system, bortezomib and other catalytic 20S inhibitors cause a concentration-dependent accumulation of the constitutively expressed reporter by blocking its intracellular degradation, as reported previously [15]. To further validate the dependence of this reporter on the 26S proteasome, siRNAs were used to deplete selected components of the 20S core particle and 19S regulatory complex in MDA-MB-231-4xUb-Luc cells. As shown in Figure 2, depletion of subunits of either the 20S (α₁ and β₆) or 19S (rpn8 and rpn11) complex resulted in a time-dependent increase in the 4xUb-Luc signal.
Identification of compound 1 as a potent selective 20S β5 inhibitor

The cell-active compounds were assessed for inhibition of purified rat brain 20S in *vivo* by measuring the activity of the β5 (chymotrypsin-like) substrate Ac-WLA-AMC in the presence of PA28α. Only a small minority of compounds inhibited 20S, showing low and sub-micromolar potencies (results not shown). Among the most potent of these were four closely related peptidic benzyl amides synthesized by combinatorial chemistry, of which one was subsequently identified by MS and re-synthesis as a C- and N-terminally capped tri-peptide derived from the unnatural amino acid S-homo-phenylalanine, and designated as compound 1 (Figure 3). In *vivo*, this compound inhibited the β5 activity of the human constitutive and immunoproteasome with low nanomolar potencies, comparable with those of bortezomib, but was essentially inactive against the β1 (caspase-like) and β2 (trypsin-like) sites of either proteasome isoform (Table 1). β5 selectivity is in contrast with that of bortezomib, which also inhibits the β1 and, to a lesser extent, the β2 sites of the constitutive and immunoproteasome (Table 1). Assays against a diverse panel of ten proteases indicated that compound 1 was highly selective for the proteasome, showing only partial (≤50%) inhibitory activity against cathepsin L, coagulation Factor β-XIIa, thrombin, tissue plasminogen activator and trypsin at concentrations approx. 10 000-fold higher than its IC_{50} value for the 20S β5 site (Supplementary Table S2 at http://www.BiochemJ.org/bj/430/bj4300461add.htm). Kinetic analysis of human 20S β5 inhibition by compound 1 showed that it behaved as a rapid-equilibrium inhibitor with a K_{i} value of 0.50 ± 0.12 nM (mean ± S.E.M., n = 3), consistent with reversible non-covalent binding to the active site of the enzyme (Figure 4). This behaviour is again in contrast with that of bortezomib, which acts as a time-dependent slow–tight binding inhibitor that interacts covalently with the active site Thr$^{1}$ residue, but which displays an equivalent K_{i} value for the β5 site of 0.56 ± 0.072 nM (mean ± S.E.M., n = 3) (Figure 4 and [25]).

Cellular inhibition of proliferation, 4xUb-Luc degradation, NFκB-Luc activation by compound 1

On the basis of the high affinity and selectivity of compound 1 for the 20S β5 site *in vitro*, its activity was characterized further in cells as compared with that of bortezomib. Compound 1 inhibited the proliferation of MDA-MB-231 cells in a concentration-dependent manner with an LC_{50} (half-maximal lethal concentration) value of 150 ± 23 nM (mean ± S.E.M., n = 3), as determined by an ATP1ite assay at 72 h (see Supplementary...
Table 1  Active-site-selectivity of the peptide boronic acid, bortezomib, the capped tri-peptide screening hit, compound 1, and a series of fifteen capped di-peptides in *vivo*

Purified human erythrocyte 20S and peripheral blood monocyte 20S (0.25 nM each) were used as a source of constitutive (c) and immunoproteasome (i) respectively to measure the peptidase activity of the β1 (caspase-like), β2 (trypsin-like) and β5 (chymotrypsin-like) sub-sites of each proteasome isoform. Assays were performed in the presence of 15 μM of the indicated fluorigenic peptide-AMC substrate, 12 nM PA28γ and a titration of each inhibitor. As shown, and unlike bortezomib, compounds 1–16 were essentially inactive against the β1 and β2 sites of the constitutive proteasome and immunoproteasome. The IC50 values of the compounds against the β5c and β5i sites are mean values for at least three independent determinations.

<table>
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<th>Compound</th>
<th>β1c Z-LLE-AMC</th>
<th>β1i Ac-PAL-AMC</th>
<th>β2c Ac-KQL-AMC</th>
<th>β2i Ac-KQL-AMC</th>
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Figure S1A at http://www.BiochemJ.org/bj/430/bj4300461add.htm. Indeed, viability assays performed across a panel of five cancer cell lines, comprising MDA-MB-231, Calu6, H460, HCT116 and HT29 cells, indicated that compound 1 has broad cytotoxic activity, with IC50 values ranging from 50 nM to 380 nM and being approx. 10–20 fold less potent than bortezomib (Table 2 and Supplementary Table S3 at http://www.BiochemJ.org/bj/430/bj4300461add.htm). In the screening assay, compound 1 caused up to a 100-fold accumulation of the 4xUb-Luc reporter at 8 h [maximal effect of 52 ± 16 fold, with an EC50 (half-maximally effective concentration) of 690 ± 72 nM (mean ± S.E.M., n = 6)], showing an approx. 3-fold higher EC50 and one-seventh of the efficacy of bortezomib [maximal effect, 340 ± 12 fold; EC50 = 240 ± 21 nM (mean ± S.E.M., n = 102)] (Supplementary Figure S1B and Table 2). The activity of compound 1 was also assessed in the Proteasome-Glo assay that measures directly the peptidase activity of the β5 site of the 26S proteasome in cells using the luminogenic substrate Suc-LLVY-amcinofel. Treatment of Calu6 cells for 1 h with compound 1 caused a concentration-dependent inhibition of β5 activity in this assay with an IC50 (half-maximally inhibitory concentration) value of 53 ± 16 nM (mean ± S.E.M., n = 3). Bortezomib was approx. 10-fold more potent, inhibiting hydrolysis of the chymotrypsin-like substrate with an IC50 of 3.9 ± 0.49 nM (mean ± S.E.M., n = 8) (Supplementary Figure S1C and Table 2). Finally, we assessed the effect of compound 1 on activation of the transcription factor NFκB by TNF-α, which is dependent on proteasomal degradation of IkBα (inhibitory protein of NFκB) following its rapid phosphorylation by the IkB kinase complex and ubiquitination by the SCF[κB] (E3 ubiquitin-protein ligase complex Skp1–Cullin–F-box) [53]. Compound 1 inhibited TNF-α-induced activation of NFκB-Luc in HEK-293 cells with an IC50 of 47 ± 7.7 nM (mean ± S.E.M., n = 7), consistent with potent proteasome inhibition in these cells. Interestingly, it did not fully inhibit NFκB-Luc, even at saturating concentrations [maximal inhibition, 79 ± 3.2 % (mean ± S.E.M., n = 7)], whereas bortezomib inhibited NFκB-Luc activity by 100% with an IC50 of 9.7 ± 0.73 nM (mean ± S.E.M., n = 14) (Supplementary Figure S1D and Table 2). Collectively, these data indicate that selective inhibition of the β5 site of the proteasome by compound 1 in cells is sufficient to inhibit the degradation of the 4xUb-Luc reporter, TNF-α-dependent NFκB activity and the proliferation of cancer cells with potencies that are approximately one order of magnitude lower than those of bortezomib.

X-ray crystallography of compound 1 in complex with the yeast 20S proteasome

To determine its binding mode, compound 1 was soaked into crystals of the yeast 20S core particle. X-ray diffraction data obtained at 3.1 Å resolution indicated that it only occupied the β5 sites of the proteasome (Figure 5A), consistent with selective inhibition of this active site in *vivo*. The C-terminal 4-methylbenzyl group (P1) was seen to occupy a well-defined S1 pocket, with the first homo-phenylalanine residue (P2) pointing toward solvent, the second (P3) occupying a well-defined S3 pocket and the third (P4) projecting into a much larger and ill-defined S4 pocket (Figure 5B). No electron density was observed corresponding to the N-terminal cap, presumably because this region of the molecule is disordered. The structure of compound 1 overlaid with that of bortezomib, as determined previously [26], suggests that, although the side chains of the two compounds differ in their orientations, most of the backbone hydrogen-bond interactions within the β5 site are the same (Figure 5C). Of note, in this structure as in others, residual electron density corresponding to Mes, the buffer in which the crystals were prepared, was observed near Thr1 in the β5 active site (Figure 5A). The main interaction between Mes and compound 1 involves hydrophobic contacts between the morpholino group of Mes and the homo-phenylalanine residue in the P2 position. For structures without ligand in the active site, no density for the morpholino group was observed (M.D. Sintchak, unpublished work), rather electron density at the position of the sulfur atom of Mes was seen. This sits
Table 2  Cell-based activity of bortezomib, compound 1 and fifteen capped di-peptide proteasome inhibitors

The cell-based potencies of bortezomib and compounds 1–16 were assessed in a diverse panel of assays that monitor the short- and longer-term effects of proteasome inhibition in cells, as indicated. In particular, inhibition of the β5 activity of the proteasome was measured directly with the Proteasome-Glo™ assay using Suc-LLVY-aminoluciferin as substrate following incubation of Calu6 cells (10,000 per well) with compound for 1 h. Inhibition of NFκB luciferase activity in HEK-293 cells was determined by pre-incubation of the cells (10,000 per well) with compound for 1 h followed by stimulation with 10 ng/ml TNF-α in the continued presence of compound for 3 h. The IC50 and percentage inhibition of NFκB luc activity are given. Accumulation of the 4xUb-Luc reporter in MDA-MB-231 cells was determined by incubation of the cells (10,000 per well) with compound for 8 h and expressed as the fold accumulation of luciferase activity together with an EC50 value for this effect. The effects of the compounds on the viability of Calu6 cells (2000 per well) and H460 cells (1000 per well) were assessed by the ATPlite assay following incubation of the cell with compound for 72 h. The results are mean values for at least three independent experiments.

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<th>Compound</th>
<th>Calu6 Proteasome-Glo™ β5</th>
<th>HEK-293 NFκB-Luc</th>
<th>MDA231 4xUb-Luc</th>
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Optimization of capped di-peptide 20S inhibitors

The partial crystal structure of compound 1 (Figure 5) together with the report of a potent related tri-methoxy-phenylalanine-containing tri-peptide 20S inhibitor (Figure 1 and [38]) suggested that one homo-phenylalanine residue could be deleted, provided that a compensating hydrophobic N-terminal capping group could be incorporated in place of the pyrazine. A series of di-peptide analogues were less potent than the trimer compound 5, with small polar P3 residues, in this case a threonine residue (Figure 7), retained reasonable potency in vitro (β5 IC50 values of approx. 500 nM). Significant further improvements were effected by varying the capping group, with the indanone in compound 6 being among the optimal residues at P4 with this compound showing a β5 IC50 of 25 nM (Figure 7 and Table 1) and a K50 value of 13 ± 1.4 nM (mean ± S.E.M., n = 3).

The crystal structure of compound 6 bound to the β5 site of yeast 20S was determined at 2.6 Å resolution (Figure 8) and compared with that of bortezomib determined at 2.7 Å, a resolution comparable with that determined previously [26]. In the structure of bortezomib, in addition to the covalent and hydrogen-bonding interactions involving the boronate, backbone hydrogen-bonding interactions with the β5 site can be seen (Figure 8A). Interestingly, the same hydrogen-bonding interactions between the backbone of compound 6 and the β5 site are observed, including the presence of the bridging water molecule between the P3 carbonyl group and Ala50 and Ala50 (Figure 8A). Figures 8(B) and 8(C) show the occupancy of the binding pockets by compound 6, whereas Supplementary Figure S2 (at http://www.BiochemJ.org/bj/430/bj4300461add.htm) shows an alternative view to include the electron density of the water molecule. The 4-methylbenzyl P1 residue occupies S1, with the methyl group occupying a small sub-pocket at the bottom of the cavity. The P2 homo-phenylalanine residue occupies S2 and the P3 threonine residue projects, to a small degree, towards the deep S3 binding pocket. The P2 homo-phenylalanine residue does not fill the S2 binding pocket, which is quite shallow, but likely confers potency by contributing a hydrophobic residue [38] at P2 gave compound 3 (Figure 7) with a β5 IC50 value of 130 nM (Table 1). In the next library, smaller replacements for the P3 homo-phenylalanine residue were sought in combination with alternative N-terminal P4 capping groups that provided compensating hydrophobic interactions within the large S4 binding pocket. Numerous compounds, such as compound 4 and the 4-benzyloxybenzoyl derivative compound 5, with small polar P3 residues, in this case a threonine residue (Figure 7), retained reasonable potency in vitro (β5 IC50 values of approx. 500 nM). Significant further improvements were effected by varying the capping group, with the indanone in compound 6 being among the optimal residues at P4 with this compound showing a β5 IC50 of 25 nM (Figure 7 and Table 1) and a K50 value of 13 ± 1.4 nM (mean ± S.E.M., n = 3).
Figure 5  Electron-density map of compound 1 and occupancy of the specificity pockets of the 20S β5 subunit

(A) A 3.1 Å sigmaA-weighted 2Fo–Fc electron-density map contoured at 1.0σ covering compound 1 (PDB entry 3MG4) and Mes in the β5/β6 active site of wild-type 20S is shown in stereo view, with residue positions P1–P4 indicated. Atoms are in ball-and-stick representation with carbon in yellow (compound 1) or salmon (Mes), nitrogen in blue and oxygen in red. The side chain atoms for Thr1 (β5) and Asp114 (β6) are shown in ball-and-stick representation, coloured as above, except for carbon in green. Protein atoms from 20S are shown in cartoon representation coloured by secondary structure with helices in cyan, β-strands in magenta and loops in salmon. The Figure was made using the PyMOL Molecular Graphics System (DeLano Scientific, Palo Alto, CA, U.S.A.). (B) The β5/β6 active site with compound 1 bound (coloured as in A), showing the molecular surface of 20S. Specificity pockets S1–S4 are indicated. (C) Superposition of compound 1 and bortezomib bound to the β5/β6 active site of 20S is shown, with positions P1–P4 indicated. Colour scheme is as described above, except bortezomib carbon atoms are coloured grey.

interaction at a ‘ledge’ formed from Gly47-Gly48 (Figure 8C). Of note, the P4 indanone in compound 6 is closely related to inhibitors reported previously [35,36], for example, CVT-659 (Figure 1B). Whereas the indanone carbonyl in these molecules was originally designed as a ‘putative electrophilic head group’ [35,36], the crystal structure of compound 6 places this moiety in the S4 binding pocket remote from the β5 catalytic Thr1 residue, thereby precluding such an interaction. In addition, the water molecule appears to be present in all higher-resolution structures (<2.6 Å), including those without ligand bound, such as that reported originally [54]. This water molecule was also observed in structures with inhibitors having IC50 values greater than 1 μM (M.D. Sintchak, unpublished work). Therefore it is unlikely that this single water-mediated hydrogen bond contributes significantly to potency.

In the third library, optimization of the C-terminal P1 residue was explored. The requirement of the hydrogen-bonding interaction with Gly47 can be seen from N-methylation of P1 to give compound 7 (Figure 7), which diminished activity greatly (β5 IC50 = 9.1 μM; Table 1). However, comparable potency with compound 6 was observed for the 2′-chlorobenzyl derivative compound 8 (β5 IC50 = 13 nM; Table 1 and Figure 7). A crystal of compound 8 bound to 20S indicated an additional hydrophobic interaction between the ortho-halogen and a second ‘sub-pocket’ within the S1 site that cannot be accessed by the less active meta-substituted analogue ([42] and see Supplementary Figure S3 at http://www.BiochemJ.org/bj/430/bj4300461add.htm).

In the final library, the influence of a hydrophobic P3 residue was assessed, with emphasis on asparagine derivatives. As with the potency-enhancing N-terminal P4 capping groups described above, several low nanomolar inhibitors were identified, such as compounds 9 (β5 IC50 = 6.8 nM, Table 2) and 10 (β5 IC50 = 4.6 nM, Table 2) (Figure 7). Potent compounds such as 11 (β5 IC50 = 2.4 nM, Table 2) and 12 (β5 IC50 = 4.4 nM, Table 2) in which the homo-phenylalanine residue was replaced by an alanine residue and compound 13 (β5 IC50 = 3.9 nM, Table 1), in which 3-pyridyl was incorporated, were also identified (Figure 7). Similarly, the neopentyl-asparagine residue was able to compensate for sub-optimal interactions in S1, with compounds 14 and 15 showing good inhibitory activity (β5 IC50 = 11 and
Optimization of compound 1 involved contracting the chain to a di-peptide of the general structure shown and systematically modifying the central amino acids and capping groups, as denoted by residues P1–P4 in a series of compound libraries prepared by solution-phase parallel synthesis (see the Experimental section). 12 nM respectively (Table 1 and Figure 7). Like compound 1, these di-peptides at 100 μM had negligible to modest activity against ten unrelated proteases (Supplementary Table S2), indicating that the most potent compounds are selective for the chymotrypsin-like site of the proteasome by 4 or 5 orders of magnitude.

The most potent proteasome inhibitor identified was compound 16 (Figure 7) with a β5 IC50 = 1.2 ± 0.35 nM for the human enzyme (Table 1) and a Kd below the 20S concentration of 250 pM in the assay, indicating that it behaved as a non-covalent active-site titrant (F.J. Bruzzone, unpublished work). The X-ray structure of this compound bound to yeast 20S resolved at 2.6 Å is shown in Figure 9. The potentially electrophilic α-ketoamide N-terminal cap does not form any covalent interactions; it is positioned in the S4 binding pocket which is located away from the catalytic Thr1 and it also does not appear to interact with any residues in S4 (Figure 9A). Importantly, the high potency of compound 16 and all compounds with a P3 neopentyl-asparagine group can be accounted for by the near-optimal fit of this residue in the S3 binding pocket (Figure 9B). As with compound 8 (Supplementary Figure S3), the P1 ortho-chlorine of compound 16 is also likely to contribute to the potency by accessing the S1 sub-pocket (Figure 9A). Of note, however, the aromatic P2 contains only one methylene and therefore the ledge interaction cannot take place with the pyridyl projecting toward solvent (compare with Figure 8C).

Relationship between 20S β5 potency and cellular activity of capped di-peptides

The effects of the di-peptides on 26S activity in cells were measured (Table 2) and compared with their 20S β5 IC50 values determined in vitro (Table 1 and Supplementary Figure S4 at http://www.BiochemJ.org/bj/430/bj4300461add.htm). Inhibition of the β5 site of the 26S proteasome was measured directly in
Figure 8  Hydrogen-bonding interactions and crystal structure of compound 6 bound to the chymotrypsin-like site of the 20S proteasome with reference to bortezomib

(A) Schematic representation of the β5/β6 active site of 20S with compound 6 (left-hand panel) (PDB entry 3MG6) and bortezomib (right-hand panel) (PDB entry 3MG6) bound. Key hydrogen bonds between the inhibitors and the protein are shown as dashed lines coloured magenta, with distances indicated in Å. Thus in the structure of bortezomib, the NH of the P1 leucine residue forms a hydrogen bond with the carbonyl of Gly47, whereas the carbonyl and amino groups of the P2 residue form hydrogen-bond interactions with Thr21. The carbonyl of the pyrazinoyl cap is involved in a network of hydrogen-bond interactions with Ala49, Ala50 and Asp114 that includes a bridging water molecule. Similar hydrogen-bonding interactions between the backbone of compound 6 and the β5 site are shown, including the presence of the bridging water molecule between the P3 carbonyl and Ala50 and Ala51; the NH of P3 makes the hydrogen-bond interaction with Asp114 that is formed by the pyrazine N in bortezomib. (B) 2.6 Å sigmaA-weighted 2Fo-Fc electron-density map contoured at 1.0σ covering compound 6 (PDB entry 3MG6) and MES is shown in the β5/β6 active site of yeast 20S open-gated mutant in stereo view, with positions P1–P4 indicated. The colour scheme is as described in Figure 5. (C) β5/β6 active site with compound 6 bound (coloured as described above), showing the molecular surface of 20S. Specificity pockets S1–S4 are indicated. The P2 binding ‘ledge’ is shown by shading of the molecular surface.

Calu6 cells using the Proteasome-Glo™ assay and corresponded well with the inhibitory potencies determined in vitro with purified 20S. There was also a striking correlation between the IC50 values of the compounds for inhibition of NFκB-Luc activity in cells and 20S β5 activity in vitro, with five of the analogues, specifically compounds 10–13 and 16, showing inhibitory NFκB potencies in the range 6.6–12 nM, equivalent to or greater than that of bortezomib [NFκB-Luc IC50 = 13 ± 2.0 nM (mean ± S.E.M., n = 6)]. Interestingly, the activity of NFκB-Luc was not fully inhibited even at saturating compound concentrations (Table 2). The di-peptides also stabilized the 4xUb-Luc reporter in MDA-MB-231 cells with EC50 values that tracked closely with their in vitro 20S β5 potencies (Table 2), although the maximal stabilization effects of approx. 100-fold were lower than those observed for bortezomib (Table 2), other boronates (J.L. Blank, unpublished work) and covalent inhibitors, such as
salinosporamide A [15]. Finally, the cytotoxic LC₅₀ values of these compounds also correlated well between cell lines (Table 2) and with their 20S β5 potencies (Table 1). Interestingly, none of the compounds were as potent as bortezomib in this assay, although several gave LC₅₀ values that were approx. 100 nM or less in at least one cell line, specifically compounds 9–13 and 16 (Table 2). Significantly, each of these contained the neopentyl-asparagine residue of compound 16 in the β5/β6 active site of the 20S core particle (coloured as described above).

Constitutive and immunoproteasome selectivity of di-peptide proteasome inhibitors

In addition to monitoring the β5 inhibitory potency of the capped di-peptides using purified human erythrocyte constitutive 20S proteasomes, IC₅₀ values were also obtained for the β5i site of the immunoproteasome from human peripheral blood monocytes using the selective substrates Ac-WLA-AMC and Ac-ANW-AMC respectively (Table 1). Bortezomib, compound 1 and the majority of the capped di-peptides were non-selective in these assays, inhibiting the constitutive and immunoproteasome with similar IC₅₀ values, although they tended to inhibit β5i with slightly greater potencies (Table 1). In common with compound 1, the di-peptides were essentially inactive with respect to inhibition of the β1i or β2i sites of the immunoproteasome. However, a limited number of compounds showed significantly greater selectivity for the immunoproteasome, with compounds 4 and 5 being the best examples, showing ~10-fold β5i selectivity [compound 4, β5i IC₅₀ = 41 ± 3.3 nM and β5c IC₅₀ = 470 ± 96 nM; compound 5, β5i IC₅₀ = 27 ± 2.7 nM and β5c IC₅₀ = 340 ± 31 nM (means ± S.E.M., n = 3)]. Conversely, compounds 14 and 15 showed the reverse selectivity, the former of these two compounds being 18-fold more selective for the constitutive proteasome [β5c IC₅₀ = 11 ± 2.3 nM; β5i IC₅₀ = 200 ± 62 nM (means ± S.E.M., n = 3)] and the latter 3.6-fold more selective [β5c IC₅₀ = 12 ± 2.8 nM (mean ± S.E.M., n = 4); β5i IC₅₀ = 44 ± 9.2 nM (mean ± S.E.M., n = 3)] (Table 1).

To determine whether this selectivity was maintained in cells, three B-cell lymphoma subtypes were used that differ in their proteasome isoform expression. As shown in Figure 10(A), OCI-Ly10 cells do not express the immunoproteasome, as demonstrated by the negligible hydrolysis of the β1i- and β5i-specific substrates Ac-PAL-AMC and Ac-ANW-AMC respectively, whereas Karpas-1106P express substantial β1i and β5i activities, indicating considerable enrichment of the immunoproteasome. The hydrolysis of the β5c substrate Ac-WLA-AMC supported this expression profile, although it is not entirely selective for β5c over β5i and therefore over-represents the amount of constitutive proteasome in Karpas-1106P cells. The chymotrypsin-like substrate Suc-LLVY-AMC is hydrolysed approximately equally well by the β5i and β5c sites in vitro, although cells enriched in the immunoproteasome can show approx. 2–3-fold higher activity with this substrate. On the basis of these data, Karpas-1106P cells are highly enriched for the immunoproteasome, whereas OCI-Ly10 cells only express the constitutive proteasome. WSU-DLCL2 cells are intermediate in this activity profile, indicating that they express a mixed population of the proteasome isoforms.

We assessed the potency of the β5i- and β5c-selective compounds 5 and 14 respectively in the three B-cell

Effect of 20S β5 inhibition on bulk protein turnover

The majority of intracellular proteins are degraded by the proteasome, as demonstrated using inhibitors that irreversibly inactivate each of the 20S active sites [4]. The effect of selectively inhibiting the β5 site alone on the degradation of short-lived protein was assessed using compounds 10 and 16, two of the most potent cell-active di-peptides. As shown previously [41], bortezomib inhibits approx. 50% of bulk protein turnover in HCT116 cells (see Supplementary Figure S5 at http://www.BiochemJ.org/bj/430/bj4300461add.htm), an effect that is comparable with that observed with ML858, a synthetic version of salinosporamide A that irreversibly inhibits each of the 20S active sites [15] (T.A. Soucy, unpublished work). Under identical conditions, the di-peptides reduced protein turnover by approx. 20% (Supplementary Figure S5), indicating that inhibition of the β5 site alone results in partial inhibition of protein degradation.

Figure 9 Crystal structure of compound 16 bound to the chymotrypsin-like site of the 20S proteasome

(A) β5/β6 active site with compound 16 (PDB entry 3MG8) bound to yeast open-gated 20S, showing the molecular surface of 20S (coloured as described in Figure 5). Selectivity pockets S1–S4 are indicated. (B) Close-up view of the S3 specificity pocket to illustrate occupancy by the neopentyl-asparagine residue of compound 16 in the β5/β6 active site of the 20S core particle (coloured as described above).

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of compound 14 in cells that exclusively express the constitutive proteasome as compared with those that predominantly express the immunoproteasome. Importantly, bortezomib, which does not discriminate substantially between β5c and β5i sites in vitro (Table 1), gave IC50 values that were within 2-fold of each other in the three B-cell lymphomas (Table 3). These data therefore provide the first description of non-covalent inhibitors of the proteasome that can discriminate between the immunoproteasome and constitutive proteasome in vitro and in cells.

**DISCUSSION**

The present study has identified a new series of potent non-covalent proteasome inhibitors on the basis of a capped trimeric peptide derived from the unnatural amino acid S-homophenylalanine, first identified by a large-scale high-throughput cell-based screen for small-molecule inhibitors of the UPS. These capped di-peptides, prepared by high-throughput liquid-phase peptide synthesis methods, are entirely selective for the capping groups that are likely to contribute to potency by picking up further hydrophobic interactions. This binding mode broadly corresponds to that proposed on the basis of a homology model [38], except that the crystal structures obtained in the present study place the N-terminal

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**Table 3** Proteasome inhibition in B-cell lymphoma subtypes expressing differing levels of immunoproteasome by β5i- and β5c-selective compounds 5 and 14 respectively compared with bortezomib

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**Figure 10** Proteasome-subtype selectivity of compounds 5 and 14 in B-cell lymphomas expressing different levels of the immunoproteasome

(A) The activities of the β1 and β5 subunits of the constitutive (c) and immunoproteasome (i) were assessed in diffusible B-cell lymphoma extracts (10 μg of protein per reaction) using recombinant PA28α (12 nM) and subtype-selective fluorogenic peptide substrates (100 μM), as indicated. As shown, the rank order of immunoproteasome expression in these cell lines was Karpas-1106 > WSU-DLCL2 > LY10. (B and C) The B-cell lymphoma lines (20 000 cells per well) were treated with the indicated concentrations of immunoproteasome-selective compound 5 (B) or constitutive-selective compound 14 (C) for 1 h under cell culture conditions. Inhibition of the β5 site of the constitutive and immunoproteasome was then assessed in situ using the Proteasome-Glo™ assay with the chymotrypsin-like lumogenic substrate Suc-LLVY-aminoluciferin, as described in the Experimental section. As shown, the IC50 values of compound 5 in Karpas-1106, WSU-DLCL2 and LY10 cells were 200, 380 and 620 nM respectively; the IC50 values of compound 14 in Karpas-1106, WSU-DLCL2 and LY10 cells were 870, 120 and 19 nM respectively. Results are means ± S.E.M. for triplicate determinations.
cap in a large S4 binding pocket rather than in small ‘accessory hydrophobic pockets’, designated previously AS1 and AS2 [38]. Importantly, the most potent compounds identified each contained a neopentyl-asparagine substituent in P3, which appears to provide a near-optimal fit for the S3 binding pocket of the β5 site (Figure 9B). This residue can also be found in the tri-peptide 2-keto-1,3,4-oxadiazoles (Figure 1A) that are slowly reversible and presumably covalent in nature [11].

Characterization of these compounds in cells demonstrated that they can functionally inhibit the 26S proteasome with potencies that correlated well with those determined in vitro using the purified enzyme (Table 2 and Supplementary Figure S4). Furthermore, since the analogues are essentially inactive against the β1 (caspase-like) and β2 (trypsin-like) sites of the proteasome (Table 1), these data indicate that inhibition of the β5 (chymotrypsin-like) site of the proteasome alone is sufficient to potently inhibit the degradation of the 4xUb-Luc reporter, activation of NFκB in response to TNF-α and the proliferation of cancer cells. Interestingly, despite the fact that the cellular IC50 values of these compounds spanned three orders of magnitude, with the most potent being in the single- or double-digit nanomolar range, neither the stabilization of 4xUb-Luc nor the inhibition of the NFκB pathway was complete, even at saturating compound concentrations (Table 2 and Supplementary Figure S1). These observations suggest that non-covalent inhibition of 20S β5 activity alone is not sufficient to fully inhibit the proteasomal degradation of either 4xUb-Luc or 16KDa, at least over the timescale of these cell-based assays. This is in contrast with the covalent inhibitors bortezomib and salinosporamide A, which completely block the degradation of these proteins in cells and can minimally inhibit both the β1 and β5 sites of the proteasome. Furthermore, estimates of bulk protein turnover in cells suggest that the overall catalytic rate of proteasome is reduced, but not abolished, by non-covalent inhibition of the β5 site (Supplementary Figure S5), although it remains a possibility such inhibition completely blocks the degradation of a subset of proteins that are only substrates of the β5 site and not the β1 and/or β2 sites of the proteasome. Importantly, however, prolonged inhibition of the β5 site alone appears to sufficient to inhibit cancer cell proliferation.

Proteasome inhibitors that are currently in clinical use do not discriminate between the chymotrypsin-like activities of the constitutive or immunoproteasome, and the potential therapeutic benefit of selective inhibition of either of these activities has not been demonstrated. However, recent evidence from preclinical studies with PR-957, a β5i-selective peptide epoxyketone analogue of carfilzomib [28], suggests that the anti-inflammatory effects of proteasome inhibitors, such as bortezomib, may be mediated by their inhibition of the chymotrypsin-like site of the immunoproteasome [8]. Our studies have identified compounds that differentially inhibit the β5 sites of the constitutive and immunoproteasome in vitro and in B-cell lymphomas. Of note, compound 5 that preferentially inhibited the β5i site contained the small threonine residue in P3 and the larger 4-methylbenzylamine aromatic cap in P1. Conversely, the two β5c-selective compounds 14 and 15 each contained a smaller residue at P1 and the bulkier potency-enhancing neopentyl-asparagine in P3 (Figure 8). This structure–activity relationship, albeit limited, is entirely consistent with selectivity data obtained from an approx. 6000 tri-peptide library screen for substrates that are preferentially hydrolysed by each active site of the constitutive and immunoproteasome ([55] and C. T. unpublished work). For example, the tri-peptide substrate Ac-ANW-AMC, where P1 is a tryptophan residue and P3 is an alanine residue, is preferentially cleaved by the β5i subunit of the immunoproteasome, whereas Ac-WLA-AMC, where the P1 and P3 residues are reversed, is preferentially hydrolysed by the constitutive β5c subunit. The crystal structure of the immunoproteasome has not yet been determined to provide an explanation for these selectivity differences. However, the primary sequences of the mature forms of the human β5c and β5i subunits are 60.7% identical (see Supplementary Figure S7 at http://www.BiochemJ.org/bj/430/bj4300461add.htm). We have therefore mapped the sequence differences between the β5c and β5i subunits on to the bovine 20S crystal structure [56] and modelled compound 1 into the active site (see Supplementary Figure S8 at http://www.BiochemJ.org/bj/430/bj4300461add.htm). The majority of the sequence differences between β5c and β5i lie outside of the active site, whereas the residues that make contacts with compound 1 appear to be mainly the same, with some relatively minor exceptions, such as the replacement of Ala36 and Ser53 with Ser46 and Gln53 within the S1 binding pocket, Gly48 with Cys48 in the S2 ‘ledge’, and the reversal of the Ala12-Ser32 sequence with Ser12-Ala32 in the S3 pocket. Since only subtle differences exist between the ligand-interacting residues that form the β5c and β5i active sites, it is not possible to rationalize selectivity differences with respect to substrate preference or inhibitor sensitivity based on such an analysis. Clearly, crystal structures of the constitutive and immunoproteasomes in complex with selective inhibitors will help identify the structural features of each sub-site that contribute to selectivity. Finally, although a covalent immunoproteasome-selective inhibitor has been described recently [8], the function of the immunoproteasome in cancer cells is unknown. The identification of a new class of proteasome inhibitors, such as compounds 14 and 5, that preferentially inhibit the 20S β5c and β5i sites respectively and can thus discriminate between the constitutive and immunoproteasome in cells may help address their functional roles in various cellular models of disease including cancer.

AUTHOR CONTRIBUTION

Christopher Tsu, Paul Hales and Frank Bruzzone designed and performed the biochemical characterization of the compounds. Cynthia Barrett, Jane Liu and Kristofer Garcia performed the cell-based assays and Teresa Sowry performed the protein turnover assays. Darshan Sappal generated the MDA231-4xUb-Luc cell line and performed the high-throughput library screen. Edward Olhava performed the re-synthesis of compound 1, and Christopher Blackburn, Kenneth Gigstad and Matthew Jones designed and performed the chemical syntheses of the capped di-peptides. Nancy Bump purified yeast 20S core particles for crystallization and Michael Sintchak prepared the crystals for X-ray crystallography and analysed the data. Paul Fleming and Lawrence Dick provided project oversight, participated in experimental design and data interpretation, and reviewed the manuscript. Jonathan Blank performed the high-throughput screen and the initial characterization of compound 1 in cells, contributed to experimental design and data analysis, and wrote the manuscript with Christopher Blackburn.

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U.S.A.) for providing cell pellets derived from a panel of B-cell lymphoma subtypes to profile for immunoproteasome expression. Finally, we thank Mark Williamson and Dr Mark Rolfe for valuable discussions.

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REFERENCES


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SUPPLEMENTARY ONLINE DATA

Characterization of a new series of non-covalent proteasome inhibitors with exquisite potency and selectivity for the 20S β5-subunit

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Figure S1 Activities of compound 1 and bortezomib in cells

(A) The concentration-dependent effects of compound 1 and bortezomib on the proliferation of MDA-MB-231-4xUb-Luc reporter cell line were determined by the ATPlite assay at 72 h. (B) Accumulation of the 4xUb-Luc reporter in the stable MDA-MB-231 cell line was determined by incubation with the indicated concentrations of compounds for 8 h followed by luciferase assay. (C) The β5 activity of the 26S proteasome was measured in Calu6 cells by the Proteasome-Glo™ assay using Suc-LLVY-aminoluciferin as a substrate following incubation of the cells with the indicated concentration of compounds for 1 h. (D) Inhibition of NFκB–luciferase activity in the stable HEK-293 cell line was determined by pre-incubation of the cells with the indicated concentration of compounds for 1 h followed by stimulation with 10 ng/ml TNF-α in the continued presence of compound for a further 3 h. Results are means ± S.E.M. from replicate determinations and are representative of several independent experiments.

SUPPLEMENTARY EXPERIMENTAL

Protease selectivity assays

Protease selectivity of the compounds was assessed using a panel of purified human enzymes (all from Calbiochem, EMD Chemicals) by monitoring hydrolysis rates of peptide-AMC substrates (all from Bachem) using fluorescence measurements as described for ‘In vitro assays of purified 20S’ in the main paper, with modifications as follows. To measure the activities of coagulation Factor β-XIIa (200 nM), chymotrypsin (2 nM), elastase (4 nM), plasmin (4 nM), tissue plaminogen activator (25 nM) and trypsin (4 nM) (final concentrations), reactions were performed at 30°C in 50 μl of buffer containing 50 mM Tris/HCl, pH 8.0, 100 mM NaCl, 5 mM CaCl2, 0.01% Tween 20 and their respective substrates Boc-QGR-AMC (50 μM), Suc-AAPF-AMC (40 μM), MeOSuc-AAPV-AMC (50 μM), H-AFK-AMC (65 μM), Bz-FVR-AMC (20 μM), Boc-E(OBzl)GR-AMC (50 μM) and Z-FR-AMC (12 μM). The activities of cathepsin B and L were measured using 2 nM enzyme in 50 μl of 100 mM sodium acetate, pH 5.5, containing 5 mM DTT (dithiothreitol), 1 mM EDTA, 0.01% Brij 35 and the respective substrates Z-RR-AMC (60 μM) and Z-FR-AMC (37.5 μM). Calpain I activity was measured using 200 nM enzyme in 50 μl of 100 mM sodium acetate, pH 7.4, containing 75 μM CaCl2, 1.5 mM DTT and 100 μM Suc-LY-AMC. Percentage inhibition of the proteases was

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The structural co-ordinates of the yeast 20S proteasome with the indicated ligand bound reported will appear in the PDB under accession codes: 3MG0 (bortezomib); 3MG4 (compound 1); 3MG6 (compound 6); 3MG7 (compound 8); 3MG8 (compound 16).
calculated relative to DMSO and either 2–20 μM E64 [trans-epoxysuccinyl-1-leucylamido(4-guanadino)butane; Sigma] for the cysteine proteases (cathepsins and calpain) or complete EDTA-free protease inhibitor cocktail (1 tablet/ml diluted 1:10 (v/v) in assay buffer, Roche) for the remaining serine proteases.

**Protein turnover assay**

Bulk protein turnover was measured in HCT-116 cells as described previously [1]. Briefly, HCT116 cells (100,000 cells/well in 12-well plates) were labelled for 20 min under cell culture incubation conditions in methionine- and cysteine-free DMEM (Dulbecco’s modified Eagle’s medium; Invitrogen), supplemented with 10% dialysed FBS (fetal bovine serum), 2 mM L-glutamine and 50 μCi/well of EasyTag™ EXPRESS 35S-labelled methionine/cysteine mix (NEN Radiochemicals, PerkinElmer). The cells were then washed three times with the same medium containing the unlabelled 2 mM methionine and 0.2 mM cysteine, and then incubated with 3 μM compound in this medium for the indicated times. Medium (50 μl) was removed from the cells and subjected to liquid scintillation counting to monitor the release of 35S-labelled peptides. At the end of the experiment, cells were washed in PBS and then solubilized in 1 ml of 0.2 M NaOH and subjected to liquid scintillation counting. Percentage protein turnover at each time point was calculated as: [(total counts released into medium)/(total counts released into medium + total counts in NaOH-solubilized cells)] × 100.

**Synthesis of compound 1, the capped tri-peptide 5-methyl-\(N-(S)-1-((S)-1-(4\text{-methylbenzylamino})-1\text{-oxo-4-phenylbutan-2-ylamino})-1\text{-oxo-4-phenylbutan-2-ylamino})-1\text{-oxo-4-phenylbutan-2-yl}pyrazine-2-carboxamide**

To a mixture of (S)-2-(tert-butoxycarbonylamino)-4-phenylbutanoic acid (0.634 g, 2.26 mmol) and 4-methylbenzylamine (0.306 ml, 2.40 mmol) in dichloromethane (10.0 ml) was added HBTU (O-benzotriazol-1-yl-N,N,N′,N′-tetramethyluronium hexafluorophosphate) (0.984 g, 2.6 mmol) and N-methylmorpholine (0.50 ml, 4.54 mmol). The reaction mixture was stirred at room temperature overnight and then washed with saturated aqueous NaHCO3. The organic layer was dried over anhydrous MgSO4 and evaporated and the residue dissolved in tetrahydrofuran (20 ml), treated with 4.0 M HCl in 1,4-dioxane (20 ml) and stirred at 16 h at ambient temperature. The solvents were removed in vacuo to give (S)-2-amino-N-(4-methylbenzyl)-4-phenylbutanamide hydrochloride as an oil (0.70 g, 98%).

A solution of (S)-2-amino-N-(4-methylbenzyl)-4-phenylbutanamide hydrochloride (0.50 g, 1.57 mmol) and (S)-2-(tert-butoxycarbonylamino)-4-phenylbutanoic acid (0.439 g, 1.57 mmol) and 2,4,6-collidine (0.571 mg, 4.71 mmol) in DMF (10 ml) was treated with TBTU [O-(benzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium tetrafluoroborate] (0.556 g, 1.73 mmol) and N-methylmorpholine (0.50 ml, 4.54 mmol) and stirred at ambient temperature for 3 h. The reaction mixture was diluted with water and extracted with dichloromethane. The extracts were dried over MgSO4 and evaporated. The residue was re-dissolved in dichloromethane containing 1% MeOH and hexane was added to precipitate tert-butyl (S)-1-[(S)-1-(4-methylbenzylamino)-1-oxo-4-phenylbutan-2-ylamino]-1-oxo-4-phenylbutan-2-ylcarbamate (0.546 g, 90%).

A solution of tert-butyl (S)-1-[(S)-1-(4-methylbenzylamino)-1-oxo-4-phenylbutan-2-ylamino]-1-oxo-4-phenylbutan-2-ylcarbamate (0.754 g, 1.4 mmol) in dichloromethane (10 ml) was treated with 4 M HCl in dioxane (2.1 ml, 10.4 mmol) and stirred at room temperature for 4 h. The solvent was evaporated and the residue triturated with ether to give the corresponding amine hydrochloride (0.65 g, 100%) which was used without further purification. The next S-homo-Phe residue was coupled as described for the previous step and the Boc protecting group removed as described above to give (S)-2-amino-N-[(S)-1-[(S)-1-(4-methylbenzylamino)-1-oxo-4-phenylbutan-2-ylamino]-1-oxo-4-phenylbutan-2-yl]-4-phenylbutanamide as the hydrochloride salt (81% yield). The amine described above was coupled to 5-methylpyrazine-2-carboxylic acid using the coupling conditions described above and worked up in the usual way. The crude product was purified by flash chromatography.
Correlation between in vitro and cellular potencies of bortezomib and compounds 1–16

Data presented in Tables 1 and 3 in the main paper are expressed in μM concentrations and compared to show the correlation between in vitro potency of the compounds against the β5 site of the constitutive 20S proteasome determined with Ac-WLA-AMC as substrate and Calu6 cell Proteasome-β5 IC₅₀, HEK293-NFκB-Luc IC₅₀, MDA-MD-231-4xUb-Luc EC₅₀ and Calu6 cell ATPlite viability LC₅₀. The viability effects of the compounds in Calu6 cells and H460 cells, as shown in Table 3 in the main paper, shown as LC₅₀ values are expressed in μM concentrations and correlated. The open symbol represents bortezomib and the closed symbols are numbered to indicate compounds 1–16.

on silica gel eluting with a gradient of 0 to 5% methanol in dichloromethane to give 5-methyl-N-[[(S)-1-[(S)-1-[(S)-1-(4-methylbenzylamino)-1-oxo-4-phenylbutan-2-ylamino]-1-oxo-4-phenylbutan-2-yl]pyrazine-2-carboxamide (compound 1) in 73% yield.

Compound 1

1H NMR (300 MHz, d₆ DMSO) δ: 1.91 (m, 5H), 2.10 (m, 2H), 2.21 (s, 3H), 2.59 (m, 8H), 3.31 (s, 1H), 4.19 (m, 2H), 4.25 (m, 1H), 4.66 (m, 1H), 7.01 (d, 2H, J = 7.8 Hz), 7.17 (m, 17H), 8.21
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Figure S5 Effect on intracellular protein turnover of potent di-peptide proteasome inhibitors 11 and 16 compared with bortezomib

HTC116 cells were metabolically labelled for 20 min with [35S]methionine/cysteine and then chased with excess unlabelled amino acids in the presence of compound 11, 16 or bortezomib (each at 3 μM) for the times indicated. Intracellular degradation of [35S]-labelled proteins was then determined as described in the Experimental section in the main paper and expressed as a percentage of total label incorporated into the proteins. The results are means ± S.E.M. (n = 6) and are representative of two similarly performed experiments.

(d, 1H, J = 7.9 Hz), 8.34 (d, 1H, J = 7.1 Hz), 8.45 (d, 1H, J = 7.8 Hz), 8.65 (m, 1H), 8.81 (d, 1H, J = 8.3 Hz), and 9.05 (d, 1H, J = 11.6 Hz). HRMS (high-resolution MS; ESI+): calculated for C44H48N6O4 [M + H] 725.3815; found 725.3799.

Synthesis of (S)-2-(tert-butoxycarbonylamino)-4-(neopentylamino)-4-oxobutanoic acid (compound 18)

Compound 18 was prepared according to Scheme S1. A solution of Boc-Asp-OBn (0.937 g, 2.9 mmol), neopentylamine (0.267 g, 2.9 mmol), HBTU (O-benzotriazole-N,N,N′,N′-tetramethyluronium-hexafluorophosphate) (1.33 g, 3.5 mmol) and N-methylmorpholine (0.44 g, 4.4 mmol) in anhydrous methylene chloride (15.0 ml) was stirred at ambient temperature for 16 h. The solvent was evaporated and the residue obtained was partitioned between ethyl acetate (35 ml) and saturated aqueous NaHCO3 (25 ml). The organic layer was dried over anhydrous MgSO4, concentrated and purified by flash chromatography on silica gel to afford (S)-benzyl 2-(tert-butoxycarbonylamino)-4-(neopentylamino)-4-oxobutanoate (17) as a white solid (0.84 g, 96%) which was dissolved in methanol (3 ml) and stirred under 20 psi (1 psi = 6.9 kPa) H2 for 2 h in the presence of 10% palladium on carbon (10 mol%). The Pd/C was removed via filtration through celite and the solvent evaporated to afford 18 in quantitative yield.

(s, 9H), 0.91 (s, 9H). MS (ESI+): calculated for C14H26N2O5 [M + H] 303; found 303.

Synthesis of capped di-peptide proteasome inhibitors

A representative procedure for the parallel synthesis of compound arrays (denoted IV in Scheme 2 in the main paper) follows: a deep-well polypropylene synthesis plate was charged with the appropriate amine salts II (0.25 mmol) in one dimension and a solution of the amino acid (0.25 mmol) constituting the P3 coupling partner in DMF (2.0 ml) added in the second dimension by use of a Tecan liquid handler. HBTU (0.5 ml, 0.5 M solution in DMF) and N-methylmorpholine (0.9 mmol) were then added to each well by means of the liquid handler. The plate was shaken at room temperature for 16 h. The residue in each well obtained after evaporation of the solvent in a Genevac was partitioned between CHCl3/THF (3:1) (3 ml per well) and saturated aqueous NaHCO3 (2 ml) with each solution delivered by the liquid handler. The phases were separated by centrifugation and the aqueous layer was extracted with additional CHCl3/THF (3:1) (3 ml per well) and saturated aqueous NaHCO3 (2 ml) with each solution delivered by the liquid handler. The phases were separated by centrifugation and the aqueous layer was extracted with additional CHCl3/THF (3:1) (3 ml per well) and the combined organic layers were evaporated. The residue thus obtained was treated with 4 M HCl in dioxane (2 ml), shaken at room temperature for 16 h and concentrated in a Genevac evaporator to remove solvent and residual HCl. The N-terminal cap was then coupled and the final products isolated as in the

Scheme S1 Synthesis of compound 18

(i) Neopentyl amine, HBTU, NMM, DCM, 25 °C, 24h; (ii) H2, Pd/C, MeOH.

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Figure S7 Sequence alignment of the human β5 subunits of the constitutive and immunoproteasome

PSB5_HUMAN (PSMB5, β5 constitutive subunit, upper sequence) is compared with PSB8_HUMAN (PSMB8, β5 immunoproteasome subunit, lower sequence), with regions of sequence identity shaded black. Residues forming the inhibitor-binding pockets are indicated by circles below the aligned sequences and coloured as follows: S1 pocket in blue, S2 pocket in magenta and S3 pocket in green. Note that Ala49 sits between the S1 and S3 pockets and has been assigned to both. Residues from the β6 subunit that form the S4 pocket, as well as a portion of the S3 pocket, are not shown because this subunit does not differ between the constitutive and immunoproteasome.

Figure S8 Residue conservation between the β5/β6 active site of the constitutive and immunoproteasome

Surface representation of the β5 (light blue) and β6 (grey) subunits of bovine 20S proteasome (PDB entry 1IRU [56]). Residues that are non-identical between constitutive and immunoproteasome are coloured red. The structure of compound 1 has been modelled into the active site on the basis of a superposition of the β5 subunit structure from yeast 20S, reported in the present study, with the corresponding subunit of the bovine 20S structure.

previous step. All final compounds IV (some 1500 in total) were purified by preparative reverse phase HPLC using mass-directed fraction collection and their purities and identities established by LC-MS. Preparative HPLC was conducted on an Agilent 1100 series LC/MSD instrument using a Waters SunFire C18 5 μm Prep OBD column (19 mm×150 mm). The compounds were eluted with a water–MeCN gradient (0.1 % formic acid) optimized by the A2Prep Agilent software. Compounds 2–16 described herein were further characterized by 1H NMR spectroscopy and HRMS (see below).

HRMS

Molecular mass measurements were performed using a QSTAR® XL quadrupole TOF (time-of-flight) mass spectrometer (Applied Biosystems) coupled to an 1100 series HPLC system (Agilent Technologies). An isocratic flow of 50 % solvent A (20 % CH3CN/80 % H2O with 0.1 % CH3COOH) and 50 % solvent B (100 % CH3CN with 0.1 % CH3COOH) at 200 μl/min was used to deliver each sample to the electrospray source of the mass spectrometer, tuned in ESI TOF-MS positive mode with a 3 min acquisition time for each analysis. The mass spectrometer was externally calibrated between m/z 400 and 800 by using API Calibration (NaCsI) Solution (Waters Corp). For each analysis, approx. 5 scans were summed and the centroid m/z value of the protonated monoisotopic molecular ion [M + H]+ was recorded.

The relative deviations from the calculated exact masses based on the expected formulae were also calculated. For each compound, the experimental and calculated [M + H]+ values were within ±4 ppm, supporting their expected elemental composition (see the Supplementary Results section).

SUPPLEMENTARY RESULTS

1H NMR and HRMS data for synthetic compounds

Compound 2

1H NMR (400 MHz, d6 DMSO) δ: 1.82 (m, 2H), 1.92 (m, 2H), 2.22 (s, 3H), 2.56 (m, 2H), 3.45 (d, 1H), 3.55 (d, 1H), 4.20 (d,
Table S1  Crystallographic data and refinement statistics

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<td>8.6/475</td>
<td>8.1/445</td>
</tr>
<tr>
<td>Disallowed (%/number)</td>
<td>0.3/14</td>
<td>0.5/27</td>
<td>0.4/23</td>
<td>0.6/31</td>
<td>0.4/21</td>
</tr>
</tbody>
</table>

* Rmerge = Σᵢσᵢ|Iᵢ(hkl)−<|I(hkl)>|/Σᵢσᵢ|Iᵢ(hkl)| for n independent reflections and i observations of a given reflection. <|I(hkl)|> is the average intensity of the iᵗʰ observation.
† Numbers in parenthesis are for highest resolution shell.
‡ Rfree = Σᵢ|Fᵢ(h)||Fc(h)||/Σᵢ|Fo(h)||Fc(h)|, where Fo and Fc are the observed and calculated structure factors respectively.

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Table S2  Protease selectivity of compounds
Protease selectivity of compounds was assessed using fluorogenic peptide-AMC substrates and a panel of purified human enzymes, as described in the Experimental section of the main paper. Percentage inhibition of the proteases was determined in the presence of compound 1 or bortezomib for 72 h under cell culture conditions. IC50 data are means ± S.E.M. for 3–11 determinations.

<table>
<thead>
<tr>
<th>Enzyme inhibition at 100 μM compound (%)</th>
<th>Activity against compound 1</th>
<th>Activity against bortezomib</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin B</td>
<td>91 ± 7</td>
<td>86 ± 10</td>
</tr>
<tr>
<td>Cathepsin L</td>
<td>89 ± 7</td>
<td>85 ± 10</td>
</tr>
<tr>
<td>Coagulation Factor ß-XII</td>
<td>92 ± 7</td>
<td>87 ± 10</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>93 ± 7</td>
<td>88 ± 10</td>
</tr>
<tr>
<td>Elastase</td>
<td>94 ± 7</td>
<td>90 ± 10</td>
</tr>
<tr>
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<td>95 ± 7</td>
<td>91 ± 10</td>
</tr>
<tr>
<td>Thrombin</td>
<td>96 ± 7</td>
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</tr>
<tr>
<td>Tissue plasminogen activator</td>
<td>97 ± 7</td>
<td>93 ± 10</td>
</tr>
<tr>
<td>Trypsin</td>
<td>98 ± 7</td>
<td>94 ± 10</td>
</tr>
<tr>
<td>Calpain I</td>
<td>99 ± 7</td>
<td>95 ± 10</td>
</tr>
</tbody>
</table>

Table S3  Anti-proliferative effects of compound 1 and bortezomib in cancer cell lines
The indicated cell lines (1000–2000 cells per well) were incubated with a range of concentrations of compound 1 or bortezomib for 72 h under cell culture conditions. Cell viability was assessed by monitoring cellular ATP levels using the luminescence-based ATPlite assay as described in the Experimental section of the main paper. IC50 data are means ± S.E.M. for 3–11 determinations.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>ATPlite cell viability LC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>150 ± 23</td>
</tr>
<tr>
<td>Calu6</td>
<td>130 ± 10</td>
</tr>
<tr>
<td>H460</td>
<td>380 ± 110</td>
</tr>
<tr>
<td>HCT116</td>
<td>50 ± 6.1</td>
</tr>
<tr>
<td>HT29</td>
<td>55 ± 6.9</td>
</tr>
</tbody>
</table>

Table S4  Protease selectivity of compounds
Protease selectivity of compounds was assessed using fluorogenic peptide-AMC substrates and a panel of purified human enzymes, as described in the Experimental section of the main paper. Percentage inhibition of the proteases was determined in the presence of compound 1 or bortezomib for 72 h under cell culture conditions. IC50 data are means ± S.E.M. for 3–11 determinations.

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<td>95 ± 10</td>
</tr>
</tbody>
</table>

Compound 9
1H NMR (400 MHz, d6 DMSO) δ: 0.78 (s, 9H), 1.83 (m, 1H), 2.01 (m, 1H), 2.25 (s, 3H), 2.72 (m, 5H), 4.21 (m, 3H), 4.76 (d, 1H), 5.17 (s, 2H), 7.16 (m, 1H), 7.39 (m, 6H), 7.84 (m, 3H), 8.21 (d, 1H, J = 8.1 Hz), 8.42 (m, 1H), and 8.53 (d, 1H). HRMS (ESI+): calculated for C41H46N4O4 [M + H] 677.3703; found 677.3729.

Compound 10
1H NMR (400 MHz, d6 DMSO) δ: 0.78 (s, 9H), 1.82 (m, 1H), 2.03 (m, 1H), 2.24 (s, 3H), 2.44 (m, 3H), 2.63 (m, 3H), 2.80 (m, 4H), 4.14 (m, 2H), 4.25 (m, 1H), 4.60 (m, 1H), 6.98 (m, 3H), 7.11 (m, 7H), 7.25 (m, 3H), 7.85 (t, 1H, J = 6.1 Hz), 8.22 (d, 2H, J = 5.4, 7.7 Hz), and 8.45 (t, 1H, J = 6.1 Hz). HRMS (ESI+): calculated for C36H45FN4O4 [M + H] 617.3503; found 617.3527.

Compound 11
1H NMR (400 MHz, d6 DMSO) δ: 0.75 (s, 9H), 1.25 (d, 3H, J = 7.4 Hz), 2.71 (m, 2H), 2.80 (d, 2H, J = 6.2 Hz), 4.27 (m, 3H), 4.67 (m, 1H), 7.02 (m, 1H), 7.19 (m, 1H), 7.26 (m, 2H), 7.35 (m, 1H), 7.53 (m, 1H), 7.89 (t, 1H, J = 6.3 Hz), 8.22 (m, 1H), 8.32 (d, 1H, J = 7.3 Hz), 8.46 (t, 1H, J = 6.1 Hz), 8.71 (d, 1H, J = 8.1 Hz), 8.75 (s, 1H), and 12.25 (s, 1H). HRMS (ESI+): calculated for C36H45F2N4O4 [M + H] 570.2528; found 570.2510.

Compound 12
1H NMR (400 MHz, d6 DMSO) δ: 0.75 (s, 9H), 1.27 (d, 3H, J = 6.9 Hz), 2.41 (m, 3H), 2.71 (m, 5H), 4.24 (t, 1H, J = 7.4 Hz), 4.32 (m, 2H), 4.58 (d, 1H, J = 6.8 Hz), 7.18 (m, 3H), 7.27 (m, 5H), 7.41 (m, 1H), 7.84 (t, 1H, J = 5.7 Hz), 8.14 (d, 1H, J = 8.1 Hz), 8.33 (d, 1H, J = 6.9 Hz), and 8.51 (t, 1H, J = 5.3 Hz). HRMS (ESI+): calculated for C35H44ClN4O4 [M + H] 529.2582; found 529.2602.
Compound 13

$^1$H NMR (400 MHz, d$_6$ DMSO) $\delta$: 0.75 (s, 9H), 2.36 (m, 4H), 2.56 (m, 1H), 2.74 (m, 3H), 2.89 (m, 1H), 3.18 (m, 1H), 4.35 (m, 2H), 4.54 (m, 2H), 7.17 (d, $J = 6.5$ Hz), 7.28 (m, 6H), 7.42 (m, 1H), 7.72 (d, $J = 7.9$ Hz), 7.84 (t, $J = 3.9$ Hz), 8.08 (d, $J = 7.7$ Hz), 8.36 (d, 1H, $J = 7.9$ Hz), 8.44 (m, 2H), and 8.60 (t, $J = 5.4$ Hz). HRMS (ESI$^+$): calculated for C$_{33}$H$_{40}$ClN$_5$O$_4$ [M$+H^+$/2] 606.2847; found 606.2866.

Reference