Characterization of peptidyl boronic acid inhibitors of mammalian 20 S and 26 S proteasomes and their inhibition of proteasomes in cultured cells

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Proteasomes are large multisubunit proteinases which have several distinct catalytic sites. In this study a series of di- and tri-peptidyl boronic acids have been tested on the chymotrypsin-like activity of purified mammalian 20 S and 26 S proteasomes assayed with succinyl-Leu-Leu-Val-Tyr-amidomethylcoumarin (suc-Leu-Leu-Val-Tyr-AMC) as substrate. The inhibition of 20 S proteasomes is competitive but only slowly reversible. The K_i values for the best inhibitors were in the range 10–100 nM with suc-Leu-Leu-Val-Tyr-AMC as substrate, but the compounds tested were much less effective on other proteasome activities measured with other substrates. Free boronic acid inhibitors exhibited equivalent potency to their pinacol esters. Both benzoyl (Bz)-Phe-boroLeu and benzyloxycarbonyl (Cbz)-Leu-Leu-boroLeu pinacol ester inhibited 20 S and 26 S proteasomes with non-ideal behaviour, differences in inhibition of the two forms of proteasomes becoming apparent at high inhibitor concentrations (above $3 \times K_i$). Both of these compounds were also potent inhibitors of 20 S and 26 S proteasomes in cultured cells. However, gel filtration of cell extracts prepared from cells treated with radiolabelled phenacetyl-Leu-Leu-boroLeu showed that only 20 S proteasomes were strongly labelled, demonstrating differences in the characteristics of inhibition of 20 S and 26 S proteasomes. The usefulness of peptidyl boronic acid inhibitors for investigations of proteasome-mediated protein degradation was confirmed by the observation that Bz-Phe-boroLeu and Cbz-Leu-Leu-boroLeu pinacol ester inhibited NFκB activation with IC_{50} values comparable to their K_i values for purified proteasomes. The latter result supports the view that the chymotrypsin-like activity of proteasomes assayed with suc-Leu-Leu-Val-Tyr-AMC is a critical one for protein degradation in cells.

Key words: chymotrypsin-like activity, tight binding inhibitors, NF-κB.

INTRODUCTION

The proteasome is a large multisubunit proteinase complex located in the nucleus and cytoplasm of all eukaryotic cells [1,2]. The 20 S proteasome is composed of four rings, each containing seven subunits. Yeast proteasomes contain seven different α-type subunits and seven different β-type subunits arranged as a complex dimer [3]. In mammalian cells there are 10 β-type subunits; the expression of three of these is induced by the antiviral cytokine interferon-γ [4]. The 20 S proteasome can combine with two different types of regulatory protein complexes. One of these is the 19 S regulatory complex. One 19 S complex binds to each end of the 20 S proteasome to form the 26 S proteasome [5]. The 26 S proteasome is responsible for the ATP-dependent degradation of ubiquitinated proteins and also for the ATP-dependent, but ubiquitin-independent, degradation of proteins such as ornithine decarboxylase [6]. Another regulator protein called PA28, or the 11 S regulator, is a hexameric complex which also binds to the two ends of the 20 S proteasome cylinder and has been implicated in antigen processing [7].

Eukaryotic 20 S proteasomes exhibit multiple catalytic activities each having different specificities. The activities can be distinguished using various synthetic peptide substrates and inhibitors. Proteinases can cleave peptide bonds on the carboxyl side of basic, hydrophobic and acidic amino acid residues. These activities have been termed trypsin-like, chymotrypsin-like and peptidylglutamylpeptide hydrolase activities respectively. Proteinase inhibitors have been used to characterize further activities [8–10], although it is becoming clear that there must be some overlap between some of these peptidase activities [11]. Many inhibitors of serine and cysteine proteinases were found to be rather poor inhibitors of proteasomes. Proteasomes are now known to have an unusual catalytic mechanism in which the N-terminal threonine residue of β-subunits is the catalytic nucleophile [12,13]. The proton donor/acceptor role in catalysis may be performed either by a conserved lysine residue (Lys-33 in the *Thermoplasma* proteasome) or a water molecule bonded to the α-amino group of the N-terminal threonine [12,3]. The yeast proteasome contains two copies of the three catalytic subunits β1/PRE3, β2/PUP1 and β5/PRE2 [3,14]. Homologous catalytic β-subunits are constitutively expressed in mammalian cells. In addition, the interferon-γ-inducible subunits LMP2, LMP7 and MECL1 are also catalytically active.

The precise substrate specificity of different catalytic sites is not well understood. Although the kinetically distinct activities are generally defined by the amino acid in the P1 position of a

Abbreviations used: Boc, *N*-t-butoxycarbonyl; Bz, benzoyl; Cbz, benzyloxycarbonyl; AMC, 7-amido-4-methylcoumarin; NAP, β-naphthylamide; suc, succinyl; TNF-α, tumour necrosis factor-α

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Figure 1 Peptidyl boronic acids that act as inhibitors of the proteasome

For details of the compounds **1–4**, see the text.

synthetic peptide substrate, it is well recognized that the specificity determinants go well beyond the P1 position. In fact, the catalytic sites must overlap in specificity to some extent since there are more activities described than there are different catalytic β-subunits. Characterization of the effects of novel proteasome inhibitors is useful in unravelling the specificity of the different sites. Selective inhibitors of proteasomes are also important for establishing proteasome functions in animal cells and may be useful in the treatment of certain diseases. Lactacystin has proved to be very useful and highly, but not completely, specific for proteasomes. It is an irreversible inhibitor of proteasomes which was originally suggested to label only one subunit [15] but was later found, using higher concentrations, to covalently modify all of the catalytic subunits [16]. Peptide aldehyde inhibitors are still commonly used but they are only effective at quite high concentrations and are relatively non-specific [17,12]. Therefore there is still a need for well characterized membrane-permeable proteasome-specific inhibitors for studies of proteasome function in animal cells.

Recently Bogyo et al. [18] have reported the use of peptidyl vinyl sulfone compounds as inhibitors of proteasomes. Peptidyl boronic acids have also been shown to be potent inhibitors of the proteasome [19,20]. Boronic acids act as transition-state analogues for serine proteinases because the boron can accept the oxygen lone pair of the active site serine residue. It seems likely that these compounds react similarly with the catalytic N-terminal threonine residue of the proteasome catalytic subunit(s). The tripeptide (Figure 1, compound **1**) and dipeptides (**2**) and (**3**) are reported to have sub-nanomolar potency (K_i 0.03, 0.62 and 0.18 nM respectively) against rabbit muscle 20 S proteasome [20]. A peptidyl boronic acid (compound **4**) with an N^{γ}-nitro-Arg P2 residue has also been reported to inhibit human liver and brain proteasome (IC_{50} 8 nM) [19] (Figure 1).

We have synthesized a series of novel di- and tri-peptidyl boronic acids to characterize their inhibition of different forms of proteasomes and to explore their structure–activity relationships. Many of the analogues are potent inhibitors of the chymotrypsin-like activity, while having relatively little effect on the other proteasome activities. Moreover, we have shown that proteasomes immunoprecipitated from cultured cells are inhibited, that there are differences in their reaction with 20 S and 26 S proteasomes, and that they can be used to effectively inhibit proteasome function in the activation of NFκB.

EXPERIMENTAL PROCEDURES

Materials

Wistar rats were obtained from B&K Universal Ltd. (Hull, U.K.). *N*-t-Butoxycarbonyl-Leu-Ser-Thr-Arg-4-amidomethylcoumarin (Boc-Leu-Ser-Thr-Arg-4-AMC) and succinyl (suc)-Leu-Leu-Val-Tyr-AMC were purchased from the Peptide Institute (Osaka, Japan). Ala-Ala-Phe-AMC, benzyloxycarbonyl (Cbz)-Leu-Leu-Glu-β-napthylamide (NAP), ATP, Protein A–agarose and BSA were obtained from Sigma. Solutions for cell culture were obtained from Gibco BRL. Peptidyl boronic acids were synthesized by the general method of Kettner and Shenvi [21] and Matteson and Sadhu [22], and characterized by proton NMR and mass spectrometry. [4-^{3}H]Phenacetyl-(*S*)-Leu-(*S*)-Leu-(*R*,*S*)-boroLeu was prepared by palladium-catalysed tritiodehalogenation of para-bromophenylacetyl-(*S*)-Leu-(*S*)-Leu-(*R*,*S*)-boroLeu pinacol ester; the ester group was removed to give the free acid. The resulting compound had 97.3 %

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radiochemical purity by HPLC, with a specific activity of 6.4 Ci/mmol.

Purification and assay of 20 S and 26 S proteasomes

20 S and 26 S proteasomes were purified to apparent homogenity from fresh or frozen rat livers, respectively, as described previously in [23,24]. Protein concentrations were determined according to [25], and the purity of the preparations was confirmed by non-denaturing and SDS/PAGE.

Peptidase activities of the 20 S and 26 S proteasome were assayed using 1–2 μg and 0.1–1 μg of protein, respectively, in 50 mM Hepes buffer, pH 7.5, as described previously in [23]. ATP (0.5 mM) was added to assays of 26 S proteasomes. Trypsin-like activity was assayed with 40 μM Boc-Leu-Ser-Thr-Arg-AMC; chymotrypsin-like activities with 40 μM suc-Leu-Leu-Val-Tyr-AMC, Ala-Ala-Phe-AMC and Cbz-Gly-Gly-Leu-AMC; and peptidylglutamylpeptide hydrolase activity with 100 μM Cbz-Leu-Leu-Glu-NAP as substrate. Inhibitor studies with 20 S and 26 S proteasomes were carried out by assaying peptidase activities after pre-incubation of enzyme with inhibitor for 15 min at 25 °C. Stock solutions of inhibitors were made in DMSO such that the final concentration of DMSO in the assay was less than 1 %, which does not inhibit proteasome activity.

Kinetic analysis

K_i values for the inhibition of proteasome chymotrypsin-like activity were determined using the Henderson equation for tight-binding competitive inhibitors [26]:

$$I_t/(1-v_i/v_0) = E_t + K_i[(S_t + K_m)/K_m]v_0/v_i$$

where I_t = total inhibitor concentration, v_i = inhibited velocity, v_0 = uninhibited velocity, E_t = total enzyme concentration, K_i = dissociation constant for the binding of inhibitor to enzyme, S_t = total substrate concentration and K_m = the dissociation constant for the binding of substrate to enzyme. The data analysis was conducted using Scientist version 2.0 program (MicroMath; Salt Lake City, UT, U.S.A.).

Cell culture

L-132 human embryonic lung cells were grown in Dulbecco's modified essential medium supplemented with 10 % (v/v) newborn bovine serum and penicillin/streptomycin (50 units/ml and 50 μg/ml respectively) in a humidified atmosphere of 5 % (v/v) CO_2/air.

Inhibition of 20 S and 26 S proteasomes in cultured cells

Growth medium was removed from subconfluent cells and replaced with fresh medium containing selected concentrations of peptidyl boronic acid inhibitors. Control flasks were set up containing fresh growth medium and appropriate control concentrations of DMSO instead of the inhibitors. After 2 h, cell extracts were prepared. Monoclonal antibody MCP20 [27] was used for immunoprecipitation of 20 S and 26 S proteasomes under two different conditions to favour recovery of either 20 S or 26 S proteasomes. For the immunoprecipitation of 20 S proteasomes (including those derived from 26 S proteasomes), cells were lysed using RIPA buffer as described previously [28]. Immunoprecipitation of 26 S proteasomes (and also any free 20 S proteasomes) was carried out following lysis in 20 mM Tris/HCl buffer, pH 7.5, containing 5 mM ATP, 10 % (v/v) glycerol, 0.2 % Nonidet P40 [29]. The protein content of the cell lysates was determined using the Lowry method [30] following precipitation of protein with 0.02 % sodium deoxycholate and 8 % (w/v) trichloroacetic acid. Immunoprecipitation was carried out for the two sets of samples, using the same amount of protein. The 20 S and 26 S proteasome immunoprecipitates were washed with 50 mM Hepes/KOH (pH 7.5), and 50 mM Hepes/KOH (pH 7.5) containing 2 mM ATP, respectively, prior to determination of peptidase activity using 50 μM suc-Leu-Leu-Val-Tyr-AMC as substrate in these buffers. Although 26 S proteasome immunoprecipitates could contain free 20 S proteasome, the activity measured would only be that of 26 S proteasomes because 20 S proteasomes are latent under these conditions and require SDS for activation [31]. In 20 S proteasome immunoprecipitates, those derived from 26 S complexes would only account for about 50 % of the total.

The effect on proteasomes in cells was also determined using a radiolabelled inhibitor. Subconfluent L-132 cells were cultured in the presence of 100 nM or 1 μM [^{3}H]phenacetyl-(*S*)-Leu-(*S*)-Leu-(*R*,*S*)-boroLeu for 2 h, then lysed in 200 μl of 20 mM Tris/HCl buffer, pH 7.5, containing 5 mM ATP, 10 % (v/v) glycerol and 0.2 % Nonidet P40 [29]. The cell extract was fractionated by gel filtration using a Pharmacia Superose 6 column equilibrated in 20 mM Tris/HCl buffer, pH 7.5, containing 5 mM ATP, 10 % (v/v) glycerol and 150 mM KCl. Samples of fractions were analysed by scintillation counting and by measuring enzyme activity. Assays for 20 S proteasome activity in these fractions contained 0.02 % SDS to activate the 20 S proteasome [31].

Assay of NFκB processing

A luciferase reporter plasmid containing the IL-8 'core' promoter was engineered and stably transfected into U937 cells as previously described [32]. Transfected U937 clones were twice centrifuged at 300 ***g*** for 5 min and resuspended in RPMI 1640 with 10 % foetal bovine serum to a density of 1×10^6 cells/ml. 200 μl aliquots were added to the wells of an opaque 96-well filter bottom plate. Inhibitor or DMSO carrier (1 μl) was added to the appropriate wells in triplicate and the plates were incubated at 37 °C, 5 % CO_2, for 30 min. The stimulus was added (5 ng/ml TNF-α) and the samples incubated for 5 h at 37 °C, 5 % CO_2. At the end of the incubation the medium was removed by filtration and the cells washed twice with PBS without Ca^{2+} and Mg^{2+}. The resulting cell pellets were lysed in 20 μl of 1 × lysis buffer (Promega, Madison, WI, U.S.A.) and incubated for 15 min at room temperature. Luciferase activity was measured in a MicroLumat LB96P luminometer (EG-G Berthold, Bad Wilbad, Germany) which dispensed 100 μl of luciferase assay reagent (Promega) into each well and recorded the integrated light output for 20 s. Light output was measured in relative light units.

RESULTS

Effect of di- and tri-peptidyl boronic acid inhibitors on the chymotrypsin-like activity of purified rat liver 20 S and 26 S proteasomes

A range of peptidyl boronic acids were tested on the activity of 20 S proteasomes assayed with suc-Leu-Leu-Val-Tyr-AMC. Benzoyl (Bz)-Phe-boroLeu was found to be an effective inhibitor in the nanomolar range and analysis of the time course for inhibition showed that maximal inhibition was achieved only after a 10–15 min incubation of enzyme with inhibitor (results not shown). This behaviour is characteristic of slow-binding inhibition [33]. Subsequent experiments were therefore performed following a 15 min pre-incubation of proteasome and inhibitor prior to addition of substrate. Measurement of proteasome

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Table 1 Effect of Bz-Phe-boroLeu (compound 14) on distinct peptidase activities of rat liver 20 S proteasomes

Assays were performed as described in the Experimental procedures section using 1 μg of 20 S proteasome. The substrate concentration was 40 μM for all substrates except Cbz-Leu-Leu-Glu-NAP which was used at 0.1 mM. Values are given as the average of two separate experiments, each performed in duplicate. Values are expressed as the percentage of control activity in samples containing no inhibitor. The effect of this inhibitor on activity assayed with suc-Leu-Leu-Val-Tyr-AMC under the same conditions is shown in Figure 2.

	Activity (% control)	
Substrate	20 nM Bz-Phe-boroLeu	80 nM Bz-Phe-boroLeu
Suc-Leu-Leu-Val-Tyr-AMC	42	26
Boc-Leu-Ser-Thr-Arg-AMC	113	124
Cbz-Gly-Gly-Leu-AMC	93	82
Ala-Ala-Phe-AMC	89	82
Cbz-Leu-Leu-Glu-NAP	96	61

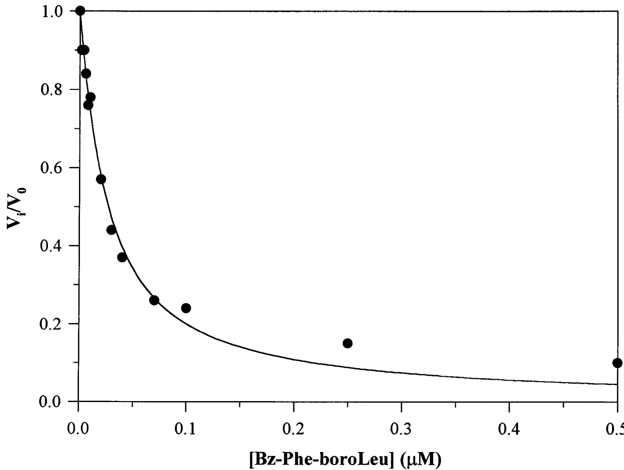

Figure 2 Inhibition of 20 S proteasomes by Bz-Phe-boroLeu (compound 14)

20 S proteasome (1 μg) was incubated in the presence of a range of Bz-Phe-boroLeu (compound **14**) concentrations in 50 mM Hepes/KOH buffer, pH 7.5, for 15 min at 25 °C. Substrate (40 μM suc-Leu-Leu-Val-Tyr-AMC) was added and the assays were incubated at 37 °C for 15 min. Activities are expressed as the fraction of control activity in samples containing no inhibitor.

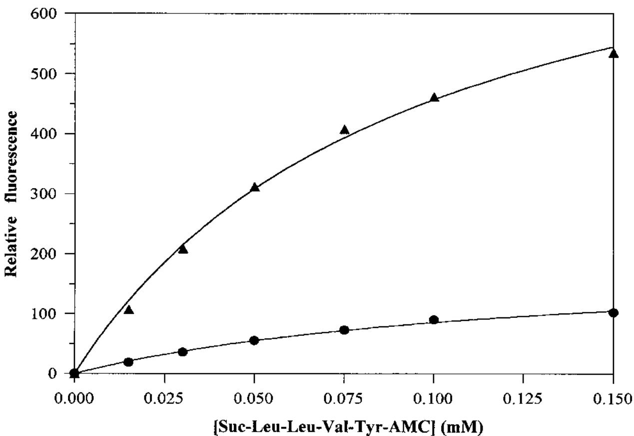

Figure 3 Hydrolysis of suc-Leu-Leu-Val-Tyr-AMC for the 20 S and 26 S proteasomes

The 20 S (circles) or 26 S (triangles) proteasomes were assayed with a range of suc-Leu-Leu-Val-Tyr-AMC concentrations in 50 mM Hepes/KOH buffer, pH 7.5. Assays were conducted for 15 min at 37 °C, using 1 μg of either proteasome form. Results from a typical experiment are shown. Mean K_m values, determined by fitting the data from separate experiments to the Michaelis–Menten equation, were 130 μM (S.D. $\pm$23.3, $n = 4$) for 20 S proteasomes, and 95 μM (range $\pm$2.1, $n = 2$) for 26 S proteasomes.

activity at varying Bz-Phe-boroLeu concentrations showed that this compound was a tight-binding inhibitor with 50 % inhibition being observed at concentrations approximately equal to that of the enzyme. Inhibition was only very slowly reversible as judged by the recovery of activity following dialysis of the enzyme (results not shown).

Experiments with other fluorogenic peptide substrates showed that other activities of proteasomes measured with different substrates were much less susceptible to inhibition by Bz-Phe-boroLeu than the chymotrypsin-like activity measured with suc-Leu-Leu-Val-Tyr-AMC (Table 1). A slight activation of trypsin-like activity measured with Boc-Leu-Ser-Thr-Arg-AMC was observed, comparable with some other inhibitors of chymotrypsin-like activity [34,35]. There was little inhibition of activity assayed with Ala-Ala-Phe-AMC and Cbz-Gly-Gly-Leu-AMC, which supports earlier suggestions that these substrates are not hydrolysed at the same site as suc-Leu-Leu-Val-Tyr-AMC [24,35]. The effects on trypsin-like and peptidylglutamyl peptide hydrolase activities were readily reversed by dialysis.

Kinetic analysis of the inhibition data obtained for the 20 S proteasome with various concentrations of Bz-Phe-boroLeu (Figure 2) gave a K_i value of 17 nM. This value was determined using the Henderson equation for competitive tight-binding inhibition with a K_m value of 130 μM as determined in Figure 3. Further assays with a few different concentrations of the suc-Leu-Leu-Val-Tyr-AMC substrate confirmed that the inhibition was competitive, as expected. However there was some evidence for the abnormal kinetic behaviour of proteasomes at high inhibitor concentrations (from $3\times$ to $50 \times K_i$) where the extent of inhibition observed was less than would be predicted by the Henderson equation for competitive inhibition (Figure 2). Fitting data to other possible kinetic models was tested but proved to be inappropriate. The data were fitted to a non-competitive equation but this model was ruled out on the basis that the K_i value obtained was different at different substrate concentrations, whereas with the competitive model there was no significant difference in the K_i value determined using different ranges of inhibitor concentration or different substrate concentrations. Since it is possible that not all proteasomes could be inhibited, a third model taking into account the possibility that not all proteasomes are inhibited was tested. This was also ruled out on the basis of poor fits to experimental data.

Inhibitor constants were calculated for a range of di- and tri-peptidyl boronic acids (Tables 2 and 3). The compounds all contained C-terminal α-aminoalkylboronic acid residues (or their labile pinacol boronate esters) to form a reversible tetrahedral adduct with the active site threonine residue in the proteasome [21,22]. All compounds except **14** (Bz-Phe-boroLeu) were racemic at the P1 position (Schechter and Berger nomenclature, see [36]). The free boronic acid **5** and its boronate ester **6** exhibited almost identical potency *in vitro* (K_i 25 and 35 nM respectively), indicating that under the assay conditions boronic acids and their boronate esters were equivalent [21]. The dipeptide boroLeu-based inhibitors **6** and **7** with a P2 Leu residue were potent

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Table 2 K_i values for the inhibition of rat liver 20 S proteasome chymotrypsin-like activity by peptidyl boronic acids

Activities were determined with 1 μg 20 S proteasome and various inhibitor concentrations in the range indicated. Numbers in parentheses are the number of determinants. Enzyme was preincubated with inhibitor for 15 min at 25 °C, in 50 mM Hepes/KOH (pH 7.5). Substrate (40 μM suc-Leu-Leu-Val-Tyr-AMC) was added to start the assays that were incubated for 15 min at 37 °C. Assays were performed in duplicate. K_i values, which are given as the mean±S.D., were determined using the Henderson equation for competitive inhibition, as described in the Experimental procedures section.

Compound number	Inhibitor	Concentration range (nM) and number of determinations	K_i value (nM)	Compound number	Inhibitor	Concentration range (nM) and number of determinations	K_i value (nM)
5	Cbz-Leu-boroLeu (free acid)	0–50 (3)	25.4 ± 9.9	**10**	Cbz-Leu-boroNle (pinacol ester)	0–200 (4)	96.8 ± 20.1
6	Cbz-Leu-boroLeu (pinacol ester)	0–60 (3)	34.7 ± 4.6	**11**	Cbz-Phe-boroLeu (pinacol ester)	0–60 (3)	24.5 ± 10.9
7	Bz-Leu-Leu-boroLeu (pinacol ester)	0–50 (3)	23.4 ± 4.7	**12**	Cbz-nitroArg-boroLeu (pinacol ester)	0–100 (3)	38.9 ± 3.8
8	Cbz-Leu-boroLeu (pinacol ester)	0–200 (4)	99.1 ± 19	**13**	Cbz-Lys(boc)-boroLeu (pinacol ester)	0–200 (3)	116.4 ± 9.7
9	Cbz-Leu-boroPhe (pinacol ester)	0–200 (3)	83.7 ± 11.6	**14**	Bz-Phe-boroLeu (free acid)	0–40 (3)	17.4 ± 4.1

inhibitors with similar activity, indicating that Cbz and benzoyl N-capping groups were equally acceptable. In contrast, compound **15**, with a phenylsulphonyl N-capping group, was poorly active (54 % of control activity at 1 μM). The tripeptide inhibitor **8**, with an additional P3 Leu residue, was less potent (K_i 99 nM). This additional residue, therefore, conferred no obvious advantage.

The effect of varying the P1 substituent was studied with inhibitors **6**,**9**,**10** and **16**, bearing *iso*-butyl, benzyl, *n*-butyl and *tert*-butylmethyl side chains respectively. The order of potency was $\mathbf{6} > \mathbf{9} = \mathbf{10} \gg \mathbf{16}$, thus the leucyl iso-butyl side-chain is preferred and the S1-binding site will not tolerate the more bulky tert-butylmethyl substituent. The effect of varying the P2 substituent was studied with inhibitors **6**,**11**,**12**,**13** and **17**, bearing Leu, Phe, N^{γ}-nitro-Arg, Boc-Lys and Pro P2 residues respectively. The order of potency was $\mathbf{6} = \mathbf{11} = \mathbf{12} > \mathbf{13} \gg \mathbf{17}$. Thus lipophilic Leu and Phe residues are equally preferred and the more polar N^{γ}-nitro-Arg residue is also acceptable. This result, therefore, agrees with the work of Iqbal et al. [19,37] on the related compound **4**. A Pro residue is obviously not accommodated at the P2 position, unlike inhibitors of classical serine proteinases [38]. The most potent inhibitor of the series was the (*R*)-boro-Leu derivative **14**, with a Phe residue at the P2 position (K_i 17.4 nM).

Inhibition of 26 S proteasomes, which have a lower K_m and a higher V_{max} for suc-Leu-Leu-Val-Tyr-AMC (Figure 3) than 20 S proteasomes, was also characterized using two of the inhibitors, Bz-Phe-boroLeu (compound **14**) and Cbz-Leu-Leu-boroLeu pinacol ester (compound **8**). The K_i value determined with compound **14** for 26 S proteasomes was 11.3 ± 6.1 nM ($n = 3$), compared to the value of 17.7 ± 4.1 nM for 20 S proteasomes, but at high concentrations of inhibitor these compounds were both found to be more effective for 26 S than for 20 S proteasomes (Figure 4). It is still not clear why this should be, although we

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Table 3 Percentage inhibition of rat liver 20 S proteasome chymotrypsin-like activity by peptidyl boronic acids

Activities were determined with 1 μg of 20 S proteasome and 1 μM inhibitor concentration. Numbers in parentheses are the number of determinants. Enzyme was preincubated with inhibitor for 15 min at 25 °C, in 50 mM Hepes/KOH (pH 7.5). Substrate (40 μM suc-Leu-Leu-Val-Tyr-AMC) was added to start the assays, which were incubated for 15 min at 37 °C.

Compound number	Compound structure	Concentration range (nM) and number of determinations	Activity (% of control) with compound at 1 μM
15	PhSO2-Leu-boroLeu (pinacol ester)	0–1000 (2)	54
16	Cbz-Leu-t-butylboroLeu (pinacol ester)	0–1000 (2)	73
17	Cbz-Pro-boroLeu (pinacol ester)	0–1000 (2)	91

have previously observed the non-ideal behaviour of 20 S proteasomes with peptidyl chloromethyl ketone inhibitors [24,35].

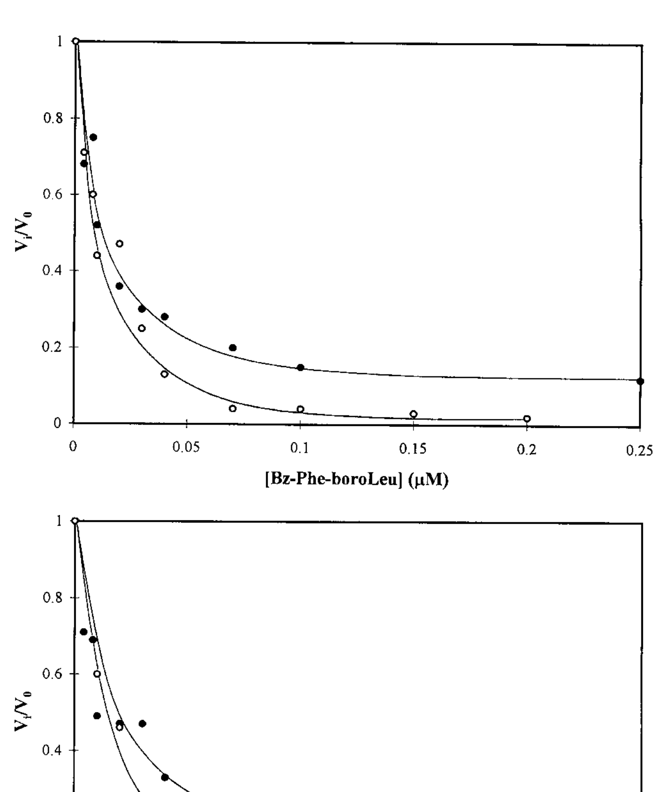

Figure 4 Effects of peptidyl boronic acid inhibitors on 20S and 26S proteasome activities

Incubations of proteasomes and inhibitor were carried out as described in the legend to Figure 2 using 0.1 μg of 26 S proteasome per assay. The inhibitors used were Bz-Phe-boroLeu (compound **14**, top panel) and Cbz-Leu-Leu-boroLeu pinacol ester (compound **8**, bottom panel). Activities of the 20 S (closed circles) or 26 S (open circles) proteasomes are expressed as percent of control activity in samples containing no inhibitor.

Inhibition of proteasome activity and functions in cultured cells

Two of the inhibitors, Bz-Phe-boroLeu (compound **14**) and Cbz-Leu-Leu-boroLeu pinacol ester (compound **8**), were also tested for their effects on proteasomes in cultured cells. Cells were grown in the presence or absence of inhibitor and then proteasomes were immunoprecipitated and assayed under conditions that would distinguish between 20 S and 26 S activities. Immunoprecipitated proteasomes were inhibited (Table 4), confirming that these inhibitors are effective in cells. Interestingly the inhibition of proteasomes, isolated under conditions chosen to favour recovery of 26 S proteasomes, was consistently greater than that of 20 S proteasomes in each experiment with Bz-Phe-boroLeu (compound **14**; Table 4) but also in similar experiments carried out with Cbz-Leu-Leu-boroLeu pinacol ester (compound **8**). These results are consistent with the data obtained with purified proteasomes. However, studies carried out with the radiolabelled inhibitor, [^{3}H]phenacetyl-Leu-Leu-boroLeu, again showed differences in the results obtained for 20S and 26S proteasomes. In these experiments, extracts of cells which had been treated with [^{3}H]phenacetyl-Leu-Leu-boroLeu (100 nM or 1 μM) were fractionated by gel filtration (Figure 5). The majority of the label was associated with 20 S proteasomes. The apparent lack of a separate label peak in fractions containing 26 S proteasome activity cannot be explained by the low amount of 26 S compared to 20 S proteasome, but suggests that this inhibitor does not bind very tightly to 26 S proteasomes and is readily removed during the gel-filtration chromatography procedure. The immunoprecipitation procedure (Table 4) is a more rapid process. A small peak of label in fraction 17 (less than 6 % total counts) at the high concentration of inhibitor used in this experiment (1 μM, more than $10 \times K_i$) was not observed in experiments with 100 nM inhibitor.

The effect of peptidylboronic acid inhibitors on proteasome function was tested in a cell-based assay for NFκB activation, which involves conjugation of ubiquitin and partial degradation by proteasomes [39]. The relative IC_{50} values for inhibition of proteasome-dependent activation of NFκB with Bz-Phe-boroLeu (compound **14**; 96.0 and 74.0 nM in two separate experiments) and Cbz-Leu-Leu-boroLeu pinacol ester (compound **8**; 140 and 200 nM in two separate experiments) were comparable to relative K_i values for rat liver proteasomes (17.7 ± 4.1 and 99.1 ± 19 nM respectively).

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Table 4 Inhibition of 20 S and 26 S proteasomes by Bz-Phe-boroLeu (compound 14) in human L-132 cells

L-132 cells were cultured in growth medium containing selected concentrations of Bz-Phe-boroLeu (compound **14**) for 2 h. Control flasks were cultured with growth medium containing appropriate amounts of DMSO instead of inhibitor. Monoclonal antibody MCP20 was used to immunoprecipitate (a) 20 S proteasomes and (b) 20 S and 26 S proteasomes, as described in the Experimental Procedures section. Immunoprecipitates were washed and then assayed (a) for 20 S proteasome activity in the presence of 0.02% SDS to activate latent 20 S proteasomes and (b) for 26 S proteasome activity (20 S inactive in the absence of SDS) using 50 μM suc-Leu-Leu-Val-Tyr-AMC as substrate. Activities of the 20 S and 26 S proteasomes are expressed as percentages of control activity in samples containing no inhibitor.

Inhibitor concn. (nM)	20 S proteasomes (% of control)	26 S proteasome (% of control)
10	72.3 ± 42.0	53.7 ± 34.3
100	33.3 ± 11.1	19.0 ± 5.3

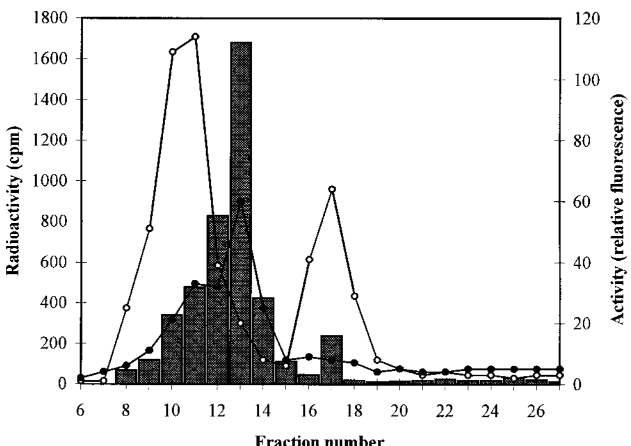

Figure 5 Inhibition by [^{3}H]phenacetyl-Leu-Leu-boroLeu

Subconfluent L-132 cells were incubated with 1 μM [^{3}H]phenacetyl-Leu-Leu-boroLeu in Dulbecco's modified Eagle's medium for 2 h at 37 °C. Cell extracts were loaded onto a Pharmacia Superose 6 column, and fractions collected for scintillation counting and assays of proteasome activity. Assays were conducted in the presence (filled circles, 20 S) or absence (open circles, 26 S) of 0.02% SDS to activate latent 20 S proteasomes. The second peak of activity under 26 S proteasome assay conditions is due to a lower molecular mass proteinase. Shaded bars represent the radioactivity in the different fractions.

DISCUSSION

Peptide boronic acid inhibitors can act as tight-binding inhibitors of 20 S proteasomes and also inhibit the 26 S form of proteasomes. Of the di- and tri-peptides tested, the best inhibitor was Bz-Phe-boroLeu (compound **14**). From the results presented here, there seems to be little difference in K_i values with 20 S and 26 S proteasomes. However, there are clear differences at high inhibitor concentrations where 26 S proteasomes are completely inhibited, while 20 S proteasomes are not. There may also be differences in the reversibility of the reaction. Since several groups have reported kinetic differences between proteasomes containing interferon-inducible subunits and other 20 S proteasomes not containing these subunits, one possibility is that a fraction of the 20 S proteasomes is not affected. This plausible explanation, for the incomplete inhibition of rat liver 20 S proteasomes, has been ruled out because the kinetic data do not fit this model. Furthermore, we know that the variable subunits LMP2 and LMP7 are present in 26 S as well as 20 S proteasomes (R. Z. Murray, K. B. Hendil and A. J. Rivett, unpublished work), so this model would not explain differences in kinetic behaviour of 20 S and 26 S proteasomes. A more likely explanation is that inhibitor binding to 20 S proteasomes may cause some aggregation and non-ideal behaviour at high inhibitor concentrations, as found with peptidyl chloromethyl ketone inhibitors [24].

Peptidyl boronic acid inhibitors are potent transition-state analogue inhibitors of a number of serine proteinases. These inhibitors act in a reversible, competitive manner with K_i values that are often in the low nanomolar range and they often exhibit slow-binding inhibition. Slow-binding kinetics can be explained by a slow conformational change which occurs over several minutes following inhibitor binding to the catalytic site and yields the fully inhibited state [40,41]. The mechanism of inhibition of the threonine proteinase is similar to that for serine proteinases and therefore consistent with the modification of the N-terminal threonine as proposed from studies with the *Rhodococcus* proteasome [42]. The difference between the K_i value reported for compound **1** [20] and our determined value for the closely related compound **8** (0.03 and 99 nM respectively) cannot be explained by a difference between free acid and ester, or by the racemic nature of the compound. There is no clear explanation for the difference in K_i values obtained, but it is possible that proteinase purification protocols and assay conditions (cf. with 0.035% SDS [20]) could influence the K_i values. Results of independent measurements of IC_{50} values for rat liver proteasomes, using a microtitre plate assay with significantly lower enzyme concentrations [43], gave results consistent with the K_i values reported here.

The most effective inhibitors of chymotrypsin-like activity assayed with suc-Leu-Leu-Val-Tyr-AMC were much less effective on proteasome activities measured with other substrates. Radiolabelling of proteasomes with irreversible inhibitors has proved useful for the identification of catalytic subunits reacting with inhibitors of the different activities. Lactacystin predominantly inhibits chymotrypsin-like activity and modifies subunit X (MB1) [15], but at higher concentrations can also react with the other catalytic subunits [16]. Radioactive 3,4-dichloroisocoumarin modifies more than one subunit [44] and X and LMP7 are modified by peptidyl chloromethyl ketone inhibitors [24] and peptidyl vinylsulphone inhibitors [45] of chymotrypsin-like activity. 20 S proteasome subunits MECL1 and Z seem to be associated with cleavage after basic residues and LMP2 and Y with cleavage after acidic residues [45].

The results presented here demonstrate that these compounds can be useful inhibitors of proteasome function in mammalian cells. The observed K_i values for the inhibition of rat liver 20 S and 26 S proteasomes were similar, which is not surprising in view of the fact that they share the same catalytic components. Both 20 S and 26 S proteasomes were inhibited in experiments with cultured cells, but inhibition of 26 S proteasomes was more readily reversed. The fact that inhibition of immunoprecipitated proteasomes was detected is consistent with the tight binding and slow reversibility of the inhibition. The cell permeability of these compounds and their specificity for the chymotrypsin-like activity of proteasomes makes them useful for investigations of proteasome function in many aspects of cellular regulation. Their usefulness was confirmed by the demonstration that they inhibit NFκB activation which is known to require 26 S proteasome activity [39]. A recently published study with another peptidylboronic acid inhibitor reports inhibition of NKκB activation in a different cellular system [46]. Thus the peptidyl boronic acids are useful as reversible cell permeable inhibitors which are more potent for proteasomes than many of the commonly used peptide aldehyde inhibitors.

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