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Development of lipopeptides for inhibiting 20S proteasomes

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Abstract—Proteasomes are responsible for the cytoplasmic turnover of the vast majority of proteins including regulatory proteins. We have synthesized lipopeptides a new class of non-covalent inhibitors of the 20S proteasome and assayed their inhibitory capacities. Their ability to inhibit at micromolar concentrations chymotrypsin-like and post-acid activities depends on peptide length (3 or 6 amino acids), sequence (presence of a positively or negatively charged amino acid), and alkyl chain length (C6–C18). These structural features could be varied to selectively inhibit one or more of the three proteasome activities.

Proteasome inhibitors are new potential therapeutic agents for treating disorders such as cancer, inflammation, immune diseases, muscle wasting, and certain myopathies.[1] The ubiquitin–proteasome system is the major pathway of proteolysis in eukaryote cells. In addition to removing damaged and unneeded proteins, it degrades key regulatory proteins that are crucial for many intracellular processes, including cell progression, apoptosis, NF-κB activation, and antigen presentation.[2] The eukaryotic proteasome is a multicatalytic protease consisting of a 20S proteolytic core particle and a 19S regulatory subunit at one or both ends.[3] The 20S proteasome is a hollow barrel-shaped structure formed of four stacked $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ multiprotein rings. The β1, β2, and β5 subunits are present in duplicate and are associated with post-acidic (PA) or caspase-like, trypsin-like (T-L), and chymotrypsin-like (ChT-L) activities, respectively.[4]

Most proteasome inhibitors are short peptides linked at their C-terminal end to a reactive group responsible for the chemical modification of the catalytic Oγ-Thr1 of the 6 catalytic sites.[1b] The peptide boronate bortezomib (formerly PS341) or Velcade® has been used to treat incurable multiple myeloma since May 2003.[5] Some non-peptide threonine modifiers have also been described.[1,6] Non-covalent inhibitors, which should theoretically have fewer side-effects, have been investigated less extensively. They include ritonavir,[7] benzylstatine derivatives,[8] and TMC-95A derivatives.[9] We postulated that adding a large aliphatic blocking group to the N-terminus of a short peptide that is known to inhibit proteasomal activities would make it a better inhibitor and favor targeting of the cell membrane.

This report describes the synthesis, enzyme inhibitory activity, and metabolic stability of a series of tripeptides and hexapeptides linked at their N-end to an aliphatic chain. Several natural hexapeptides **1**–**11** (Table 1) were first synthesized to obtain peptide sequences suitable for use as reference sequences for lipopeptides. These hexapeptides are small peptides that mimic peptides released by the proteasome during protein breakdown, and which modulate proteasome activities.[10] Some of them are positively charged (lysine at position 5 in **1**–**3**, **5**, and **8**) and others negatively charged (aspartate in **4**, **6**, **7**, **10**, **11** and glutamate in **9** at position 5), as the inhibitory action of peptides on proteasome activities appears to depend on their overall charge.[10,11] Basic and acidic hexapeptides had a leucine residue or an aromatic residue at the C-terminus to mimic major cleavages induced by the ChT-L activity. The influence of an alkyl chain added at the N-terminal end of representative acidic and basic hexapeptides was also analyzed (**12**–**27**). Peptidic length was decreased by linking tripeptides to an alkyl chain

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Table 1. Structure and inhibitory (or activatory) activities of compounds **1**–**42** at pH 7.5 and 30 °C

	Compound	*n*	K_i (μM), or activation factor (x)		
			ChT-L	T-L	PA
1	TVTFKF	—	139	ni	148
2	TVTYKY	—	>625	ni	>625
3	TVTYKL	—	545	ni	ni
4	TVTYDY	—	ni	ni	ni
5	TVSYKF	—	>625	ni	>625
6	TITYDF	—	ni	ni	> 625
7	TITFDY	—	>625	ni	>625
8	TITYKF	—	151	ni	260
9	TITYEY	—	ni	ni	ni
10	TISYDY	—	>625	ni	>625
11	TITYDL	—	619	ni	>625
12	CH_3–$(CH_2)_n$–CO-TVTYKF	4	80	315	ni
13		6	20	x 1.2	x 1.5
14		8	72	ni	x 1.2
15		10	>80	68	x 1.2
16		12	0	80	x 1.2
17		14	x 1.6	—	—
18	CH_3–$(CH_2)_n$–CO-TVTFKF	4	43	ni	ni
19		6	>80	—	—
20		8	28	—	—
21	CH_3–$(CH_2)_n$–CO-TITFDY	4	34	ni	ni
22		6	43	ni	>80
23		8	37	—	—
24		10	>80	>80	>80
25		12	ni	78	x 1.3
26		14	80	ni	x 1.5
27		16	39	x 1.6	x 1.3
28	Pam-YDL		20	>80	15
29	CH_3–$(CH_2)_n$–CO-YEL	4	>80	ni	59
30		6	>80	x 1.9	ni
31		8	ni	ni	72
32		10	ni	>80	62
33		14	18	>80	15
34	Pam-YNT(0)		30	—	—
35	Pam-YET(0)		20	—	21
36	Apam-YET(0)		5	—	8
37	Pam-FKF		30	>80	ni
38	Pam-YKF		81	>80	ni
39	YED		ni	ni	ni
40	YEL		ni	ni	ni
41	Pam-OMe		ni	ni	ni
42	Apam		ni	ni	ni

The peptidase activities of yeast 20S proteasome were determined by following the hydrolysis of the appropriate fluorescent substrate for 30 min. ni, no inhibition (< 10% inhibition) at 625 μM (**1**–**11**) or 80 μM (**12**–**42**). $K_i > 625$ or 80 μM: <30 or 20% inhibition at the highest inhibitor concentration used (625 or 80 μM, respectively).

instead of hexapeptides, starting from the tripeptide sequence YDL derived from compound **11** and varying its amino acid composition (**29**–**36**). Two positively charged lipotripeptides were also obtained and studied, Pam-FKF (**37** from compound **1**) and Pam-YKF (**38**) from compound **8**.

Pam-YET(0), Pam-YNT(0), and Apam-YET(0) were purchased from Biosynthan GmbH (Berlin, Germany) (T(0), thyronine; Pam, palmitoyl [CH_3–$(CH_2)_{14}$–CO–]; Apam, 2-aminopalmitoyl). They were 95% pure. Pam-OMe was from Sigma, Apam from Fluka, and MG132 from Calbiochem. The Fmoc-amino acid-Wang resins, Fmoc-amino acids, 1-hydroxybenzotriazole (HOBt), *O*-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyl-uronium tetrafluoroborate (TBTU), and diisopropylethylamine (DIEA), were purchased from Senn Chemicals or Novabiochem.

Peptides and lipopeptides were obtained by solid phase synthesis using classical Fmoc/tBu methodology on a multichannel peptide synthesizer.[12] The peptide chains were assembled using the in situ neutralization protocol previously described.[12] The side chains of tyrosine, lysine, threonine, serine, aspartic, and glutamic acids were protected by *t*-Bu (*tert*-butyl) or Boc groups (Boc, *tert*-butoxycarbonyl) as appropriate. Asparagine was protected by a trityl group. Each coupling step employed 3 equivalents of Fmoc-amino acid plus 3 equivalents of HOBt and TBTU, and 9 equivalents of DIEA in DMF, at room temperature. Coupling was usually complete within 1 h, as determined by the

2,4,6-trinitrobenzenesulfonic acid (TNBSA) test. The Fmoc group was deprotected with 25% (v/v) piperidine in DMF. The lipopeptides were obtained by capping the peptide sequence with the acyl chloride CH_3–$(CH_2)_n$–CO–Cl (n = 4–16). The peptides or lipopeptides were cleaved from the resin, and the side chains deprotected by incubation with a mixture of 0.75 g crystalline phenol, 0.25 mL 1,2-ethanedithiol, 0.5 mL thioanisole, 0.5 mL deionized water, and 10 mL trifluoroacetic acid (TFA) for 1.5 h. Crude peptides, which were generally >85% pure as assessed by analytical reverse-phase HPLC, were purified by preparative RP-HPLC, giving final purities >97%. The purification conditions were: (1) for peptides: linear gradient of acetonitrile (Carlo-Erba) in Ultra-High Quality water containing 0.1% (v/v) trifluoroacetic acid (TFA, sequencing grade, Sigma) for 30 min at a flow rate of 4 mL/min with UV detection at 214 nm on an Interchrom UP5ODB.25M 5-μm column (250 × 10 mm); (2) for lipopeptides: linear gradient of acetonitrile (Carlo-Erba) in Ultra-High Quality water containing 0.1% (v/v) TFA (sequencing grade, Sigma) for 40 min at a flow rate of 4 mL/min with UV detection at 214 nm on an Macherey-Nagel Nucleosil 300-7C4 column (250 × 10 mm). ^{1}H NMR spectra for peptides and lipopeptides were fully consistent with the assigned structures.

The abilities of peptides (**1**–**11**, **39**, and **40**) and lipopeptides (**12**–**38**) to inhibit the three catalytic activities of yeast 20S proteasome were tested. The enzyme was obtained using the Sdl135 strain kindly provided by David Leggett (Harvard Medical School, Boston, USA) and purified.[13] Some experiments were performed with reticulocyte rabbit proteasome (Boston Biochem, Cambridge, USA). The ChT-L (Suc-LLVY-AMC with Suc, succinyl; AMC, aminomethylcoumaride), the T-L (Boc-LLR-AMC), and the PA (Z-LLE-β-NA with Z, benzyloxycarbonyl; β-NA, β-naphthylamine) activities of 20S proteasome were determined by monitoring the hydrolysis of the appropriate fluorescent substrate for 30 min at 30 °C using a BMG Fluostar microplate reader, in the presence of untreated proteasome (control), or proteasome that had been incubated with the test compound (0.1–1000 μM) (λ_{exc} = 360, λ_{em} = 465 nm for AMC substrates, and λ_{exc} = 340, λ_{em} = 405 nm for the βNA substrate). The buffers were (pH 7.5): 20 mM Tris, 1 mM DTT, 10% glycerol, 0.02% (w/v) SDS, and 3% (v/v) DMSO (ChT-L and PA activities); 20 mM Tris, 1 mM DTT, 10% glycerol, 0.01% Triton X-100, and 3% (v/v) DMSO (T-L activity). The control experiments were run with the same amount of DMSO solvent (3.5% (**1**–**27**) or 3% (**28**–**40**), v/v) as in the samples. Initial rates determined in control experiments (V_0) were considered to be 100% of the peptidase activity; initial rates (V_i) that were above 100% in the presence of a test compound were considered to be activations (expressed as activation factor), while initial rates below 100% were considered to be inhibitions. The results were obtained by calculating the average of at least two independent experiments; the variability was less than 10%. The inhibitory activity of compounds is expressed either as IC_{50} or apparent K_i. The values of IC_{50} were calculated by fitting the experimental data to equation 1: % inhibition = $100 \times (1 - V_i/V_0) = 100\ [I]_0/(IC_{50} + [I]_0)$ (compounds **28**, **33** for ChT-L and PA activities), or equation 2: % inhibition $= 100[I]_0^{n_H}/(IC_{50}^{n_H} + [I]_0^{n_H})$, n_H is the Hill number (compounds **1**, **8**, and **36** for ChT-L and PA activities) (Fig. 1). The inhibition constants K_i were determined using the equation $V_i/V_0 = 1/(1 + [I]/K_{i(app)}$ with $K_{i(app)} = K_i/(1 + [S]/K_m)$ or the equation $IC_{50} = K_i(1 + [S]/K_m)$. The stability of the new inhibitors in culture medium RPMI containing 20% fetal calf serum was assessed by studying their kinetics of breakdown by incubation in the culture medium at 37 °C for up to 2

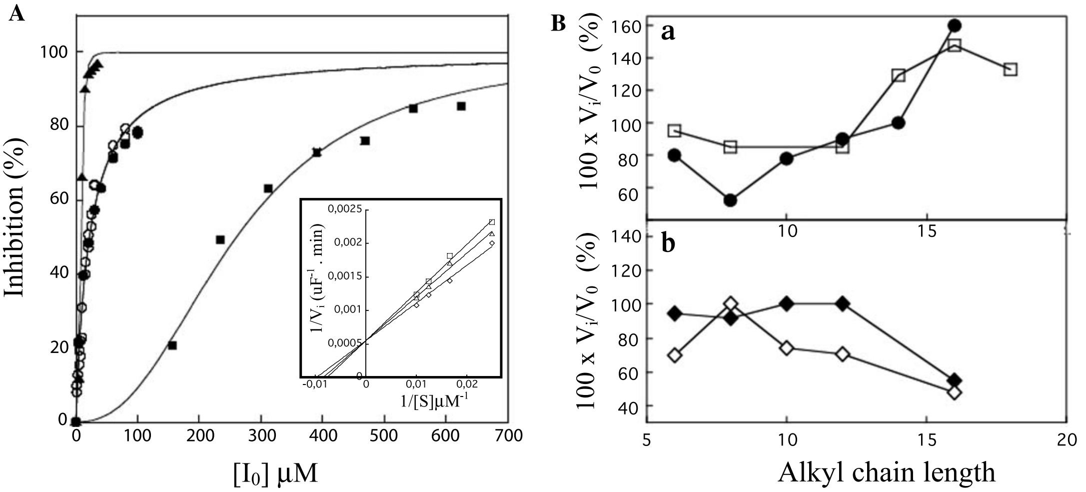

Figure 1. Effects of peptides and lipopeptides on ChT-L and PA activities of yeast 20S proteasome at pH 7.5 and 30 °C. (A) Inhibition of ChT-L by peptide **8** (■) and lipopeptide **36** (▲) and PA activity by lipopeptides **28** (○) and **33** (●). The experimental data were adjusted to equation 1 (% inhibition = $100[I]_0/(IC_{50} + [I]_0)$ (**28** and **33**) or 2 (% inhibition $= 100[I]_0^{n_H}/(IC_{50}^{n_H} + [I]_0^{n_H})$ with n_H = 2.4 (**8**) and 3.9 (**36**) giving: IC_{50} = 260 ± 12 (**8**, ChT-L), and 21.8 ± 0.8 (**28**, PA), 8.4 ± 0.2 (**36**, ChT-L), 21.8 ± 1.3 (**33**, PA). Inset: double reciprocal Lineweaver–Burk plot for the inhibition of ChT-L activity by **33** with [**33**] = 0 (◇), 20 (△), and 40 (□) μM. Experimental points are adjusted to the equation: $1/V_i = (1 + [I]/K_i) \times (K_m/V_m[S]) + 1/V_m$. (B) Influence of the alkyl chain length on the efficiency of alkyl-TVTYKF (●), alkyl-TITFDY (□) (a), and alkyl-YEL (◆,◇) (b) as inhibitors or activators of 20S proteasome. Full symbols are used for ChT-L activity and empty ones for PA activity.

days. Incubation was terminated by addition of ethanol. The mixture was poured at 4 °C and centrifuged (10,000 rpm for 10 min). Aliquots (20 μL) of the clear supernatant were injected onto the RP-HPLC column. The degradation half-life was obtained by a least-squares linear regression analysis of a plot of the log[I] versus time using a minimum of five points.

Natural hexapeptides (**1**–**11**) were poor inhibitors of ChT-L and PA activities (Table 1). Positively charged peptides (**1**–**3**, **5**, and **8**) inhibited the ChT-L activity (Fig. 1A), and most surprisingly, the PA activity, whose active sites are thought to accommodate acidic residues best. The presence of an aromatic Phe or Tyr residue at the C-terminal extremity appeared to be a major feature of their inhibitory efficiency. Three negatively charged peptides (**7**, **10** and **11**) also inhibited ChT-L and PA activities to a similar extent. Positively charged peptides were better inhibitors of both activities than were the negatively charged ones. Only negatively charged peptides (**6**, **7**, and **11**) activated the T-L activity of the latent proteasome by a factor of 2 or more. The motif YEY (**9**) was unfavorable, even for PA activity. Alkyl chains of varying lengths were attached to the N-terminal end of three hexapeptides to study the influence of alkyl chains on the interaction of peptides with the three active sites: (i) TVTYKF a competitive inhibitor of *Xenopus* 26S proteasome (K_i = 0.6 mM);[10] (ii) the related peptide TVTFKF (**1**) which exerts a kinetically complex inhibitor effect on proteasome activities (data not shown); (iii) the negatively charged TITFDY (**7**) which inhibits weakly the ChT-L and PA activities but stimulates the T-L activity 3-fold. A short alkyl chain (C8 for **13** and C10 for **20**) enhanced inhibitory activity of positively charged hexapeptides (about 18-fold for C8 and 8-fold for C10), whereas longer alkyl groups (**15**–**17**) did not enhance the inhibition of ChT-L activity by the positively charged TVTYKF (activation for **17** with C16 chain) but favored inhibition of T-L activity (**15**, **16**) (Table 1 and Fig. 1B; a). Consistent with these results, short alkyl groups (C6–C10 for **21**–**23**) also favored inhibition by the negatively charged hexapeptide TITFDY, which was a very poor inhibitor alone (about 23-fold) (compare **27** and **7**). We then decreased the peptide character of lipopeptides by shortening the peptide moiety. Our previous study on hexapeptides highlighted the influence of the three amino acids at the peptide C-end.[10] Consequently, the positively charged lipotripeptides Pam-FKF (**37**) and Pam-YKF (**38**) were synthesized and studied. The tripeptide YDL was selected to obtain negatively charged lipotripeptides because of the inhibition of *Xenopus* 26S proteasome by TISYDL and TVSYDL,[10] together with that of yeast 20S proteasome by TITYDL (**11**). The effect of replacing aspartic acid by glutamic acid or asparagine was investigated, as was the influence of the alkyl chain length (**29**–**33**); we also introduced the unnatural amino acid L-thyronine (**34**–**36**) and the 2-aminopalmitoyl group (**36**). Short lipid chains (C6–C12) did not favor inhibition by lipotripeptides (**29**–**33**) in contrast to hexapeptide alkyl derivatives (Fig. 1B; b): long alkyl chains produced better inhibitors acting at micromolar concentrations (K_i of 5 for Apam-YET(0) **36** and K_i of 18 for Pam-YEL **33** against ChT-L activity). The competitive inhibitor **33** (Fig. 1A, inset) inhibited at 35 μM T-L and PA activities as efficiently as did the established proteasome inhibitor MG132 tested at the same concentration, whereas ChT-L activity was poorly inhibited (decrease 3-fold). Compound **33** also inhibited rabbit 20S proteasome with an inhibitory effect increased (ChT-L and T-L) or decreased (PA) by a factor of 2 compared to yeast 20S proteasome. The three proteasomal activities were all inhibited to about the same extent by alkyl-tripeptides **28** and **33**. Conversely, the positively charged Pam-FKF (**37**) and Pam-YKF (**38**) did not inhibit PA activity like the corresponding hexapeptides TVTFKF (**1**) and TITYKF (**8**). They were poor inhibitors of T-L activity. Their inhibition of CT-L activity (at 35 μM) was about 5- to 10-fold better than that of the corresponding hexapeptides **1** and **8**. YED (**39**), YEL (**40**), Pam-OMe (**41**), and Apam (**42**) had no inhibitory potency, demonstrating that the whole lipopeptide structure is necessary for inhibition. Finally, the metabolic stability of some lipopeptides in culture medium RPMI containing fetal calf serum was studied; the half-life of Pam-YDL (**28**) was 23.8 h and that of Pam-YEL (**33**) was 19 h. These results show that lipopeptides are valuable candidates for the development of new proteasome inhibitors that do not form a covalent bond with the catalytic threonine-1 O^{γ}. The nonspecific lipid group may act in a dual way by improving inhibition and by targeting the inhibitor to the membrane (an interface directing group). The peptide sequence may also be varied to confer specificity toward the β1, β2 or β5 subunits.

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