Brief Articles

Peptidyl Vinyl Ester Derivatives: New Class of Selective Inhibitors of Proteasome Trypsin-Like Activity

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The proteasome is a multicatalytic proteinase complex which plays a central role in intracellular protein degradation. We report here the synthesis and biological activities of a new class of specific proteasome inhibitors selective for trypsin-like activity. These tripeptide-based compounds bearing a C-terminal vinyl ester are nontoxic, and do not affect cell proliferation, but are able to modulate the generation and presentation of immunogenic peptides presented by MHC class I molecules.

Introduction

26S proteasome represents the multicatalytic proteinase of the ubiquitin/adenosine triphosphate-dependent proteolytic pathaway.[1] This large enzymatic complex is found in the cytosol and nucleus of eukaryotic cells and plays a central role in the selective degradation of intracellular proteins.[2−4] In a modular structure of the enzyme, the 20S proteasome is the proteolytic chamber formed by four stacked rings, where each of the two inner rings is composed of seven different β subunits. The β1 subunit contains a post-acidic-like (PGPH) active site, β2 has trypsin-like (T-L) activity, and β5 is associated with a chymotrypsin-like (ChT-L) function. All the proteolytic sites utilize the γ-hydroxyl group as nucleophile and the α-amine as proton donor/acceptor of the N-terminal threonine residue in the catalytic cycle.

Proteasomes removed abnormal proteins and play a role in cell-cycle progression and apoptosis, both in transcription factor activation and in the generation of peptides presented by the major histocompatibility complex I (MHC-I). Thus, the proteasome represents a potential target for the development of therapeutic agents for the treatment of pathologies such as cancer, inflammation, immune diseases, and others.[5−7] Several classes of inhibitors have been identified, and these compounds represent a valuable tool for the identification of the role of the ubiquitin−proteasome pathway in cellular processes.[8] In addition, proteasome inhibitors could be used as therapeutic agents to arrest tumor cell proliferation and as modulators of antigen presentation. The most common inhibitors are peptide-based compounds with a C-terminal function, able to interact with enzymatic catalytic threonine.[9−18]

We recently reported the identification of some tripeptidic sequences functionalized with arecoline derivatives.[19,20] The more effective of these molecules, which carry an N-terminal 1,2,5,6-tetrahydropyridine-3-carbonyl (Guv, guvacine) group, have shown an interesting inhibition of the tryptic- and chymotryptic-like activities of the proteasome and favorable pharmacokinetic properties. In this report, we describe the synthesis and biological characterization of a new series of peptide-based inhibitors bearing a C-terminal leucine vinyl ester (Leu-VE) as pharmacophore, potentially able to interact with catalytic threonine (Figure 1). The ethyl acrylate group can function as a substrate of the γ-hydroxy threonine side chain in Michael addition in the same way as has been suggested for the well-known peptide vinyl sulfone inhibitors.[21] The dipeptidic structural core, as reported in our previous work,[19] presents two leucine residues in derivatives **1**−**4**, while the more hydrophilic sequence Val-Ser is found in compounds **5**−**8** at positions P3 and P2, respectively. The N-terminal position is functionalized with the following six-membered

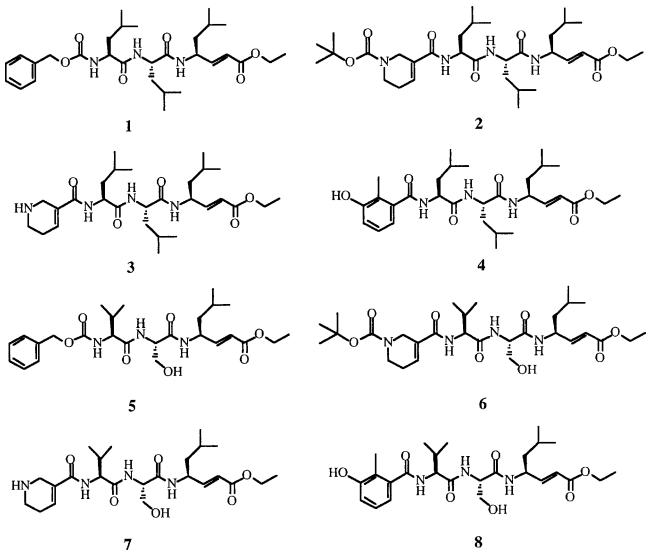

Figure 1. Structures of the vinyl ester pseudotripeptide inhibitors.

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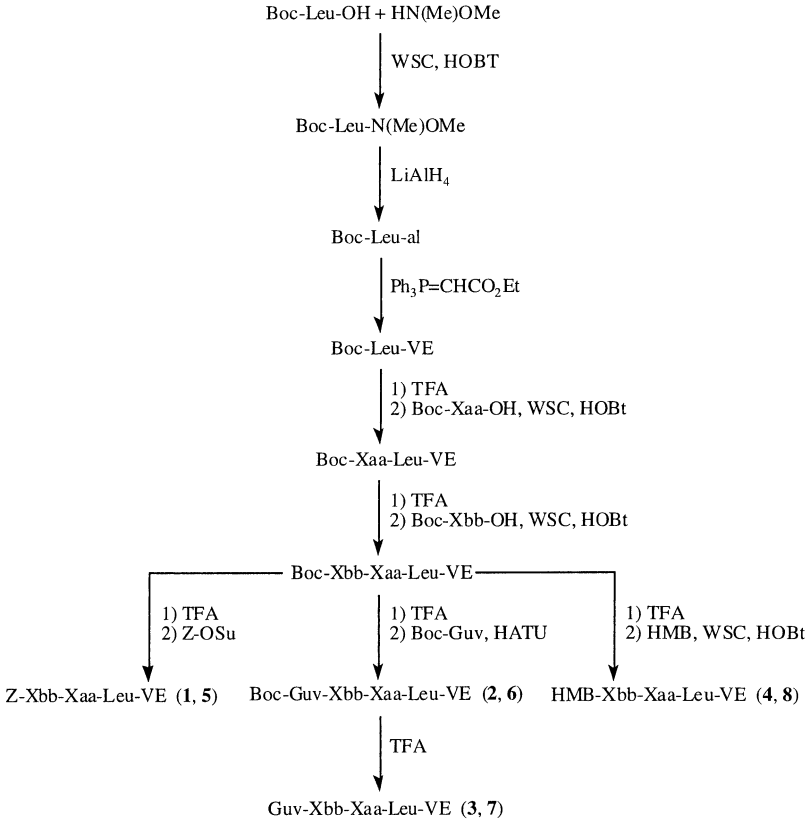
Scheme 1



rings: benzyloxycarbonyl (Z) in **1** and **5**, *N*-(*tert*-butoxycarbonyl)guvacine (Boc-Guv) in **2** and **6**, guvacine in **3** and **7**, and 3-hydroxy-2-methylbenzoyl (HMB) in **4** and **8** derivatives. These cyclic substituents were chosen for their different physicochemical characteristics, based on our previous biological evidence[19,20] and due to the fact that they are easily inserted into a peptide sequence.

Results and Discussion

Synthesis. Vinyl ester tripeptides were synthesized following the strategy reported in Scheme 1 by the classical solution method using C-terminal stepwise elongation. N_α-Boc-protected leucine vinyl ester was prepared from the corresponding aldheyde[22] by reaction with [(ethoxycarbonyl)methylidene]triphenylphosphorane without racemization.[23] WSC/HOBt or HATU were employed for the coupling steps, benzyloxycarbonyl was introduced as the succinimidyl ester (Z-OSu), and Boc was removed by TFA. All products were purified by preparative RP-HPLC, and structural verification was achieved by mass spectrometry and NMR spectroscopy.

Biological Results. We tested the inhibitory capacity of compounds **1**−**8** on purified 20S proteasome using fluorogenic substrates specific for the three major proteolytic activities of the proteasome.[24] All vinyl ester derivatives were assayed at different concentrations (from 0.001 to 10 μM) for their capacity to inhibit the tryptic-like (T-L), chymotryptic-like (ChT-L), and post-acidic (PGPH) activities in vitro of the proteasomes purified from lymphoblastoid cell lines. All compounds inhibited the tryptic activity of the proteasome (Table 1), suggesting that the pharmacophore leucine ethyl acrylate (Leu-VE) at the C-terminus may represent a functional group which could be used for the design of a new class of proteasome inhibitors. The central dipeptidic sequence seems to play a role in determining the specificity of this class of compounds. In particular, compounds **1**−**4**, containing the hydrophobic Leu-Leu, show the capacity to inhibit both tryptic- and chymotryptic-like activities, while compounds **5**−**8**, containing Val-Ser, show the capacity to inhibit only the tryptic-like activity, thereby suggesting that they preferentially bind to the β2 subunit responsible for such activity. Furthermore, it should be noted that the presence of 3-hydroxy-2-methylbenzoyl (HMB in compounds **4** and **8**) at the N-terminus increases the inhibitory potency of this class of compounds. All members of the series show negligible inhibition of the PGPH-like activity attributed to the β1 subunit of the proteasome. Vinyl ester tripeptide **8** is the most potent (IC_{50} = 33 nM) and selective for trypsin-like activity.

Table 1. Subsites of Proteasome Inhibition and Metabolic Stability of Vinyl Ester Tripeptides

		IC_{50} (μM)[a]			half-life (min)	
	compound	T-L	ChT-L	PGPH	medium	plasma
1	Z-Leu-Leu-Leu-VE	0.28	2.45	>10	>360	292
2	Boc-Guv-Leu-Leu-Leu-VE	0.19	4.36	>10	>360	198
3	Guv-Leu-Leu-Leu-VE	0.21	0.86	>10	>360	>360
4	HMB-Leu-Leu-Leu-VE	0.041	4.21	>10	>360	>360
5	Z-Val-Ser-Leu-VE	0.071	>10	>10	>360	238
6	Boc-Guv-Val-Ser-Leu-VE	0.11	>10	>10	>360	154
7	Guv-Val-Ser-Leu-VE	0.38	6.54	>10	>360	>360
8	HMB−Val-Ser-Leu-VE	0.033	>10	>10	>360	>360

[a] The values reported are the average of two independent determinations.

The cell membrane permeation of the most representative compounds, **4** and **8** were tested in live cells. After cell treatment, proteasomes were purified and assayed for proteolytic activity using specific substrate for T-L

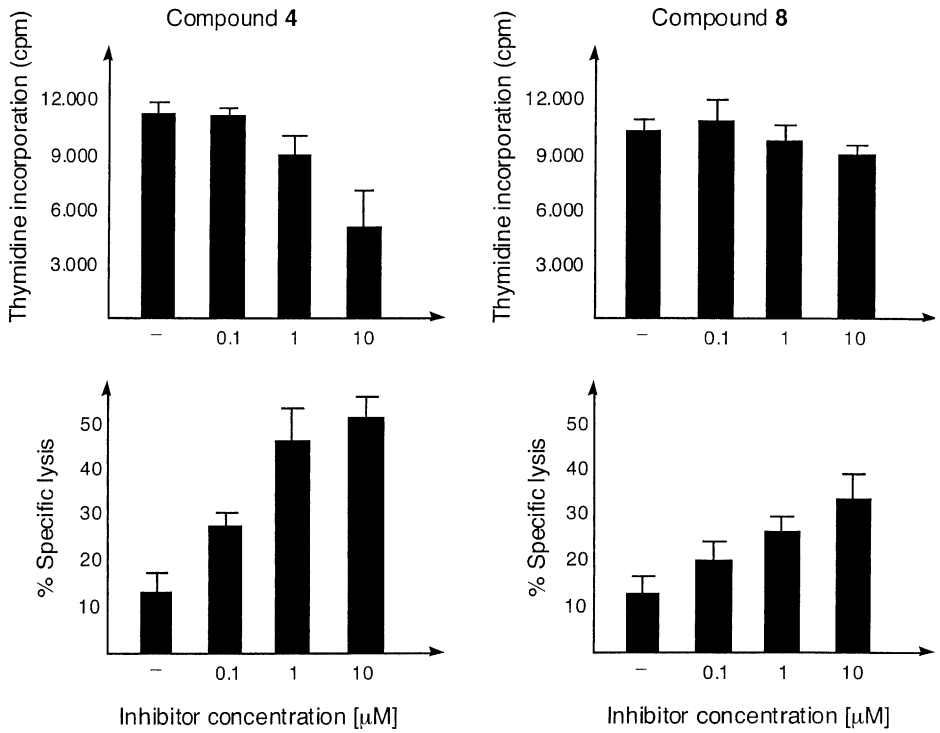


Figure 2. Panel A: Effect of compounds **4** and **8** on cell proliferation. The colon carcinoma cell line HCT116 was cultured for 3 days in the presence or not (−) of the indicated concentrations of compounds **4** and **8**. Cell proliferation was evaluated by [^{3}H]-thymidine incorporation. One representative experiment out of three performed is shown. Panel B: Effect of compounds **4** and **8** on the presentation of an EBV-derived CTL epitope. HLA-A2-positive RG lymphoblastoid cells were cultured for 18 h in the presence or not (−) of the indicated concentrations of compounds **4** and **8** and used as target of HLA-A2-restricted CLG-specific CTL cultures. Results are expressed as % specific lysis. One representative experiment out of three performed is shown.

and ChT-L activities. The results obtained were comparable to that observed in the in vitro assay (IC_{50}: 0.062 and 0.050 μM, respectively), thereby demonstrating that compounds **4** and **8** are cell-permeable and able to inhibit the proteasome in vivo.

To characterize the proteasomal interaction of vinyl ester tripeptides, derivatives **4** and **8** were analyzed for reversibility inhibition. In the first assay, the enzyme−inhibitor (0.1 μM) mixture was preincubated for 1 h, increasing concentrations of specific T-L substrate were added, and the enzyme activity was measured after each addition. In the second experiment, after enzyme−inhibitor preincubation, the mixture was diluted for 25 or 50-fold prior to the addition of substrate, and the activity was monitored. In both experiments, small shifts in the IC_{50} values were observed (from 0.030 to 0.057 μM for **4** and from 0.031 to 0.040 μM for compound **8**), indicating an irreversible inhibition. This suggests that the vinyl ester group functions as a substrate of catalytic threonine and, in particular, that the conjugate double bond is susceptible to Michael's addition of the threonine γ-hydroxyl group. Crystallographic data and molecular modeling studies, such as docking simulation, are in progress to define the inhibitor−enzyme interaction subsite pockets.

Susceptibility to enzymatic hydrolysis of compounds **1**−**8** was determined by incubation at 37°C in culture medium (RPMI) in the presence of 10% foetal calf serum or in human plasma.[25] The half-lives of the derivatives reported in Table 1 show great stability in cell culture medium and good enzymatic resistance to human plasma proteases.

Since proteasomes play a key role in cell viability and proliferation, we evaluated the pro-apoptotic and anti-proliferative activities of these compounds on tumor cell lines.[26] To this end, two colon-carcinoma cell lines (COO115, HCT116) were treated with different concentrations (from 0.1 to 10 μM) of prototypes **4** and **8** for 3 days, and the cellular effects of the compounds were evaluated by trypan blue exclusion and cell proliferation assays. No toxicity was observed (data not shown) while inhibition of HCT116 cell proliferation was noticed at elevated concentrations (10 μM) only for compound **4** (Figure 2, panel A). This suggests that inhibitors of the tryptic-like activity of proteasomes do not induce cell death or inhibit cell proliferation. Similar results were obtained with COO115 cells (data not shown).

The critical involvement of the proteasome in MHC class I-restricted antigen processing was established with the help of specific inhibitors. It is now widely recognized that treatment with membrane-permeable inhibitors of the proteasome strongly affect the generation of antigenic peptides.[27] However, the presentation of certain CTL epitopes appears to be insensitive to proteasome inhibition, and some epitopes are generated in larger amounts in cells subjected to partial inhibition of proteasomes.[28] We also evaluated the effects of compounds **4** and **8** on the presentation of an EBV-derived HLA-A2 presented CTL epitope. To this end, HLA-A2 positive lymphoblastoid cells were treated for 18 h with compounds **4** and **8** and tested in cytotoxicity assays using HLA-A2-restricted CTLs specific for the CLG epitope. CLG-specific CTLs showed low recognition of target cells (Figure 2, panel B) due to poor expression of the CLG epitope on the surface of EBV-infected B cells.[29] However, a net dose-dependent increase of CLG-specific killing was observed in cells treated with compounds **4** and **8**. Treatment of HLA-A2-negative cells

with compounds **4** and **8** did not affect killing by CLG-specific CTLs (data not shown). These results suggest that inhibition of the tryptic activity of the proteasome favors the generation and presentation of the subdominant CLG epitope.

Conclusion

In this work, we have designed, synthesized, and defined the biological profile of a new series of peptide-based proteasome inhibitors. Prototypes of these vinyl ester tripeptide derivatives are potent and selective for tripsyn-like activity, are cell permeable, and are stable in in vitro degradation assays. This new class of inhibitors may represent a useful instrument to understand the mechanisms underlying antigen production by the ubiquitin−proteasome system and, in perspective, may have applications in new therapeutic protocols aimed at increasing the generation and presentation of poorly expressed CTL epitopes.

Experimental Section

General. Amino acids, amino acid derivatives, resins, and chemicals were purchased from Bachem, Novabiochem, or Fluka (Switzerland). Crude pseudopeptides were purified by preparative reversed-phase HPLC using a Water Delta Prep 4000 system with a Waters PrepLC 40 mm Assembly column C18 (30 × 4 cm, 300 A, 15 μm spherical particle size column). The column was perfused at a flow rate of 50 mL/min with a mobile phase containing solvent A (10%, v/v, acetonitrile in 0.1% TFA), and a linear gradient from 0 to 50% of solvent B (60%, v/v, acetonitrile in 0.1% TFA) in 25 min was adopted for the elution of compounds. HPLC analysis was performed by a Beckman System Gold with a Hypersil BDS C18 column (5 μm; 4.6 × 250 mm). Analytical determination and capacity factor (K') of the peptides was determined using HPLC conditions in the above solvent system (solvents A and B) programmed at flow rates of 1 mL/min using the following linear gradients: (a) from 0% to 100% B in 25 min and (b) from 10% to 70% B in 25 min. All pseudopeptides showed less than 1% impurities when monitored at 220 and 254 nm. Molecular weight of compounds was determined by a ESI Micromass, ZMD2000 mass spectrometer. ^{1}H NMR spectroscopy was obtained on a Bruker AC 200 spectrometer.

Chemistry. General Procedures. Coupling with WSC/HOBt. The amino component (1 mmol), NMM (1 mmol), HOBt (1.1 mmol), and WSC (1.1 mmol) in this order were added to a solution of the carboxyl component (1 mmol) in DMF (10 mL) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and 18 h at room temperature; then the solution was diluted with EtOAc (100 mL) and washed consecutively with 0.1 N HCl, brine, $NaHCO_3$, and brine. The organic phase was dried ($MgSO_4$), filtered, and evaporated to dryness. The residue was treated with Et_2O, and the resulting solid was separated by centrifugation.

Coupling with HATU. The deprotected vinyl ester tripeptide (1 mmol), NMM (1 mmol), and HATU (1 mmol) were added to a solution of the Boc-Guv (1 mmol) in DMF (3 mL) at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and 24 h at room temperature; then the solution was diluted with AcOEt (70 mL) and treated as described above.

Introduction of Z. Z-OSu (0.5 mmol) was added to a solution of the tripeptide vinyl ester (0.5 mmol) in DMF (5 mL) at 0 °C. The mixture was stirred for 1 h at 0 °C and 15 h at room temperature and then evaporated. The resulting target compounds were purified by preparative HPLC.

TFA Deprotection. *tert*-Butyloxycarbonyl protection was removed by treating intermediates with aqueous 90% TFA (1:10, w/v) for 30−40 min. After evaporation, the residue was treated with Et_2O, and resulting solid was separated by centrifugation.

Purification of Proteasomes. Partially purified proteasomes were obtained from lymphoblastoid cell lines, untreated or treated for 12 h at 37 °C with the inhibitors, as previously described.[30] A subsequent purification was carried out by affinity chromatography (mAb α-subunit, Affinity). Fractions containing proteasomes were combined and dialyzed against 25 mM Tris-HCl pH 7.5. Protein concentration was determined using BCA protocol (Pierce, Rockford, IL).

Proteasome Subunit Inhibition Assays. Suc-LLVY-AMC, Boc-LRR-AMC, and Ac−YVAD-AMC (Sigma) were used to measure chymotrypsin-like, trypsin-like, and postacidic proteasome activities, respectively. Substrates were incubated at 37 °C for 30 min with proteasomes, untreated or pretreated with 0.01−20 mM of test compounds, in activity buffer. Fluorescence was determined by a fluorimeter (Spectrafluor plus, Tecan, Salzburg, Austria) using an excitation of 360 nm and emission of 465 nm. Activity was evaluated in fluorescence units, and the inhibitory activity of the compounds is expressed as IC_{50}. The data were plotted as percentage control (the ratio of percentage conversion in the presence and absence of inhibitor) vs inhibitor concentration, and fitted with the equation $Y = 100/1 + (X/IC_{50})^A$, where IC_{50} is the inhibitor concentration at 50% inhibition, and A is the slope of the inhibition curve.

Cytotoxicity Assay. HLA-A2-restricted EBV-specific CTL cultures reacting against the LMP2-derived CLGGLLTMV (CLG, aa 426−434) epitope were obtained by stimulation of lymphocytes from the HLA-A2-positive EBV-seropositive donor RG with CLG-pulsed T2 cells as previously described.[29] CTL cultures were maintained in medium supplemented with 10 U/mL rIL-2 (Chiron, Milan, Italy).

Target cells were treated or not for 18 h with different concentrations of compounds **4** and **8** and labeled with $Na_2{}^{51}CrO_4$ for 90 min at 37 °C. Cytotoxicity tests were routinely run at different effector:target ratios in triplicate. Percentage specific lysis was calculated as 100 × (cpm sample − cpm medium)/(cpm Triton X-100 − cpm medium). Spontaneous release was always less than 20%.

Cell Viability and Proliferation Assay. The colon carcinoma cell lines COO115 and HCT116 were cultured in the presence of compounds **4** and **8** at different concentrations (from 0.1 to 10 μM), and cell viability was evaluated by trypan blue exclusion. In the cell proliferation assays, 5×10^4 cells were seeded in 96-well microtiter plates in the presence or absence of different concentrations of compounds **4** and **8**, cultured for 48 h, and pulsed with [^{3}H]-thymidine (1 μCi/well) for the last 16 h. Cells were collected on glass microfiber filters using a cell harvester, and radioactivity was assessed by a β-counter.

Metabolic Stability Assays. The degradation kinetics of new inhibitors were studied in culture medium (RPMI) and human plasma. An amounr of 0.1 mL of a solution of each compound (10 mg/mL in acetonitrile/H_2O 1:1) was added to 1 mL of RPMI containing 20% FCS. Alternatively, test compounds were incubated with plasma (0.6 mL) in a total volume of 1.5 mL of 10 mM Tris-HCl buffer, pH 7.5. Incubation was performed at 37 °C for different durations: up to 360 min in the case of human plasma, and up to 2 days in the case of RPMI containing 20% FCS. The incubation was terminated by addition of ethanol (0.2 mL), the mixture poured at 21 °C, and, after centrifugation (5000 rpm for 10 min), aliquots (20 μL) of the clear supernatant were injected into RP-HPLC column. HPLC was performed as described in analytical determinations. The degradation half-life ($T_{1/2}$) was obtained by a least-squares linear regression analysis of a plot of the logarithmic inhibitor concentration versus time, using a minimum of five points.

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(progetto AIDS). Thanks are also due to Anna Forster for linguistic assistance.

Appendix

Abbreviations used: Boc, *tert*-butoxycarbonyl; HATU, *O*-(7-azabenzotriazolyl)tetramethyl uronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; NMM, *N*-methylmorpholine; TFA, trifluoroacetic acid; WSC (water soluble carbodiimide), 1-ethyl-3-(3′-dimethylaminopropyl)carbodiimide; Z, benzyloxycarbonyl-*N*-hydroxysuccinimide.

Supporting Information Available: Analytical data of intermediates and products **1**−**8** are available free of charge via the Internet at http://pubs.acs.org.

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