Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 1317–1319

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

A New Structural Class of Selective and Non-covalent Inhibitors of the Chymotrypsin-like Activity of the 20S Proteasome

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Received 22 December 2000; accepted 19 March 2001

Abstract—We describe the identification and in vitro characterization of a series of 2-aminobenzylstatine derivatives that inhibit non-covalently the chymotrypsin-like activity of the 20S proteasome. Our initial SAR data demonstrate that the 2-aminobenzylstatine core structure can effectively serve as the basis for designing potent, selective and non-covalent inhibitors of the chymotrypsin-like activity of the 20S proteasome.

Introduction

The 20S proteasome, which is the catalytic core of the 26S proteasome,[1] is a threonine protease that exhibits at least three distinct peptidase activities: chymotrypsin-like, trypsin-like, and post-glutamyl-peptide hydrolytic activities.[2] Because of the role of this enzyme in the non-lysosomal ATP-dependent degradation of proteins involved in critical intracellular regulatory cascades,[3] inhibitors of the 20S proteasome are being explored for use as potential anti-inflammatory agents and for the treatment of cancer and auto-immune diseases.[4] Our specific target in the search of novel cytotoxic and anti-proliferative agents is the chymotrypsin-like activity of the 20S proteasome. Modulation of this enzymatic activity by β-subunit-specific proteasome inhibitors may convey an anti-tumor effect by induction of cell cycle arrest and apoptosis in tumor cells.[4c,d,5] Early inhibitors of the chymotrypsin-like activity of the 20S proteasome exert their inhibitory activity via adduct formation with the N-terminal threonine on the active site of the β-subunits.[6] The inherent drawbacks of these classical protease inhibitors (e.g., non-target specific, too reactive or unstable) prompted us to the search for alternative subunit-specific proteasome inhibitors.

We describe herein the identification and in vitro characterization of a series of 2-aminobenzylstatine derivatives that inhibit non-covalently the chymotrypsin-like activity of the 20S proteasome. This new structural class shows good selectivity over the trypsin-like and post-glutamyl-peptide hydrolytic activities of the 20S proteasome.

Synthesis and Biological Assays

The synthesis of compounds **1**–**4** has been reported previously[7] and compounds **6**–**12** were prepared according to known methods.[7–9] The general route for the syntheses of 2-heterosubstituted derivatives of 4-amino-3-hydroxy-5-phenylpentanoic acid is illustrated for compound **12** in Scheme 1. Compound **6**, synthesized according to the general route (Scheme 1), was the starting point for the synthesis of compounds **7**–**12**. After removal of the *tert*-butoxycarbonyl protecting group, the amino terminus was acylated with various carbonic acid esters or carboxylic acids. The ability of these compounds to inhibit the 20S proteasome was determined in vitro using purified human placenta 20S proteasome[10] and the following fluorogenic peptides as substrates: Suc-Leu-Leu-Val-Tyr-AMC (substrate for chymotrypsin-like assay), Boc-Leu-Arg-Arg-AMC (substrate for trypsin-like assay) and Z-Leu-Leu-Glu-AMC (substrate for post-glutamyl-peptide hydrolytic-like assay). Fluorescence excitation/emission wavelengths were 355 nm/460 nm for 7-amido-4-methylcoumarin (AMC). The rates of hydrolysis were monitored by the fluorescence increase and the initial linear portions of curves were used to calculate the IC_{50} values (Tables 1 and 2).

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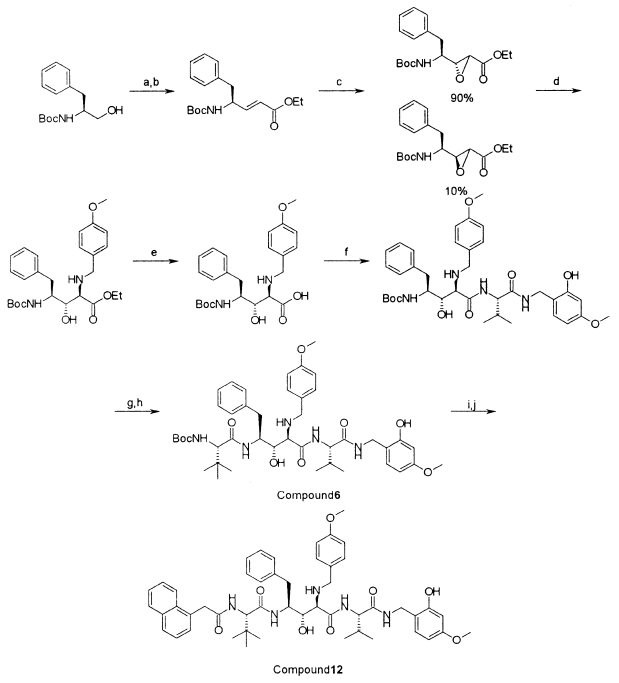
0960-894X/01/$ - see front matter
PII: S0960-894X(01)00205-0

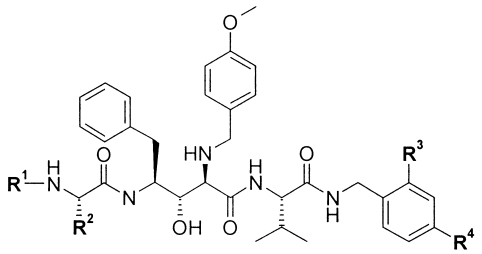
Results and Discussion

To search for novel non-covalent structures inhibiting specifically the chymotrypsin-like activity of the 20S proteasome, we screened our in-house compound archive. Among the compounds identified, compound **1** (Table 1) was selected for further profiling.[11] This compound, which was originally synthesised to target the HIV-1 proteinase,[7,12] inhibits the chymotrypsin-like activity of the 20S proteasome with an IC_{50} value of 0.9 μM (compound **1**, Table 1) and shows good selectivity over the trypsin-like and post-glutamyl-peptide hydrolytic activities ($IC_{50} > 20$ μM). Encouraged by these results, a series of available 2-aminobenzylstatine derivatives were tested in our in vitro assay to try to identify key interactions of this new structural class of inhibitors with the X and HC5 subunits of the 20S proteasome.[13] The SAR data obtained is shown in Table 1. Replacement of *tert*-leucine by the less bulky valine (**2**) resulted in a 3-fold decrease in binding affinity. A more dramatic effect on potency was observed by introducing variations at the C- and N-terminal groups. Methylation of the phenol group (**3**) or removal of the methoxy group (**4**) in the C-terminal benzylamide ring resulted in a substantial drop in binding activity (>20-fold). A similar drop in activity was observed for a derivative that lacks the bulky N-terminal benzyloxycarbonyl group (**5**). On the basis of this limited SAR data, we decided to focus our initial medicinal chemistry efforts on the N-terminal part of the 2-aminobenzylstatine template. In this modular approach, the N-terminal part of the molecule was modified while the other remained unchanged. To this end, a series of derivatives were prepared to examine the effects of N-terminal substitution on potency.[14] Representative examples of the compounds analyzed are shown in Table 2. A 27-fold decrease in potency was observed when the benzyloxycarbonyl group was replaced by the less bulky *tert*-butyloxycarbonyl group (**6**, Table 1). The possibility to increase potency by establishing additional hydrophobic or hydrogen-bonding interactions was explored by increasing the size of the N-terminal group (**7** and **12**), adding substituents to the aromatic ring (**8** and **11**) or by incorporating hydrogen-bond donors (**9** and **10**). Modifications on the N-terminal phenyl ring did not affect substantially the inhibitory activities of the



Scheme 1. Synthesis of compound **12**. Conditions: (a) $(COCl)_2$, DMSO, CH_2Cl_2, Et_3N, −55 °C → rt; (b) $Ph_3P{=}CHCO_2Et$, toluene, 80 °C; (c) MCPBA, CH_2Cl_2, rt; (d) 4-Methoxybenzylamine, EtOH, 70 °C; (e) 1 N LiOH, THF, rt; (f) HCl·(*S*)-Val-(2-hydroxy-4-methoxy)-benzylamine, TPTU, DIEA, DMF, rt; (g) 4 N HCl in dioxane, rt; (h) N^{α}-Boc-L-Tleu-OH, TPTU, DIEA, DMA, rt; (i) CF_3CO_2H, water, rt; (j) 1-naphthylacetic acid, TPTU, DIEA, DMA, rt.

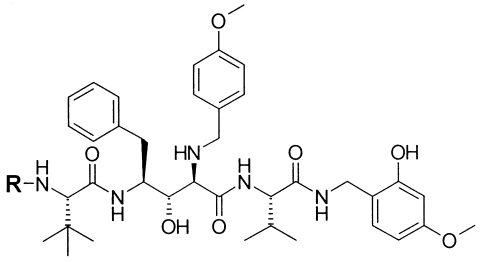
Table 1. Inhibition of the chymotrypsin-like activity of the 20S proteasome by 2-aminobenzylstatine derivatives[a]



Compound	R^1	R^2	R^3	R^4	IC_{50} (μM)
1	CO_2CH_2Ph	$C(CH_3)_3$	OH	OCH_3	0.9
2	CO_2CH_2Ph	$CH(CH_3)_2$	OH	OCH_3	3.3
3	CO_2CH_2Ph	$CH(CH_3)_2$	OCH_3	OCH_3	>20
4	CO_2CH_2Ph	$C(CH_3)_3$	OH	H	72
5	H	$C(CH_3)_3$	OH	OCH_3	70

[a]The IC_{50} value is the concentration of inhibitor at which the rate of the chymotrypsin-like activity of the 20S proteasome catalyzed hydrolysis of the substrate Suc-Leu-Leu-Val-Tyr-AMC is reduced at 50%.

Table 2. Inhibition of the chymotrypsin-like activity of the 20S proteasome by 2-aminobenzylstatine derivatives[a]



Compound	R	IC_{50} (μM)
1	CO_2CH_2Ph	0.9
6	$CO_2C(CH_3)_3$	27.0
7	CO_2CH_2(1-Naphthalene)	2.0
8	$CO_2CH_2Ph[3,5\text{-}(CH_3)_2]$	1.1
9	$CO_2CH_2Ph(3\text{-}NH_2)$	0.6
10	$C(O)CH_2CH_2Ph(3\text{-}NH_2)$	0.6
11	$CO_2CH_2Ph(3\text{-}CH_3)$	0.5
12	$C(O)CH_2$(1-Naphthalene)	0.1

[a]The IC_{50} value is the concentration of inhibitor at which the rate of the chymotrypsin-like activity of the 20S proteasome catalyzed hydrolysis of the substrate Suc-Leu-Leu-Val-Tyr-AMC is reduced at 50%.

compounds and only a major effect on potency was observed for the inhibitors containing bulkier groups. While the compound containing the carbamic acid naphthalen-1-ylmethyl ester (**7**) showed a 2-fold decrease in binding affinity relative to our reference compound (**1**), the 1-napthyl acetic acid synthon (**12**) increased 9-fold the potency of our initial compound. Compound **12** is not only a potent inhibitor of the chymotrypsin-like activity of the 20S proteasome but also very selective for this catalytic site. This compound shows at least 200-fold selectivity over the trypsin-like and post-glutamyl-peptide hydrolytic activities.

In summary, our initial SAR data demonstrate that the 2-aminobenzylstatine core structure can effectively serve as the basis for designing potent, selective and non-covalent inhibitors of the chymotrypsin-like activity of the 20S proteasome. Further modular modifications of this series hold the promise for future inhibitors with increased in vitro activity.

Acknowledgements

The authors thank D. Arz, R. Wille, V. von Arx, W. Beck and E. Boss for technical assistance.

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