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Structure-Based Optimisation of 2-Aminobenzylstatine Derivatives: Potent and Selective Inhibitors of the Chymotrypsin-Like Activity of the Human 20*S* Proteasome

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Abstract—We have identified 2-aminobenzylstatine derivatives that inhibit non-covalently the chymotrypsin-like activity of the human 20*S* proteasome. A structure-based optimisation approach has allowed us to improve the potency of this structural class of proteasome inhibitors from micromolar to nanomolar level. The new derivatives showed good selectivity against the trypsin-like and post-glutamyl-peptide hydrolytic activities of this enzyme.

Introduction

The 20*S* proteasome, which is the catalytic core of the 26*S* 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] Our specific target in the search of novel cytotoxic and anti-proliferative agents is the chymotrypsin-like activity of the 20*S* 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.[3,4]

Recently, we have described the identification and the initial optimisation of a series of 2-aminobenzylstatine derivatives that inhibit non-covalently the chymotrypsin-like activity of the 20*S* proteasome in an in vitro enzyme assay (e.g., compound **1**, Table 1).[5,6] This new structural class shows good selectivity over the trypsin-like and post-glutamyl-peptide hydrolytic activities of the 20*S* proteasome (for compound **1**, $IC_{50} > 20\,\mu M$).

In this letter, we report further efforts to improve the in vitro 20*S* proteasome inhibition of this class of compounds by creating new productive interactions with the X and HC5 subunits of the enzyme.[7] Following a modular approach, we first optimised the binding interactions through diverse substitutions of the aromatic ring of the 2-aminobenzylstatine core structure which is assumed to bind in the S3 pocket of the enzyme. We then combined the best substituents obtained for this part of the inhibitors with a modification of the N-terminal group aimed at increasing the strength of an aromatic stacking interaction with an accessory pocket of the active site.

Synthesis and Biological Assays

Compounds **1**–**19** were prepared according to known methods.[8–10] The general route for the syntheses of 2-heterosubstituted derivatives of 4-amino-3-hydroxy-5-phenylpentanoic acid is illustrated for compound **18** in Scheme 1. The ability of these compounds to inhibit the 20*S* proteasome was determined in vitro using purified human 20*S* proteasome[11] 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-methyl-coumarin (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 (Table 1).

Results and Discussion

We have identified by high throughput screening (HTS) of our in-house compound archive 2-aminobenzylstatine

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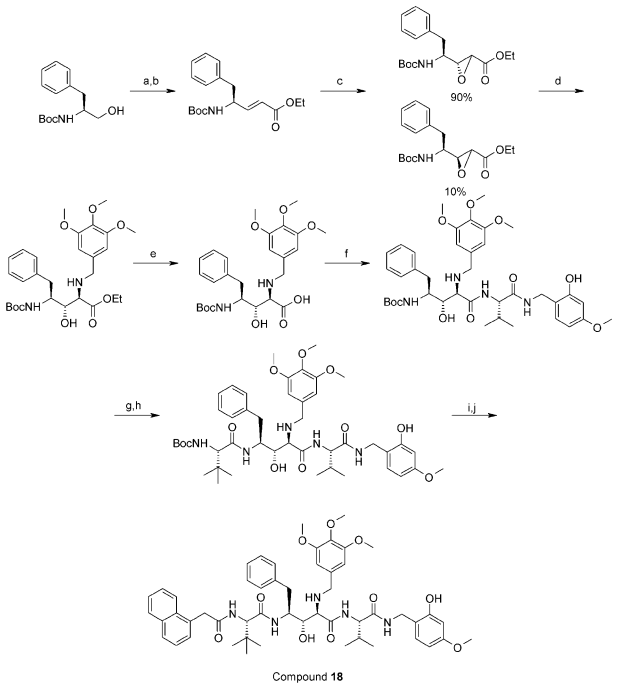
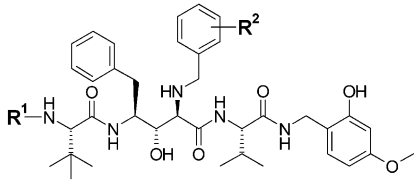


Scheme 1. Synthesis of compound **18**. Conditions: (a) $(COCl)_2$, DMSO, CH_2Cl_2, Et_3N, $-55\,^{\circ}C$ • rt; (b) $Ph_3P{=}CHCO_2Et$, toluene, $80\,^{\circ}C$; (c) MCPBA, CH_2Cl_2, rt; (d) 3,4,5-trimethoxybenzylamine, EtOH, $70\,^{\circ}C$; (e) 1 N LiOH, THF, rt; (f) HCl·(*S*)-Val-(2-hydroxy-4-methoxy)benzylamine, TPTU, DIEA, DMF, rt; (g) 4 N HCl in dioxane, rt; (h) N^{α}-Boc-*L*-Tleu-OH, TPTU, DIEA, DMA, rt; (i) 4 N HCl in dioxane, rt; (j) 1-Naphthylacetic acid, TPTU, DIEA, DMA, rt.

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



Entry	R^1	R^2	IC_{50} (μM)
1	CO_2CH_2Ph	4-OCH_3	0.9
2	CO_2CH_2Ph	H	>20
3	CO_2CH_2Ph	2-OCH_3	>20
4	CO_2CH_2Ph	3-OCH_3	1.50
5	CO_2CH_2Ph	2,4-$(OCH_3)_2$	1.60
6	CO_2CH_2Ph	3,4-$(OCH_3)_2$	0.15
7	CO_2CH_2Ph	3,5-$(OCH_3)_2$	0.19
8	CO_2CH_2Ph	2,4,5-$(OCH_3)_3$	0.20
9	CO_2CH_2Ph	2,3,4-$(OCH_3)_3$	0.10
10	CO_2CH_2Ph	3,4,5-$(OCH_3)_3$	0.05
11	CO_2CH_2Ph	4-C_6H_5	>20
12	CO_2CH_2Ph	4-$CH(CH_3)_2$	1.60
13	CO_2CH_2Ph	4-OC_6H_5	>20
14	CO_2CH_2Ph	4-$N(CH_3)_2$	0.6
15	$C(O)CH_2$(1-Naphthalene)	4-OCH_3	0.100
16	$C(O)CH_2$(1-Naphthalene)	2,4,5-$(OCH_3)_3$	0.029
17	$C(O)CH_2$(1-Naphthalene)	2,3,4-$(OCH_3)_3$	0.018
18	$C(O)CH_2$(1-Naphthalene)	3,4,5-$(OCH_3)_3$	0.007
19	$C(O)CH_2$(1-Naphthalene)	4-$N(CH_3)_2$	0.160

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

derivatives (e.g., compound **1**, Table 1) that inhibit non-covalently the chymotrypsin-like activity of the 20*S* proteasome in an in vitro assay. This discovery represents a completely new avenue for medicinal chemistry because early inhibitors of this enzyme exert their inhibitory activity via adduct formation.[12] The inherent drawbacks of these classical protease inhibitors prompted us to focus our efforts on the optimisation of the potency of the new non-covalent inhibitors. To accomplish this, we have exploited a structural model of the human proteasome in complex with compound **1**.[6]

To start with, we evaluated the SARs surrounding the substitutions on the aromatic ring of the 2-aminobenzylstatine core structure. The structures of such compounds (**2**–**14**) and their IC_{50} values are shown in Table 1. According to our model, the benzylamino group in position 2 of the statine moiety interacts with the S3 pocket of the enzyme. This pocket is formed by the side chains of subunit HC5 residues Tyr 135, Ser 151, Asp 153, Ser 157, Gln 159, Asp 161 and Lys 164 on the one hand and the side chains of subunit X residues Ala 20, Ala 22, Ala 27, Ser 28, Val 31 and Ala 49 on the other hand.[13] Two hydrogen bond interactions between this part of the inhibitor and residues of the S3 pocket are assumed: the first one between the nitrogen atom and Asp 153 and the second one between the 4-methoxy substituent of the benzylic group and Ser 151 located at the bottom of the pocket (Fig. 1). Consistent with this hypothesis, a substantial drop in activity was observed with compound **2** which lacks the 4-methoxy substituent. Examination of the model suggested that two other residues of the S3 pocket, Ser 157 and Tyr 135, could be targeted for hydrogen bonding. In modeling experiments,[14] a methoxy substituent introduced either in position 2 or 3 of the phenyl ring could accept a hydrogen bond from Ser 157 while Tyr 135 could donate a hydrogen bond to a methoxy substituent

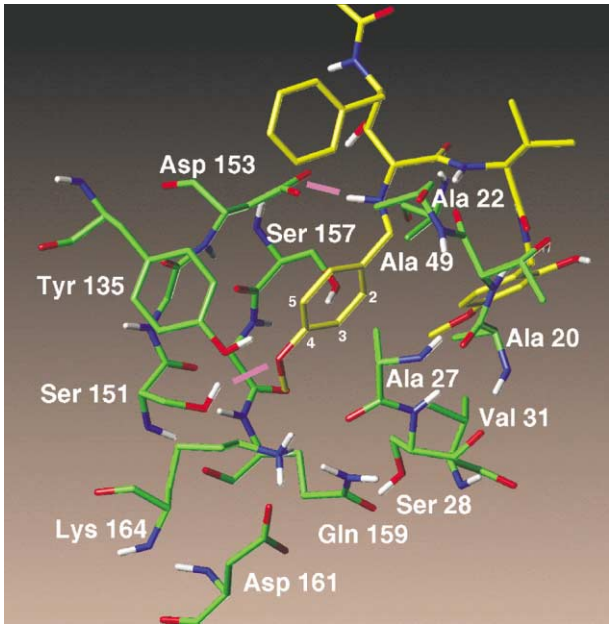


Figure 1. Model of **1** (yellow) bound to the proteasome X/HC5 site. View of the S3 pocket. Hydrogen bonds are shown in magenta.

placed in position 5. Hence, compounds **3** and **4** were synthesized, followed by compounds **5**–**10** in which combinations of methoxy substituents simultaneously targeting two or three of residues Ser 151, Ser 157 and Tyr 135 were incorporated. The relative potencies of compounds **4**, **6**, **7** and **10** show that this strategy was quite successful with the 3-methoxy substitution. The gain in potency obtained with **4** compared to **2** and with **7** compared to **4** suggests that the targeted hydrogen bonds with Ser 157 and Tyr 135 were achieved. In agreement with our concept, the most potent compound of the series **10** resulted from the addition of a third methoxy group in position 4 targeting Ser 151. In contrast, no beneficial effect was observed with the 2-methoxy substitution. In the mono-substituted variation, compound **3** is not more active than the unsubstituted compound **2** while the di- and tri-substituted derivatives **5**, **8** and **9** are equipotent to their analogues lacking the 2-substituent: **1** and **6**. A careful examination of the model suggests that in order to form a hydrogen bond with Ser 157, the 2-methoxy group has to come to a short distance of the C-terminal phenolic moiety of the inhibitor which occupies the S1 pocket. Thus, in this case an intramolecular steric hindrance may cancel out any beneficial effect obtained by forming a hydrogen bond with Ser 157.

Parallel to our efforts to establish hydrogen bond interactions, we explored the possibility to enhance potency by creating additional van der Waals interactions in the S3 pocket. The hydrocarbon parts of the side chains of Gln 159 and Lys 164 were the most appropriate protein atoms to target in this respect according to the model. Compounds **11**–**14** were synthesized following this concept. For analogues **11** and **13**, we were counting on some flexibility of the residues of the bottom of the pocket since in the model their bulky 4-substituents were causing a few steric clashes. The lack of activity of these analogues put in perspective with the substantial potency of **12**, which bears a smaller isopropyl group, clearly defines the tolerance of the S3 pocket in terms of the size of the substituent in position 4. The higher potency of **14** compared to **12** can be ascribed to the ability of the dimethylamino susbstituent to form a hydrogen bond with Ser 151 in addition to making hydrophobic contacts with Gln 159 and Lys 164.

A key finding of our earlier work was the fact that replacement of the benzyloxycarbonyl group of compound **1** by a group derived from the coupling of naphthalen-1-yl-acetic acid at the N-terminus improved potency by one order of magnitude (see compound **15** in Table 1).[5] This modification was designed to increase the strength of a postulated stacking interaction of the N-terminal group with residue Tyr 133 of subunit HC5 which forms a small accessory hydrophobic pocket together with Tyr 33 and Pro 131.[6] Consistent with this finding, we observed an increase in potency of similar magnitude when the same modification was introduced in compounds **8**–**10** and **14** to give derivatives **16**–**18** and **19**. Thus, with an IC_{50} value of 7 nM, **18** turned out to be the most potent compound of the series reported in this study. A model of its complex with the X/HC5 site of the proteasome is shown in Figure 2.

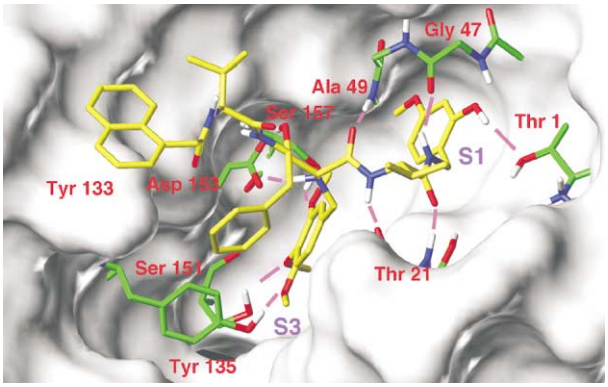


Figure 2. Model of proteasome-compound **18** complex. Hydrogen bonds are indicated as magenta lines.

The modifications introduced in compound **1** had no effect on its 20*S* proteasome speficity profile. The highly potent derivatives **16**–**18** still show good selectivity over the trypsin-like and post-glutamyl-peptide hydrolytic activities of the 20*S* proteasome (for all compounds, $IC_{50} > 20\ \mu M$).

In summary, our model of the human 20*S* proteasome in conjunction with a modular chemistry approach has allowed us to improve the proteasome chymotrypsin-like inhibitory activity of the 2-aminobenzylstatine compound class from micromolar down to nanomolar potency. The reported compounds are the most potent non-covalent inhibitors of the human proteasome described to date.[15] They open a new avenue for further investigation of the proteasome as a therapeutic target in oncology.

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