Entry into a New Class of Potent Proteasome Inhibitors Having High Antiproliferative Activity by Structure-Based Design

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Abstract: Proteasome inhibition is a therapeutic concept of current interest in anticancer research. We report here the design, synthesis, and biological characterization of prototypes of a new class of noncovalent proteasome inhibitors showing high activity in biochemical and cellular assays.

The proteasome is a multicatalytic protease complex that degrades intracellular proteins tagged for destruction by the covalent attachment of multiple ubiquitin molecules.[1,2] The ubiquitin−proteasome system is involved in the degradation of key components of the molecular machinery on which rely such important cellular functions as transcription, cell-cycle progression, tumor suppression, and apoptosis.[3−8] Given the wide range of substrates and processes that are regulated by this system, the components of the ubiquitin−proteasome pathway have become the focus of intensive biochemical research, especially in the oncology area. Although this pathway can be blocked at various steps, most inhibitors have been designed to target the proteolytic activities of the proteasome. Thus, it was shown that inhibition of proteasomal activity induced cell-cycle arrest and apoptosis in tumor cells, thereby leading to antiproliferative effects.[9−11] The observation that malignant cells were more susceptible to the proapoptotic effects of proteasome inhibition than normal cells raised the notion of proteasome inhibition as a potential new approach in cancer therapy.[12−15]

Most currently available inhibitors of the proteasome exert their inhibitory action by adduct formation with the enzyme.[16−18] The 20S proteasome, which is the catalytic core of the proteasome, is an N-terminal threonine hydrolase.[19] The hydroxyl group on the N-terminal threonine of each β-subunit is prone to react with compounds possessing functional groups receptive to nucleophilic attack. 20S proteasome inhibitors of this type include natural products and synthetic peptides belonging to the following classes of reactive compounds: epoxyketones, aldehydes, boronic acids, α-ketoamides, α-ketoaldehydes, and vinyl sulfones.[20]

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Table 1. IC_{50} Values (μM) in Enzymatic and Cellular Assays

compd	chym[a]	PGPH[a]	tryp[a]	prol[b]	cell[c]
1	0.9	>20	>20		
2	0.007	>20	>20	1.5	
3	0.015	>20	>20	0.06	0.02
4	1.0				
5	0.06	>20	>20	0.9	

[a] Inhibition of chymotrypsin-like (chym), post-glutamyl-peptide (PGPH), and trypsin-like (tryp) proteolytic activity of purified human proteasome. [b] Inhibition of proliferation of MDA-MB-435 cells. [c] Inhibition of chymotrypsin-like activity of the proteasome in cultured MDA-MB-435 cells.

Noncovalent inhibitors of the proteasome seem to have been investigated less extensively judging from the paucity of reports on this type of inhibitor.[21] In principle, such inhibitors should be devoid of the inherent drawbacks associated with the classical reactive warhead groups (i.e., lack of specificity, excessive reactivity, and instability), and we decided to explore this noncovalent strategy in our current drug-discovery activities aimed at blocking the proteasome−ubiquitin pathway in tumor cells.

We have reported in several publications the discovery and optimization of 2-aminobenzylstatine derivatives that inhibit noncovalently and with high selectivity the chymotrypsin-like peptidase activity of the human 20S proteasome (**1** and **2**).[22−24] In this class, we could improve by 2 orders of magnitude the inhibitory activity, in a biochemical assay, of the initial micromolar hit **1**. However, only modest cellular activity could be achieved with this compound class (e.g., derivative **2** in Table 1). Assuming that poor cell penetration was the reason our 2-aminobenzylstatine compounds did not express their high enzymatic inhibitory activity at the cellular level, we engaged in efforts to identify alternative inhibitor scaffolds of reduced size and attenuated peptidic character. We report herein the discovery by structure-based design of the first representatives of a new class of potent, noncovalent 20S proteasome inhibitors that, in contrast to the 2-aminobenzylstatine inhibitors, show high activity in cellular assays.

The active site responsible for the chymotrypsin-like proteolytic activity of the human 20S proteasome is formed by the association of β-subunits X and HC5.[25] Using the X-ray crystal structure of the yeast proteasome,[26] we constructed a homology model of these β-subunits.[23] The model served to establish a binding mode hypothesis for initial compound **1** and was instrumental to the optimization of this class of noncovalent 20S proteasome inhibitors.[24] The binding mode is illustrated in Figure 1 with **2**, one of the most potent noncovalent 20S proteasome inhibitors reported to date.

According to this binding model, which is supported by extensive structure−activity relationships, crucial to the affinity of the 2-aminobenzylstatine inhibitors for the X/HC5 active site is a set of hydrogen bond interactions. Four β-sheetlike hydrogen bonds are formed between the amide bonds flanking the valine residue of the inhibitor and the main chain of subunit X at residues Thr 21, Gly 47, and Ala 49. These hydrogen bonds position the inhibitors in the active site in such

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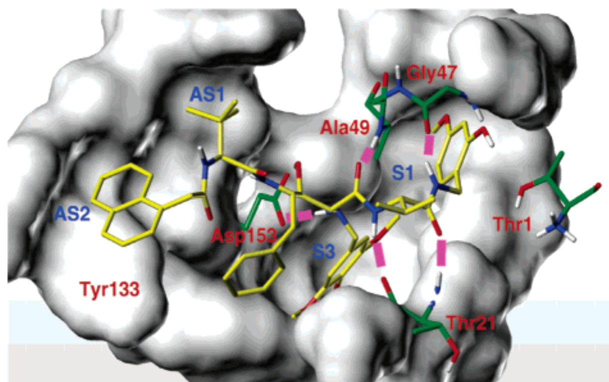


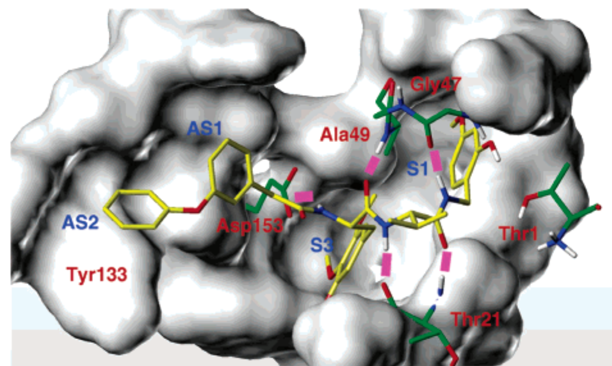
Figure 1. Model of **2** bound to the proteasome X/HC5 subunits. Hydrogen bonds are shown in magenta.

a way that their C-terminal phenol and statine 2-aminobenzyl moieties fill the enzyme's S1 and S3 pockets, respectively, where they form additional hydrophobic and hydrogen bond interactions. In particular, the statine 2-amino group establishes a hydrogen bond with residue Asp 153 of subunit HC5 located on the rim of the S3 pocket. The *tert*-leucine side chain and the lipophilic N-terminal group in the inhibitors also form favorable interactions with the active site by occupying small accessory hydrophobic pockets labeled AS1 and AS2 in Figure 1. In contrast, the two amide bonds flanking the *tert*-leucine residue and the statine 4-benzyl group have no significant interactions with the X/HC5 active site in our binding model. This hypothesis prompted us to envisage the design of new simplified proteasome inhibitor scaffolds in which these nonproductive structural features are removed while maintaining the above-described favorable interactions. Design by interactive molecular modeling led to several ideas for realizing this objective. The most appealing one was a dipeptide scaffold represented by prototype **3**.

Figure 2 shows how the important structural features of the 2-aminobenzylstatine inhibitors are incorporated or mimicked in this molecule. As can be seen, the two crucial amide bonds flanking the valine residue and the C-terminal phenol group filling the S1 pocket are preserved whereas the statine moiety is replaced by a 3,4,5-trimethoxy-L-phenylalanine residue. This nonproteinogenic amino acid is able to form exactly the same interactions with the S3 pocket as the entire 2-aminobenzylstatine moiety. The two other critical pharmacophore features, i.e., the *tert*-leucine side chain and the hydrophobic N-terminal group, are mimicked in the prototype compound by a single phenoxy substituted benzylic N-terminal group. Modeling suggested that the two phenyl rings of this bulky N-terminal group present a spatial arrangement adequate to simultaneously fill the AS1 and AS2 accessory hydrophobic pockets.

Prototypes **4** and **5** (Chart 1), which contrary to **3** do not incorporate all the favorable binding elements present in **2**, were also envisaged for synthesis to further probe the validity of our design concept.

The synthesis of **1** and **2** has been reported previously,[22-24] and **3**–**5** were prepared in a stepwise procedure by standard solution peptide chemistry. The general route for the synthesis of these new 20S proteasome inhibitors is illustrated for **3** in Scheme 1.



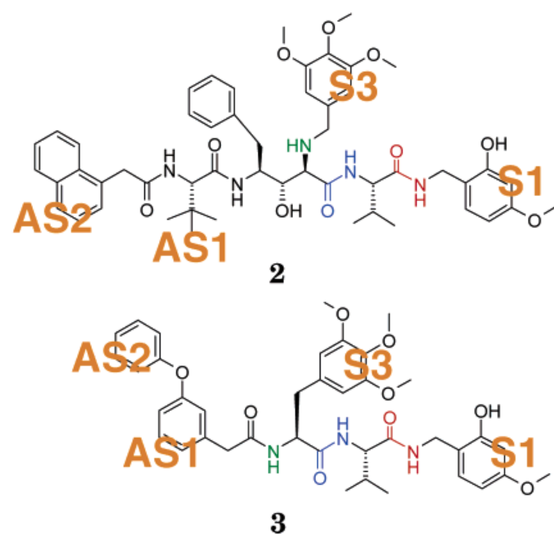
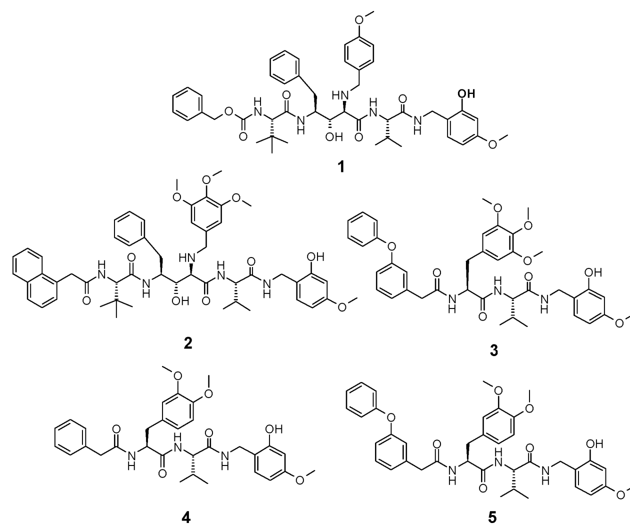


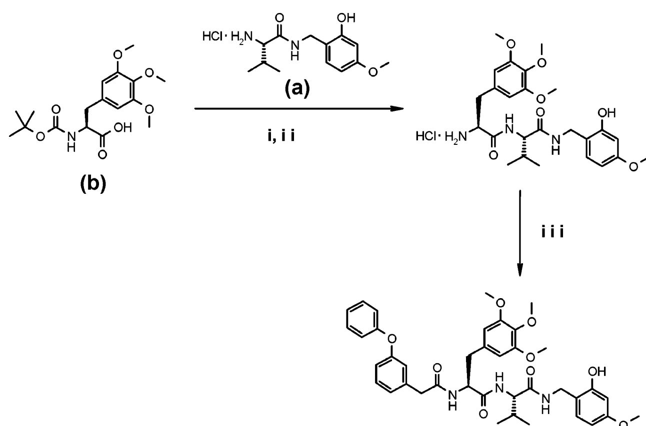
Figure 2. Top: model of designed **3** bound to the proteasome X/HC5 subunits. Hydrogen bonds are shown in magenta. Bottom: chemical structures of **2** and **3** showing the correspondence between their respective chemical moieties in terms of interaction with the proteasome binding site. Matching main chain features are represented in the same color, while matching side chain features are labeled by the name of the pocket to which they bind.

Chart 1



HCl·(*S*)-Val-(2-hydroxy-4-methoxy)benzylamine (**a**) was obtained by condensation of N^{α}-Boc-L-Val-OH with 2-aminomethyl-5-methoxyphenol followed by N^{α}-Boc deprotection with 4 N HCl in dioxane.[22-24] N^{α}-Boc-3,4,5-L-trimethoxyphenylalanine (**b**) was obtained by known

Scheme 1[a]



[a] (i) **a**, DIEA, TPTU in DMF, 3.5 h, 0 °C, room temp, 65%; (ii) 4 N HCl in dioxane, 90 min, room temp, 100%; (iii) 3-phenoxyphenylacetic acid, DIEA, TPTU in DMF, 16 h, room temp, 77%.

procedures from 3,4,5-trimethoxybenzaldehyde,[27,28] while the 3,4-dimethoxy analogue, N^{α}-Boc-3,4-L-dimethoxyphenylalanine, was from a commercial source. The phenylalanine derivatives were coupled to **a** to provide the Boc-protected dipeptides. After Boc deprotection under acidic conditions, the N-terminal capping groups designed by molecular modeling were incorporated into the corresponding derivatives to afford the target compounds.

The designed prototypes **3**−**5** were tested in biochemical assays that measure their ability to inhibit the three types of hydrolytic activities of the human 20S proteasome. Remarkably, potent and selective inhibition of the proteasome chymotrypsin-like activity was observed. As reported in Table 1, **3** turned out to inhibit the chymotrypsin-like activity with an IC_{50} of 15 nM while not affecting the trypsin-like and post-glutamyl-peptide hydrolytic activities at a concentration as high as 20 μM. Furthermore, fully validating the design concept, the structure−activity relationships observed in the 2-aminobenzylstatine inhibitor class were mirrored in the novel class, as judged from a comparison of the relative inhibitory activities of **3**−**5**. We had observed in the 2-aminobenzylstatine class the marked beneficial effect of filling the AS2 pocket where the main interaction established by the inhibitors, according to the model, is aromatic stacking with residue Tyr 133 of subunit HC5.[23,24] Consistently, the phenoxy substituent targeting the AS2 pocket present on the N-terminal benzylic group of **5** causes a 17-fold increase in potency compared to **4** whose unsubstituted N-terminal group can only interact with the AS1 pocket. Similarly, as previously verified in the 2-aminobenzylstatine series, targeting a residue of the bottom of the S3 pocket[29] for hydrogen bonding by adding a third methoxy substituent on the central benzylic moiety is beneficial. Compound **3** is significantly more potent (4-fold) than **5** in inhibiting the chymotrypsin-like activity of the proteasome.

Thus, overall, the high potency and selectivity of **2** were matched by **3** in biochemical assays. However, most importantly, in contrast to the 2-aminobenzylstatine derivative, **3** also showed good activity in a cellular setting. This compound blocks proteasome activity in cultured cells (IC_{50} = 20 nM) and inhibits in a dose-dependent manner the proliferation of different tumor cell lines (e.g., IC_{50} = 60 nM, MDA-MB-435 cells). Reduction of the molecular weight and decrease of the number of amide bonds thus appear to have been an appropriate strategy to achieve high cellular activity.

In conclusion, we have discovered a new class of potent, cellularly active inhibitors of the proteasome. Unlike most previously reported proteasome inhibitors, the new inhibitors act noncovalently and show high specificity for the chymotrypsin-like activity of the enzyme. These unique properties make **3** an interesting tool for investigations of proteasome function in many aspects of cellular regulation. In addition, the high antiproliferative activity obtained is encouraging in our efforts toward developing an anticancer drug based on the concept of proteasome inhibition.

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Supporting Information Available: Experimental procedures and analytical data for all intermediate and final compounds and description of biochemical and cellular assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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