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Modeling of the Binding Mode of a Non-covalent Inhibitor of the 20S Proteasome. Application to Structure-Based Analogue Design

Pascal Furet,* Patricia Imbach,* Peter Fürst, Marc Lang, Maria Noorani, Johann Zimmermann and Carlos García-Echeverría*

Oncology Research, Novartis Pharmaceuticals Inc., CH-4002 Basel, Switzerland

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Abstract—The 2-aminobenzylstatine derivative **1** is a 20S proteasome inhibitor of a novel chemical type identified by high throughput screening. The compound specifically inhibits the chymotrypsin-like catalytic activity of the human proteasome with an IC_{50} value in the micromolar range. Using the crystal structure of the yeast proteasome, we modeled the structure of the human proteasome in complex with **1**. As one of the first applications of the model in our oncology programme targeting the proteasome, we designed an analogue of the inhibitor having enhanced stacking/hydrophobic interactions with the enzyme. One order of magnitude in inhibitory potency was gained.

Introduction

Inhibitors of the ubiquitin-proteasome cellular pathway are considered to have therapeutic potential as novel antitumor agents.[1–3] Following this line of research, we have initiated a program aiming at the dentification of potent inhibitors of the chymotrypsin-like proteolytic activity of the human 20S proteasome. High throughput screening of the Novartis chemical archives provided a non-covalent micromolar and selective inhibitor of this enzymatic activity, **1**, that was selected for further evaluation. To assist our medicinal chemistry effort, we developed a structural model of **1** bound to the site responsible for the chymotrypsin-like activity of the 20S proteasome. In this letter, we present our binding model and report on its utilization in the design of a particular modification of the inhibitor that significantly improved its potency[4] (Fig. 1).

X/HC5 Binding Site Model

Crystal structures of the yeast 20S proteasome have clearly established that the binding site responsible for its chymotrypsin-like proteolytic activity is formed by the association of β-subunits PRE2 and C5.[5] To build a model of the corresponding binding site in the human 20S proteasome, we extracted the coordinates of β-subunits PRE2 and C5 from the overall crystal structure of the yeast proteasome in complex with the covalent aldehyde inhibitor Ac-Leu-Leu-norleucinal. After sequence alignments in the homology module of InsightII,[6] the coordinates were input in the program WHAT IF[7] to generate models of subunits X and HC5, the human homologs of PRE2 and C5.[8] The resulting model of the X/HC5 binding site ligated by the Ac-Leu-Leu-norleucinal inhibitor is shown in Figure 2 in comparison with the PRE2/C5 site. X and PRE2 have 67% sequence identity and, as can be seen in the figure, few mutations occur in the vicinity of the inhibitor. HC5 and C5 have less sequence identity (45%). However, the mutations in the region close to the inhibitor are conservative. Thus, the homology model of the X/HC5 binding site was judged reliable enough to be useful in our efforts to understand the structural basis of the proteasome inhibitory activity of **1**.

Binding Mode Model

1 is a 2-aminobenzylstatine derivative synthesized in the course of an HIV protease inhibitor project.[9] Its cocrystal structure with the HIV protease has been determined.[10] In this structure, the statine 4-benzyl group, the *tert*-leucine side chain and the N-terminal benzyloxycarbonyl group of the compound occupy respectively the S1, S2 and S3 pockets of the protease, while the statine 2-amino moiety, the valine side chain and the C-terminal benzylic group fill the S′ pockets.[11]

*Corresponding authors: Tel.: +41-61-67-990; fax: +41-61-696-6929; e-mail: pascal.furet@pharma.novartis.com; patricia.imbach@pharma.novartis.com; carlos.garcia-echeverria@pharma.novartis.com

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An initial attempt to dock **1** in the X/HC5 binding site model following a similar scheme did not lead to any convincing result because of the absence of well defined S′ pockets in the proteasome to accommodate the latter moieties.

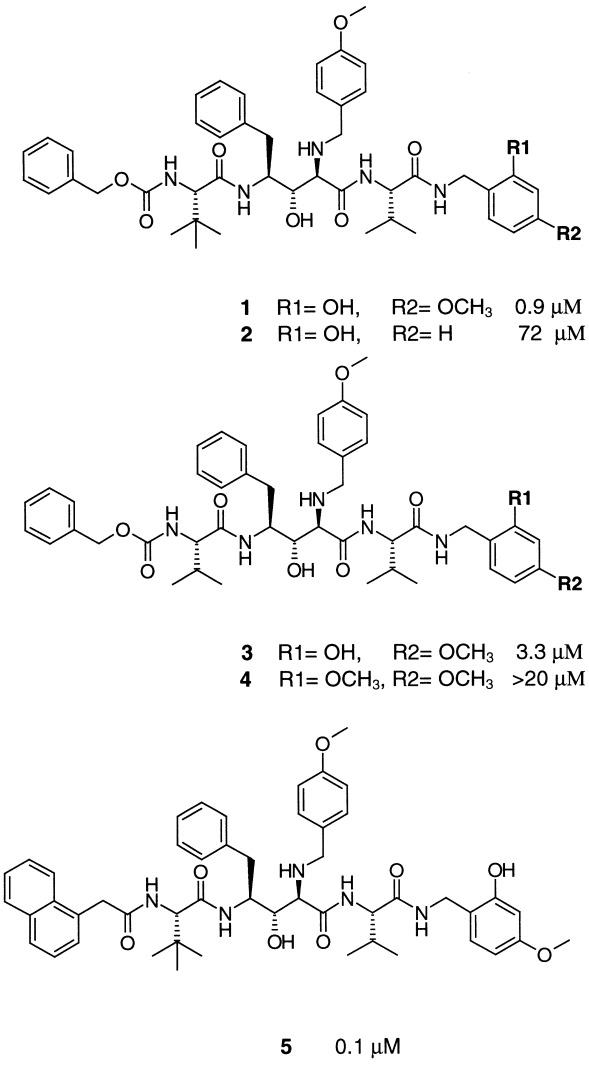

Figure 1. Chemical structures and human 20S proteasome inhibitory activities of compounds **1**–**5**.[4]

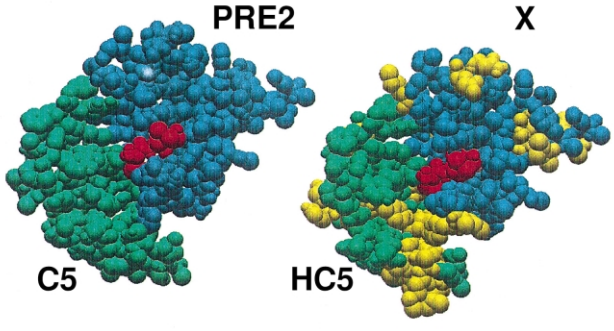

Figure 2. Homology model of the X/HC5 binding site (right) compared to the crystal structure of the yeast PRE2/C5 site (left). Subunits X and PRE2 are colored in blue, C5 and HC5 in green and the aldehyde inhibitor in red. The mutated residues appear in yellow.

Analysis of the binding mode of the Ac-Leu-Leu-norleucinal inhibitor in the crystal structure suggested another way of docking **1**. As represented in Figure 3a, the aldehyde inhibitor forms a hemiacetal adduct with the catalytic Thr 1 residue of subunit PRE2. The adduct is held in an extended conformation by four β-sheet-like hydrogen bonds involving its peptide bonds and the main chain of PRE2 at residues Thr 21, Gly 47 and Ala 49. In addition, the N-terminal acetamide group hydrogen bonds to the side chain of subunit C5 residue Asp 145.[12] These interactions position the norleucine side chain in the S1 pocket while the side chain of the N-terminal leucine partially fills the larger S3 pocket. The side chain of the central leucine, which corresponds to the P2 amino acid in a substrate, is oriented towards the solvent and does not interact with the protein. Assuming a key role of the four β-sheet-like hydrogen bonds in the recognition of substrates and peptidic molecules by the proteasome, we searched for orientations of **1** in the X/HC5 binding site model that satisfied these hydrogen bonds. Besides an orientation similar to

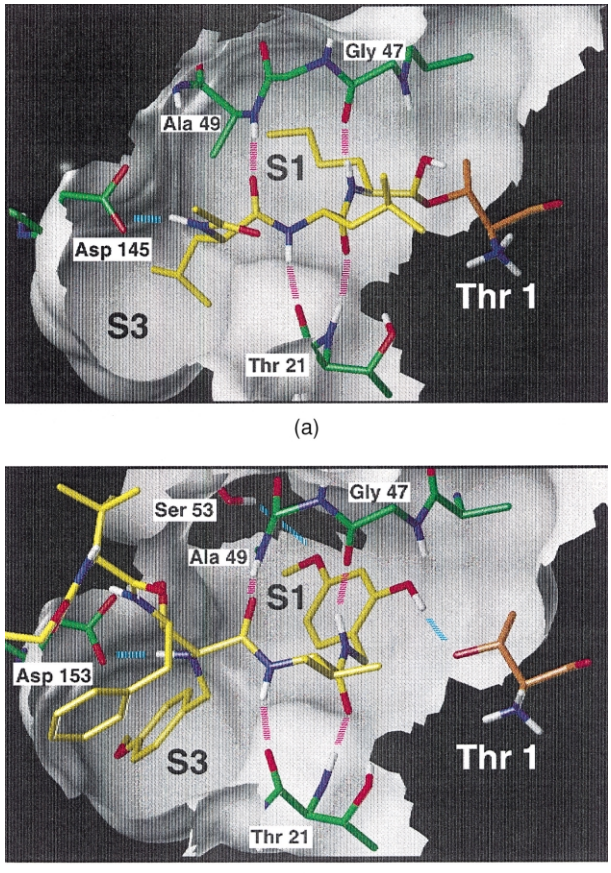

Figure 3. (a) Binding mode of Ac-Leu-Leu-norleucinal in the yeast proteasome crystal structure. (b) Modeled orientation of the valine residue of **1** and its two adjacent moieties in the X/HC5 binding site. The four β-sheet-like hydrogen bonds appear as pink dashed lines. Other hydrogen bonds discussed in the text are shown as blue dashed lines.

that observed in the crystal structure of the HIV protease, which was discarded for the reason above mentioned, only one other possibility was found. As shown in Figure 3b, in this binding mode the four β-sheet-like hydrogen bonds are formed by the peptide bonds of the inhibitor flanking its valine residue, which therefore plays the role of the P2 amino acid of proteasome substrates. In such an orientation, it is possible to fill the S1 pocket with the C-terminal benzyl group and the S3 pocket with the 4-methoxy-benzylamino group in the 2-position of the statine moiety. Moreover, the nitrogen atom of the latter can form a hydrogen bond with residue Asp 153[13] of subunit HC5 located on the rim of the S3 pocket.

The hydrogen-bond based positioning of the valine residue, followed by the manual placing of its two adjacent moieties into the S1 and S3 pockets, was completed by a Monte Carlo conformational search[14] to determine the most favorable position of the rest of the inhibitor in the binding site. We obtained a final model (Fig. 4) in which the *tert*-leucine side chain and the phenyl ring of the N-terminal benzyloxycarbonyl group nicely fit into two accessory shallow pockets. These pockets are probably involved in the binding of substrate amino acids N-terminal to P3. As for the statine 4-benzyl group, it binds at the surface of the X/HC5 subunits in a region located between the S3 pocket and the accessory pockets.

In the model shown in Figure 4, a large number of complementary hydrophobic and hydrogen bond interactions between **1** and the binding site were achieved. These could account for the ability of the compound to inhibit the 20S proteasome without possessing a reactive group capable of forming a covalent bond with the catalytic threonine. The model was also consistent with the limited structure–activity relationships we had in hand as illustrated below with a few examples.

In the model, the 4-methoxy substituent of the C-terminal benzylamide moiety of **1** is assumed to form a hydrogen bond with the side chain of Ser 53, a residue of subunit X located at the bottom of the S1 pocket (see Fig. 3b). In full consistency with this putative interaction, **1** is a weaker inhibitor of the chymotrypsin-like activity of the yeast 20S proteasome.[15] One of the few differences in the sequences of subunit X and its yeast homologue PRE2 occurs in fact at position 53 where the serine in X is replaced by a glutamine in PRE2. Modeling clearly shows that Gln 53 of the yeast proteasome is unable to form the same hydrogen bond with the methoxy substituent. Along the same line, **2**, the analogue of **1** which lacks this methoxy susbstituent, is more than one order of magnitude less potent in inhibiting the chymotrypsin-like activity of the human 20S proteasome. Another available piece of data indicated that replacement of the 2-hydroxy substituent of the same C-terminal benzylic moiety by methoxy (compound **3** compared to **4)** caused a drop in inhibitory potency. This deleterious effect is consistent with the abrogation of the hydrogen bond donated by the hydroxy susbstituent to Thr 1 according to the model (Fig. 3b).

Analogue Design

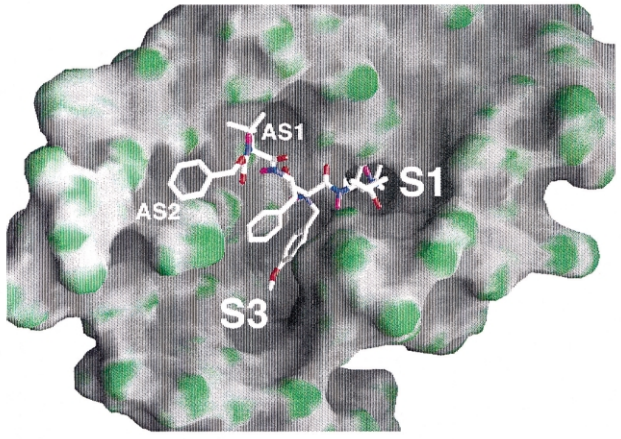

Figure 4. Model of **1** bound to the X/HC5 site (global view). The *tert*-leucine side chain and the N-terminal group of the inhibitor bind to shallow accessory pockets adjacent to S3 that are labeled AS1 and AS2, respectively.

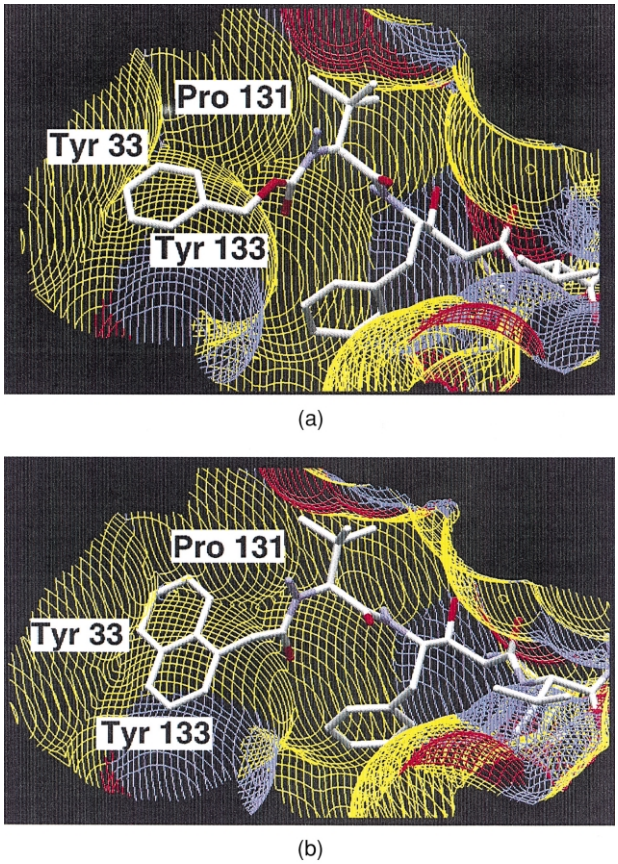

Figure 5. Color-coded Lee and Richards solvent accessible surface of the binding site.[17] Yellow, red, and blue regions correspond, respectively, to hydrophobic, hydrogen bond donor and hydrogen bond acceptor atoms of the protein: (a) with **1** bound; (b) with **4** bound.

As reported in the accompanying paper, our initial medicinal chemistry efforts to improve the potency of **1** focused on the modification of its N-terminal part. The preceding binding model served to design N-terminal groups having additional favorable interactions with the proteasome compared to the original benzyloxycarbonyl group. A successful example of application is presented in the next section.

The accessory pocket AS2 to which the benzyloxycarbonyl group binds is formed by the side chains of subunit HC5 amino acids Tyr 33, Tyr 133 and Pro 131 (Fig. 5a). Stacking between the phenyl ring of the N-terminal group and the side chain of Tyr 133 is assumed to be the main interaction between the inhibitor and the proteasome in this part of the binding site. Increasing the strength of this stacking interaction was considered as an attractive strategy in our design efforts. In particular, a modeling experiment suggested that AS2 can accommodate a larger and more electron-rich naphthyl group in stacking interaction with Tyr 133[16] provided that the spacer between the terminal aryl group and the *tert*-leucine N-terminus is one atom shorter. This idea, illustrated in Figure 5b, motivated the synthesis of compound **5** in which the benzyloxycarbonyl group of compound **1** is replaced by an N-terminal group derived from the coupling of naphthalen-1-yl-acetic acid. With an IC_{50} value of 0.1 μM, **5** turned out to be one order of magnitude more potent than **1** confirming the validity of the model and establishing its utility in the design of new proteasome inhibitors.

In summary, modeling has allowed us to give a firm structural basis to our medicinal chemistry endeavor around the new 20S proteasome inhibitor **1**. Further applications of the model to the modification of **1** are ongoing and will be reported in due course.

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11. The standard nomenclature for substrate residues and the associated protease pockets is used. Schechter, I.; Berger, A. *Biochem. Biophys. Res. Commun.* **1967**, *27*, 157.
12. The amino-acid numbering in the sequences of PRE2 and X starts at the N-terminal catalytic threonine (Thr 1). For C5 and HC5, we use the numbering of entries P23724 and P20618 of the Swiss-Prot database.
13. HC5 residue Asp 153 corresponds to C5 residue Asp 145. Therefore, according to our model, the 2-aminobenzylstatine NH of **1** mimics the N-terminal acetamide NH of Ac-Leu-Leu-norleucinal in its interaction with the proteasome.
14. The Monte Carlo (with energy minization) search was performed in Macromodel using the AMBER*/H_2O/GBSA force field. All torsion angles, except those of the C-terminal benzyl group, the valine residue and the 4-methoxybenzylamino substituent in the 2-position of the statine moiety already positioned, were varied. Macromodel: Mohamadi, F.; Richards, N. G. J.; Guida, W. C.; Liskamp, R.; Lipton, M.; Caufield, C.; Chang, G.; Hendrickson, T.; Still, W. C. *J. Comput. Chem.* **1990**, *11*, 440.
15. No significant inhibition of the chymotrypsin-like activity of the yeast proteasome is observed at a concentration of 20 μM.
16. The naphthyl ring had also additional hydrophobic contacts with Tyr 33 and Pro 131 compared to the phenyl ring of the benzyloxycarbonyl group.
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