20S Proteasome Inhibitors

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Hydroxyureas as Noncovalent Proteasome Inhibitors**

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The ubiquitin-proteasome system (UPS)[1] has been related to many different diseases including multiple myeloma, where the core protein of the UPS, the 20S proteasome (CP), has been successfully targeted by the blockbuster drug Velcade (Bortezomib).[2] The repeated emphasis on the vast therapeutic potential of CP inhibitors in different diseases and the primary resistance and ineffectiveness of current market drugs against some types of solid tumors have proven the need for not only second-generation CP inhibitors but also for the discovery of inhibitors with a new mechanism of action.

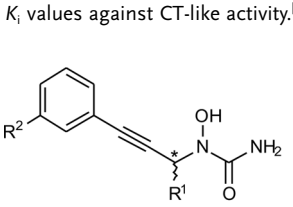
The eukaryotic CP is composed of four stacked heptameric rings, each comprising α- and β-type subunits in an $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ stoichiometry.[3] Embedded in these sandwiched β-subunits are three different active sites with distinct cleavage preferences known as caspase(C)-, trypsin(T)-, and chymotrypsin(CT)-like activities. The active-site nucleophile Thr1O$^{\gamma}$ is located in subunits β1, β2, and β5 (C-, T- and CT-like activities), respectively; however, it is the composition of active and surrounding subunits of the substrate-binding pockets which give rise to the substrate-cleaving preferences.[4]

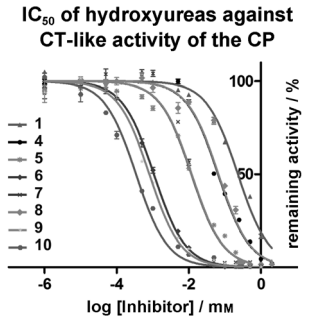
To date, all CP inhibitors are known to have either a peptidelike structure, which forms an antiparallel β sheet with the substrate-binding channel of the active sites, and/or reactive head groups which covalently bind to the threonine residue Thr1O$^{\gamma}$.[5] These characteristics have previously been related to excessive reactivity, lack of specificity, and/or instability. Furthermore, there are currently few CP inhibitors that do not simultaneously inactivate all three active sites of the CP. In fact, studies have shown that inactivation of the β5 active site alone is enough to attain therapeutic effects.[6,7]

We performed screening experiments using specific β5 fluorogenic tetrapeptide substrates in order to search for nonpeptide-based inhibitors with reversible binding to the CT-like active site. These experiments were performed using a library of inhibitors from Bayer CropScience AG. One of the hits identified was an *N*-hydroxyurea(**HU**)-based compound (**1**, Table 1). Interestingly, this inhibitor is a known 5-lipoxygenase inhibitor (U.S. Pat. No. 5,714,633) closely related to ZyfloCR (Zileuton; Figure S1 in the Supporting Information) a internationally prescribed drug for the treatment of asthma.[8] Surprisingly, **1** showed unique binding to the CT-like active site of the yeast CP with an IC_{50} of 230 μM ($K_i = 23$ μM; Table 1) with no inhibition observed for the C- and T-like activities even at an inhibitor concentration of 200 μM (Figure S2 in the Supporting Information).

Additionally this inhibitor has no structural similarities with any other known inhibitor of the CP. Moreover, it

Table 1: *N*-Hydroxyurea (**HU**) scaffold and inhibitory profile with IC_{50} and K_i values against CT-like activity.[a]





Cmpd.	R^2	R^1	IC_{50} [μM]	K_i [μM]
0	H	H	no inhibition	
1	*t*BuCH$_2$CH$_2$O	Me	229 ± 22	23.1 ± 2.2
2	CF$_3$O	Me	> 1000	–
3	*n*PentO	Me	> 1000	–
4	*t*BuMe$_2$SiO	Me	48 ± 2	4.8 ± 0.2
5	(1-Ad)O	H	12.0 ± 1.3	1.02 ± 0.13
6	(1-Ad)O	Et	1.07 ± 0.06	0.107 ± 0.006
7	(1-Ad)O	*i*Pr	10.6 ± 3.7	1.06 ± 0.37
8	(1-Ad)O	Me	0.78 ± 0.16	0.078 ± 0.016
9	(1-Ad)O	Me (*S*)[b]	57 ± 2.5	5.75 ± 0.25
10	(1-Ad)O	Me (*R*)[c]	0.34 ± 0.04	0.034 ± 0.004

[a] No inhibition against T- or C-like activity was observed at concentrations as high as 200 μM (Figure S2). [b] C* in *S* configuration. [c] C* in *R* configuration.

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fulfilled our in vitro criteria as it is not only active-site specific but it also displays reversible binding (Figure S2a,b in the Supporting Information). In previous clinical studies of this class of 5-lipoxygenase inhibitors, certain additional initial characteristics such as cell accessibility and clearance rate were reported, which make it a potential lead structure for pharmaceutical use (see Figure S3a in the Supporting Information).[9]

Following these promising results, the crystal structure of the CP:**1** complex was determined at 2.4 Å resolution (R_{free} = 0.256; Table ST1 in the Supporting Information). The $2F_O-F_C$ electron density map clearly displays **1** well defined in the proximity of the CT-like active site in subunit β5 and reveals a novel, noncovalent mode of binding (Figure S3a in the Supporting Information).

The *N*-hydroxyurea moiety does not interact with the active-site β5-Thr1O$^{\gamma}$ nucleophile, which hitherto has been considered a common principle of CP-inhibitor binding. Instead, the functional group is stabilized by hydrogen bonds with β5-Thr21NH/CO and β5-Gly47CO main-chain atoms; the binding interactions are analogous to the antiparallel β sheets observed both in substrates and peptidic inhibitors, for example, Calpain Inhibitor I (CAL I; Figure 2).

The methyl group (R^1) of **1** has hydrophobic interactions with the β5 side chains Met45 and Ala46, and the propynylbenzene unit protrudes towards a novel subpocket of the CT-like S1 pocket (S1 subpocket), which thus results in a plenitude of van der Waals interactions with Ser27, Val31, Met45, and Ala49 (Figure S3a in the Supporting Information). Furthermore, the rigid structure of the propynylbenzene reduces the entropic penalty upon binding to the CP. The 3,3-methylbutoxy group (R^2) of **1** interacts with the hydrophobic atoms of His98, Ser112, Glu122, and Arg125, all of which are located in subunit β6 and are oriented towards yet another new subpocket located near the S3 pocket (S3 subpocket) (Figure S3a in the Supporting Information). Interestingly, no inhibition of the CP was observed in the absence of a *meta* substituent (R^2 = H, **0**), proving the significance of these interactions for CP:**HU** binding. The structural data of the complex inhibitor further confirms the indispensability of this particular binding site for both subunit specificity and enthalpic stabilization of the ligand.

Subsequently, an efficient chemical synthesis of these **HU** compounds was devised, which allowed straightforward optimization through maintenance of the original propynylhydroxyurea scaffold and variation of R^1 and R^2 (Figure S4 in the Supporting Information). The *N*-hydroxyurea head group was synthesized in three steps by mesylation of but-3-yn-2-ol, nucleophilic substitution of the resulting mesylate by hydroxylamine, and subsequent reaction with potassium cyanate. *meta*-Substituted iodophenyl groups were then attached to the *N*-hydroxyurea head group through Sonogashira couplings (Pat. WO9530671).[10]

Variations in R^1 underlined the importance of a small hydrophobic side chain at this location: sterically more demanding side chains, such as ethyl (in **6**) and isopropyl (in **7**), increased the IC_{50} up to 15-fold to 1 μM (K_i = 0.1 μM) and 10 μM (K_i = 1.0 μM), respectively (Table 1). These results are in agreement with molecular modeling experiments, which

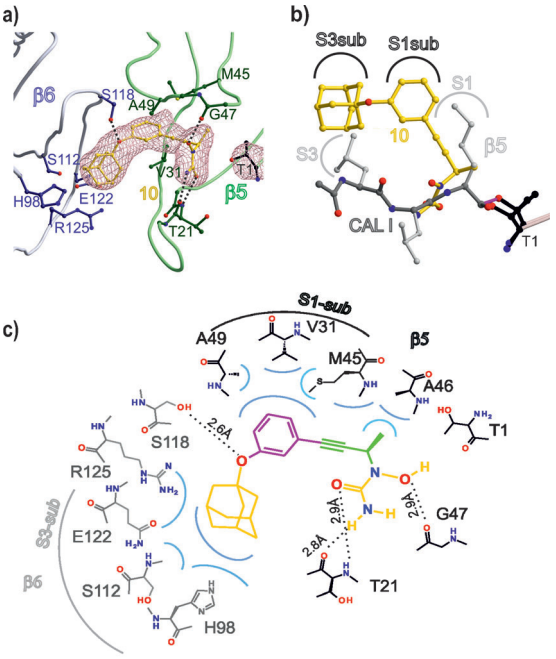

Figure 1. Crystal structure of CP in complex with the most potent **HU** derivative **10** (CP:**10**; PDB ID: 3SHJ). a) The *N*-hydroxyurea group forms a network of hydrogen bonds with β5-Thr21NH/CO and β5-Gly47CO (black dashed lines). Residues forming the S1 subpocket are highlighted in dark green and embrace the rigid propynylbenzene scaffold, whereas the S3 subpocket (blue) interacts with the R^2 group. b) Superposition of Calpain Inhibitor I (CAL I) and **10**, showing the complete new binding mode of **1** and the novel subpockets. c) Schematic overview of (a). The distinct specificity pockets S1-sub and S3-sub of the CT substrate-binding channel and their corresponding amino acids are depicted in black and gray, respectively.

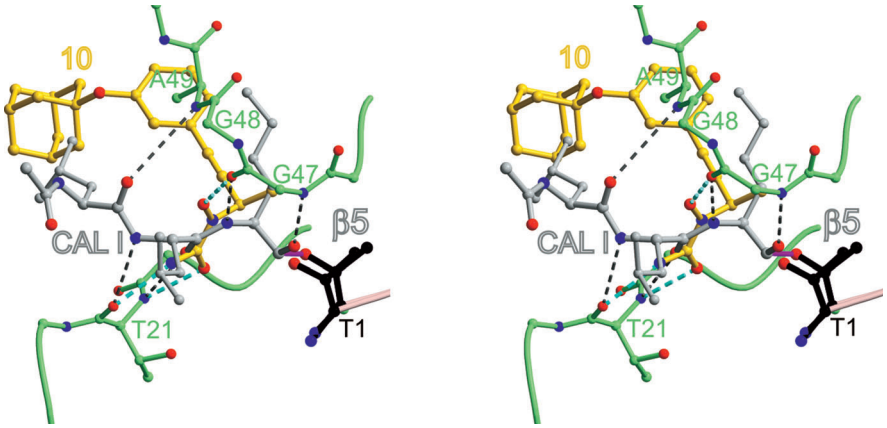

Figure 2. Structural superposition of **10** and CAL I bound to the CT-like active site, illustrating how the binding mode of **HU**s differs from that of peptide ligands. Peptide-backbone interactions between CP and CAL I are shown as gray dashed lines; **10**:CP main-chain interactions are in cyan.

indicate a clash of larger moieties with the peptide backbone of the β5 subunit and thus suggest that small hydrophobic groups are required for inhibitor stabilization.

Remarkably, slight changes in R^2 significantly influenced the IC_{50} of these **HU** compounds: small halogenated (trifluoromethoxy in **2**) and extended aliphatic R^2 side chains (*n*-pentoxy in **3**) resulted in at least a fivefold increase in the IC_{50} (>1 mM) compared to compound **1**. Interestingly, a *tert*-butyldimethylsiloxy moiety (in **4**) in this position improved affinity and resulted in a fivefold decrease in the IC_{50} (48 μM, K_i 4.8 μM) (Table 1). The crystal structure of the latter compound in complex with the CP at 3.2 Å resolution ($R_{free} = 0.228$) shows strong interactions of the *tert*-butyldimethylsiloxy side chain wiwth Met45 and Ala46 in the S1 subpocket (Table ST1 and Figure S3b in the Supporting Information). Based on these crystallographic results, molecular modeling was performed to design more appropriate side chains for R^2. A 1-adamantyloxy group in R^2 was identified to give the highest docking scores (−10.7 in GlideScore)[11,12] among a small library of 50 compounds (Figure S6 in the Supporting Information). Surprisingly, this new **HU** derivative (**8**) displayed an IC_{50} of 700 nM ($K_i = 0.08$ μM), 320 times lower than that of the starting compound (**1**) (Table 1). The crystal structure of the CP:**8** complex at 2.9 Å resolution ($R_{free} = 0.238$, Table ST1 in the Supporting Information) confirmed the modeling results (0.8 Å rmsd between experimental and modeled ligand structures; Figures S3c and S7 in the Supporting Information) and additionally revealed the importance of Ser118 in subunit β6, which formed a strong hydrogen bond to the ether oxygen atom of **8** and thus further stabilizes the position of the adamantyloxy residue in the S3 subpocket (Figure 1c).

These **HU** compounds were optimized further with respect to the configuration generated by the R^1 group. The *R* enantiomer (**10**; $IC_{50} = 300$ nM, $K_i = 34$ nM) displayed a much stronger inhibition than the *S* conformer (**9**; $IC_{50} = 56$ μM, $K_i = 5.8$ μM; Figure 1 and Table 1). The structural results of CP:**9** at 3.1 Å resolution ($R_{free} = 0.231$) and CP:**10** at 2.8 Å resolution ($R_{free} = 0.262$, Table ST1 in the Supporting Information) showed that only the orientation of R^1 in the *S* enantiomer is responsible for this decrease in binding affinity as a result of disfavored interactions with protein main chain atoms (Figure S8b,c). Accordingly, soaking proteasome crystals with a racemic mixture of **HU** compounds yielded CP:**HU** structures containing solely the *R* enantiomer, thus demonstrating a strong enantioselectivity (Figure S8a in the Supporting Information). Structural superpositions of **9** and **10** revealed that both *R* and the less active *S* enantiomer aligned almost perfectly with regard to the *N*-hydroxyurea moiety and the adamantyloxybenzene (Figure S8b in the Supporting Information). Considering the noncovalent binding mode, this observation underlines the strength of the binding motifs, which keep the inhibitor in place in spite of the disfavored orientation of the Me group in R^1 (**9**).

In addition, an *N*-morpholinoethanesulfonic acid (MES) molecule from the crystallization buffer was found in the electron density maps of most CP:**HU** complex structures (Figure S5 in the Supporting Information). The MES molecule interacts with β5-Gly47N and occupies the oxyanion hole, an area typically populated by active head groups of ligands; this phenomenon has already been described in the literature.[13,14] These two molecules, **HU** and MES, can be regarded as independent fragments that can be used in future fragment-based drug design.

In conclusion, **HU**s constitute a new class of proteasome inhibitors that are characterized by a novel mode of ligand binding and proteasome inhibition. The combination of crystallographic characterization, molecular modeling, chemical synthesis, and kinetic experiments identified a strong inhibitory binding profile of these novel proteasome ligands in defined specificity pockets that have hitherto not been explored for proteasomal drug design. This new class of compounds represents the smallest, reversibly and noncovalently bound, active-site-specific inhibitors observed to date for the 20S proteasome that do not contain a functional reactive head group. In addition, the hydroxyurea compounds present many molecular properties important for pharmacokinetics that may open new avenues for proteasomal drug development.

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