Proteasome Inhibition

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Elucidation of the α-Keto-Aldehyde Binding Mechanism: A Lead Structure Motif for Proteasome Inhibition**

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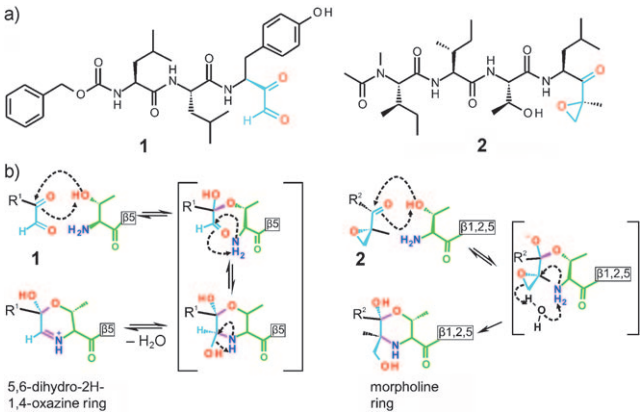
The proteasome's participation in essential biological processes such as stress response, cell proliferation, apoptosis, and antigen presentation has been well established.[1] It is, therefore, not surprising that academia and the pharmaceutical industry have made efforts to develop a range of small synthetic inhibitors against this proteolytic molecular machine (see Scheme SS1 in the Supporting Information for examples).[2] An overall structural comparison of some well-characterized inhibitors[3] implies that most of these compounds form a covalent bond with the N-terminal nucleophilic threonine (Thr1)[4] located at the active sites in the two inner heptameric β rings of the 20S proteasome, termed β1, β2, and β5 according to the subunit of their origin.[5]

MG-132, a tripeptide aldehyde, is one of the most popular proteasome inhibitors for analytical studies and was shown to induce apoptotic cell death.[6] Other inhibitors have entered human clinical trials as significant anticancer or anti-inflammatory leads. For example Velcade (Bortezomib),[7] a dipeptide boronic acid, was the first proteasome inhibitor approved by the U.S. Food and Drug Administration for the treatment of relapsed multiple myeloma and mantle cell lymphoma.[8] However, Bortezomib's boronic acid pharmacophore has been shown to produce substantial off-target activity by reacting with additional enzymes which translates to severe side effects.[9,10] Not surprisingly, competitive products have been developed with increased in vivo specificity such as the natural product salinosporamide A (NPI-0052),[11,12] Carfilzomib, a synthetic tetrapeptide α′,β′-epoxyketone, which is currently being evaluated for the treatment of multiple myeloma, non-Hodgkin's lymphoma, and solid tumors (phase I and phase II clinical trials).[13] This compound was derived from the microbial natural product epoxomicin[14] (**2**; Scheme 1 a) and was shown to be a potent, irreversible, and highly specific proteasome inhibitor.[15] The high degree of specificity was explained by the unique binding mode of the α′,β′-epoxyketone head group,[16] and this binding mode was revealed by co-crystallization experiments and structure determination of **2** bound to the yeast proteasome. In a two-step binding mechanism the inhibitor not only reacts with Thr1O$^{\gamma}$ but also binds irreversibly with Thr1N.

Another inhibitor class having a high selectivity for the proteasome is the peptidyl α-keto aldehydes (glyoxals).[17] Inhibition studies have shown that peptidyl glyoxals are cell-permeable inhibitors[18] that selectively, as well as reversibly, block one of the proteasome's active subunits (β5) with K_i values in the low nanomolar range. Interestingly, although



Scheme 1. a) Chemical structures of the tripeptide α-keto-aldehyde **1** (Z-Leu-Leu-Tyr-COCHO) and the natural product epoxomicin (**2**; α′,β′-epoxyketone). The individual functional reactive groups are shown in cyan. b) Proposed mechanisms for the formation of the 5,6-dihydro-2*H*-1,4-oxazine and the morpholine ring resulting from the binding of **1** and **2**, respectively, to Thr1. R^1 and R^2 resemble the peptide moiety of the corresponding inhibitors. Covalent bonds formed between protein and ligands are displayed in magenta. Transfer of electrons is shown as black dashed arrows.

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their function as proteasome inhibitors has been well established, the mode of action has not yet been elucidated. However, their reversible binding mode, in contrast to the irreversible binding of epoxomicin,[16] requires explanation.

We therefore co-crystallized the commercially available peptidyl glyoxal **1** (Scheme 1a) with the yeast 20S proteasome, and determined the crystal structure of the complex to a 2.7 Å resolution. Hereby, a single proteasome crystal was incubated with **1** for six hours at a final concentration of 5 mM. Crystallographic data were collected with synchrotron radiation at the Swiss Light Source (Paul-Scherrer-Institut, Villigen). Refinement of the crystal structure began using the coordinates of the yeast 20S proteasome (pdb accession code 1RYP),[5] with subsequent anisotropic temperature factor correction and positional refinement using CNS and cyclic twofold symmetry averaging using MAIN ($R_{crys}/R_{free} = 23.9/26.6\%$, see the Supporting Information). Electron density maps calculated with phases after averaging allowed a detailed interpretation of the inhibitor at both β5 sites (Figure 1a). This result is in agreement with the published data that suggest a high preference of **1** for the β5 subunit.[17] The crystallographic data demonstrate that even at concentrations as high as 5 mM, the peptidyl glyoxal only binds to the β5 subunit, which is known to be responsible for chymotrypsin-like activity. Similar to the binding mode of epoxomicin (Figure 1b) and proteasomal peptide aldehyde inhibitors [for example Calpain inhibitor I (Ac-Leu-Leu-nLeu-CHO)], the peptide backbone of **1** adopts a β conformation (Figure 1c).[4,5] It thereby fills the gap between the β strands of the proteasome and generates an antiparallel β-sheet structure that is stabilized by a set of hydrogen bonds between the peptide nitrogen atoms and the surrounding residues (see S1 in the Supporting Information).

A six-membered ring, already well defined in the unaveraged F_OF_C electron density map, indicates that binding of **1** occurs through two reaction steps (Scheme 1b) and is therefore in agreement with kinetic studies.[17] This reaction includes the formation of a covalent hemiketal, similar to the binding mechanisms of epoxomicin and peptidyl aldehydes (hemiacetal).[19] The nucleophilic N terminus of Thr1O$^{\gamma}$ attacks the carbonyl carbon atom of the α-keto moiety of the inhibitor. Furthermore, the hydroxy group of the hemiketal is hydrogen bonded to Gly47N, which acts as the oxyanion hole during peptide bond proteolysis.[5] Additionally, the reaction comprises the nucleophilic addition of the β5 terminal Thr1N to the aldehyde of the inhibitor and subsequent proton transfer, thus forming a carbinolamine intermediate. This reaction is followed by release of a water molecule and the formation of a 5,6-dihydro-2*H*-1,4-oxazine ring, a six-membered heterocycle containing a hemiketal and an imine bond (Schiff base). The electron density map clearly shows that the hydroxy group originating from the keto group still remains stabilized by Gly47N, whereas density is lacking at the position of the hydroxy group of the carbinolamine intermediate. In contrast, the adduct formed by epoxomicin is a morpholine ring (see S1 in the Supporting Information).[16] The mode of action of this natural product proceeds through the formation of a reversible hemiketal and then an irreversible intramolecular cyclization. During the cyclization Thr1N opens the epoxide by an intramolecular displacement with inversion of the carbon atom at the C2 position (Scheme 1b).

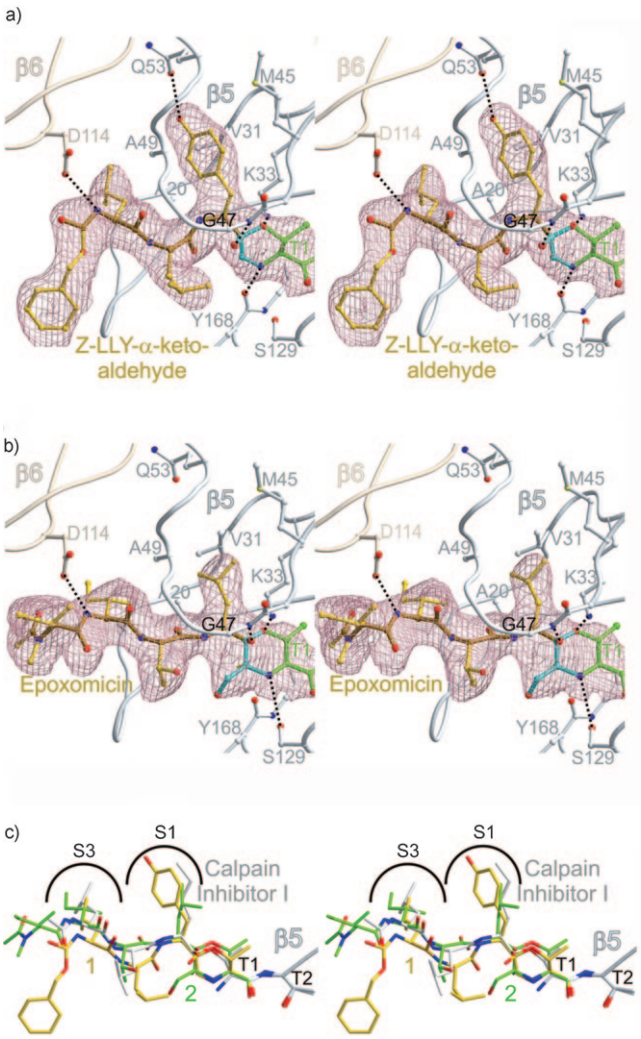

Figure 1. a, b) Stereoview of the 2 F_OF_C electron density map of the inhibitor adduct at the β5 subunit. The electron density was calculated with phases from the free enzyme structure. a) Reversible binding of **1**. The temperature factor refinement indicates full occupancy of the inhibitor of just the β5 subunit. **1** is covalently bound to Thr1 (green) forming a 5,6-dihydro-2*H*-1,4-oxazin ring. b) Covalent irreversible binding of **2**. Hydrogen bridges are indicated as black dashed lines. c) Stereorepresentation of the structural superposition of **1** (yellow), **2** (green), and the peptide aldehyde Calpain inhibitor I (Ac-Leu-Leu-nLeu-CHO, gray). In a similar mode of interaction, all inhibitors adopt an antiparallel β-sheet strand and occupy the S1 and S3 specificity pockets (black semicircles).

Such bivalent binding mechanisms, as shown for α′,β′-epoxyketones and for the α-keto aldehydes discussed herein, are unique for members of the N-terminal nucleophilic hydrolase family and explain the high selectivity of peptidyl glyoxals for the proteasome compared to peptidyl aldehydes. Inhibition of serine proteases such as chymotrypsin[20] or subtilisin[21] with peptidyl glyoxals revealed K_i values more than 1000 times higher (13 μM and 2.3 μM, respectively) because these enzymes lack the amino terminal nucleophilic residue as part of their active sites, and hence, solely react

with either the keto or the aldehyde group of the inhibitors. Furthermore, specificity for the proteasome over other proteases is enhanced by the choice of different peptide chains attached to the pharmacophore mimicking the natural substrate. The S1 and the S3 binding pockets are particularly relevant for the proteasome. Thus, modifications of the peptide portion can lead to proteasome inhibitors with improved selectivity and pharmaceutical properties as successfully shown for the generation of the Bortezomib analogue CEP-18770[22] and the epoxomicin derivative Carfilzomib.[10]

Although, peptidyl glyoxal and epoxomicin follow a similar mode of action via six-membered ring formation (Scheme 1 b), a profound difference exists in the reversibility of **1**. This result was supported with our crystallographic data, which shows that the binding mechanism of glyoxals consists of two reversible reactions resulting in an unsaturated heterocycle (Scheme 1 b). Furthermore, our structural data reveal a 3.0 Å hydrogen bond between Tyr168O and the oxazine nitrogen atom (Figure 1 a) indicating that the latter is protonated, which in turn promotes hydration of the adjacent carbon atom, and thus, reversibility of both reaction steps. This is in agreement with our kinetic studies, in which we conducted substrate competition assays after four hours of incubation time and show that the inhibition is still reversible (see Figure S2 in the Supporting Information). Thus, our data explains not only the high degree of specificity of peptidyl glyoxals with the proteasome relative to the active sites of other proteases, but also its reversible mode of action at atomic resolution. This reversible mode of action leads to temporary inhibition of the proteasome, and thus, to increased toxicity as apoptosis is not necessarily induced. In contrary, irreversible inhibition causes long-term proteasome inhibition in which proteasomal activity is regained upon resynthesis of the proteolytic machinery.

The impact of inhibitors on living cells and organisms crucially depends on the potency of the pharmacore in aqueous solution. Under these conditions, peptidyl glyoxals are hydrated,[17] and therefore, carry a much weaker functional reactive group compared to α′,β′-epoxyketone, β-lactone, boronic acid, and aldehyde groups, which leads to fewer side effects. Furthermore, reducing the number of side effects is promoted by the binding of **1** to solely the proteasome β5 subunit. It was shown that 25 % inhibition of this subunit, which harbors the chymotryptic activity, is sufficient to induce apoptosis in tumor cells; however, 80 % inhibition of the chymotryptic activity in normal cells, such as blood, liver, and spleen, is well tolerated.[23]

Previously, proteasome inhibitors have found application not only in the treatment of cancer by inducing apoptosis in fast growing tumor cells, but also as immunosuppressive agents such as the epoxomicin analogue PR-957 that demonstrates exquisite selectivity for the iβ5-immunoproteasome subunit.[24] After interferon-γ exposure, the iβ5-immunoproteasome is exchanged for the active β5 subunit in antigen presenting cells, thereby, altering the repertoire of proteasomal cleavage products and triggering immune response.[25] Thus, through side-chain modifications the selectivity of **1** could be extended to exclusively inhibit immunoproteasome subunits in a reversible manner. Thus, we expect that the inhibitor analyzed herein is a promising novel lead for the development of new anticancer or anti-inflammatory drugs.

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[1] N. Gallastegui, M. Groll, *Trends Biochem. Sci.* **2010**, *35*, 634 – 642.
[2] E. Genin, M. Reboud-Ravaux, J. Vidal, *Curr. Top. Med. Chem.* **2010**, *10*, 232 – 256.
[3] L. Borissenko, M. Groll, *Chem. Rev.* **2007**, *107*, 687 – 717.
[4] J. Löwe, D. Stock, B. Jap, P. Zwickl, W. Baumeister, R. Huber, *Science* **1995**, *268*, 533 – 539.
[5] M. Groll, L. Ditzel, J. Lowe, D. Stock, M. Bochtler, H. D. Bartunik, R. Huber, *Nature* **1997**, *386*, 463 – 471.
[6] J. Adams, R. Stein, *Annu. Rep. Med. Chem.* **1996**, *31*, 279 – 288.
[7] J. Adams, M. Behnke, S. Chen, A. A. Cruickshank, L. R. Dick, L. Grenier, J. M. Klunder, Y. T. Ma, L. Plamondon, R. L. Stein, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 333 – 338.
[8] P. G. Richardson et al., *N. Engl. J. Med.* **2005**, *352*, 2487 – 2498.
[9] A. A. Argyriou, G. Iconomou, H. P. Kalofonos, *Blood* **2008**, *112*, 1593 – 1599.
[10] S. D. Demo, C. J. Kirk, M. A. Aujay, T. J. Buchholz, M. Dajee, M. N. Ho, J. Jiang, G. J. Laidig, E. R. Lewis, F. Parlati, K. D. Shenk, M. S. Smyth, C. M. Sun, M. K. Vallone, T. M. Woo, C. J. Molineaux, M. K. Bennett, *Cancer Res.* **2007**, *67*, 6383 – 6391.
[11] R. H. Feling, G. O. Buchanan, T. J. Mincer, C. A. Kauffman, P. R. Jensen, W. Fenical, *Angew. Chem.* **2003**, *115*, 369 – 371; *Angew. Chem. Int. Ed.* **2003**, *42*, 355 – 357.
[12] M. Groll, R. Huber, B. C. Potts, *J. Am. Chem. Soc.* **2006**, *128*, 5136 – 5141.
[13] F. Parlati, S. J. Lee, M. Aujay, E. Suzuki, K. Levitsky, J. B. Lorens, D. R. Micklem, P. Ruurs, C. Sylvain, Y. Lu, K. D. Shenk, M. K. Bennett, *Blood* **2009**, *114*, 343947.
[14] M. Hanada, K. Sugawara, K. Kaneta, S. Toda, Y. Nishiyama, K. Tomita, H. Yamamoto, M. Konishi, T. Oki, *J. Antibiot.* **1992**, *45*, 1746 – 1752.
[15] L. Meng, R. Mohan, B. H. Kwok, M. Elofsson, N. Sin, C. M. Crews, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 10403 – 14408.
[16] M. Groll, K. B. Kim, N. Kairies, R. Huber, C. M. Crews, *J. Am. Chem. Soc.* **2000**, *122*, 1237 – 1238.
[17] J. F. Lynas, P. Harriott, A. Healy, M. A. McKervey, B. Walker, *Bioorg. Med. Chem. Lett.* **1998**, *8*, 373 – 378.
[18] L. J. Crawford, B. Walker, H. Ovaa, D. Chauhan, K. C. Anderson, T. C. Morris, A. E. Irvine, *Cancer Res.* **2006**, *66*, 6379 – 6386.
[19] M. Groll, R. Huber, L. Moroder, *J. Pept. Sci.* **2009**, *15*, 58 – 66.
[20] B. Walker, N. McCarthy, A. Healy, T. Ye, M. A. McKervey, *Biochem. J.* **1993**, *293*, 321 – 323.
[21] A. Djurdjevic-Pahl, C. Hewage, J. P. Malthouse, *Biochim. Biophys. Acta Proteins Proteomics* **2005**, *1749*, 33 – 41.
[22] E. Sanchez, M. Li, J. A. Steinberg, C. Wang, J. Shen, B. Bonavida, Z. W. Li, H. Chen, J. R. Berenson, *Br. J. Haematol.* **2010**, *148*, 569 – 581.
[23] S. Meiners, A. Ludwig, V. Stangl, K. Stangl, *Med. Res. Rev.* **2008**, *28*, 309 – 327.
[24] T. Muchamuel, M. Basler, M. A. Aujay, E. Suzuki, K. W. Kalim, C. Lauer, C. Sylvain, E. R. Ring, J. Shields, J. Jiang, P. Shwonek, F. Parlati, S. D. Demo, M. K. Bennett, C. J. Kirk, M. Groettrup, *Nat. Med.* **2009**, *15*, 781 – 787.
[25] L. Borissenko, M. Groll, *Biol. Chem.* **2007**, *388*, 947 – 955.

