Proteasome Inhibition

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Systematic Comparison of Peptidic Proteasome Inhibitors Highlights the α-Ketoamide Electrophile as an Auspicious Reversible Lead Motif**

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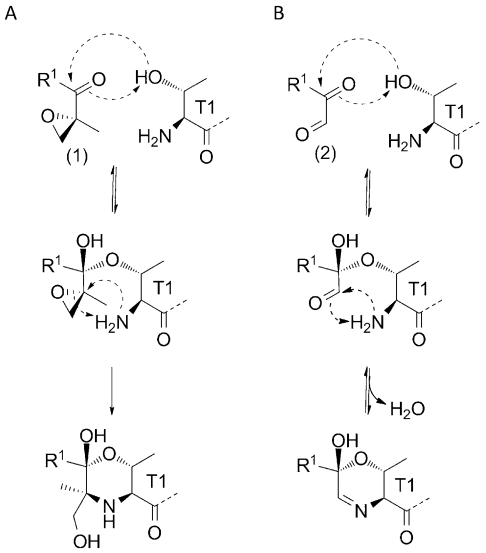
Abstract: *The ubiquitin–proteasome system (UPS) has been successfully targeted by both academia and the pharmaceutical industry for oncological and immunological applications. Typical proteasome inhibitors are based on a peptidic backbone endowed with an electrophilic C-terminus by which they react with the active proteolytic sites. Although the peptide moiety has attracted much attention in terms of subunit selectivity, the target specificity and biological stability of the compounds are largely determined by the reactive warheads. In this study, we have carried out a systematic investigation of described electrophiles by a combination of in vitro, in vivo, and structural methods in order to disclose the implications of altered functionality and chemical reactivity. Thereby, we were able to introduce and characterize the class of α-ketoamides as the most potent reversible inhibitors with possible applications for the therapy of solid tumors as well as autoimmune disorders.*

The FDA approval of the second-generation proteasome inhibitor carfilzomib (Kyprolis) for multiple myeloma and mantle cell lymphoma in 2012 once again confirmed the 20S proteasome (core particle; CP) of the nonlysosomal protein degradation system as a smart alternative for addressing oncological diseases.[1] Moreover, the development of the antiinflammatory CP inhibitor PR-957 has broken the ground for the treatment of autoimmune disorders through the modulation of the proteasomal signaling function.[2] A feature shared by carfilzomib and PR-957 is the electrophilic α′,β′-epoxyketone warhead derived from their natural product precursor, epoxomicin.[3] This lead motif, which has prevailed over the highly reactive boronic acid warhead of the first clinically applied inhibitor bortezomib (Velcade),[4] exploits the bivalent character of the catalytically active Thr1 within the proteasomal substrate binding channels (Scheme 1).[5] Its capability to thereby discriminate against other hydrolytically active enzymes such as serine-, cysteine-, aspartate-, and metalloproteases can be correlated to the vastly reduced record of side effects described in clinical studies of carfilzomib.[6] However, this second generation of proteasome inhibitors still retains an irreversible mode of action related to the reactivity of bortezomib,[5] thereby arguably preventing penetration of solid tissue and limiting their applicability to liquid tumors.[7] Therefore, we tried to adapt the bivalent lead motif of epoxyketones to a reversible mode of action by introducing the class of α-ketoaldehydes[8] (Scheme 1). Unexpectedly, however, recent experiments have demonstrated that α-ketoaldehydes exhibit a considerably impaired inhibitory potential and decreased cytotoxicity against cancer cells, thus disqualifying this class of CP inhibitors from future clinical trials.



Scheme 1. Reaction mechanisms of α′,β′-epoxyketones (A) and α-ketoaldehydes (B) with the catalytically active Thr1.

In order to understand this drop in activity, we performed a fundamental and systematic analysis of the biochemical implications of different C-terminal electrophiles in vitro and in vivo. To achieve this, the Z-Leu-Leu-Leu backbone of the thoroughly characterized proteasome aldehyde inhibitor MG132[9] (**1**) was linked to different electrophilic headgroups:

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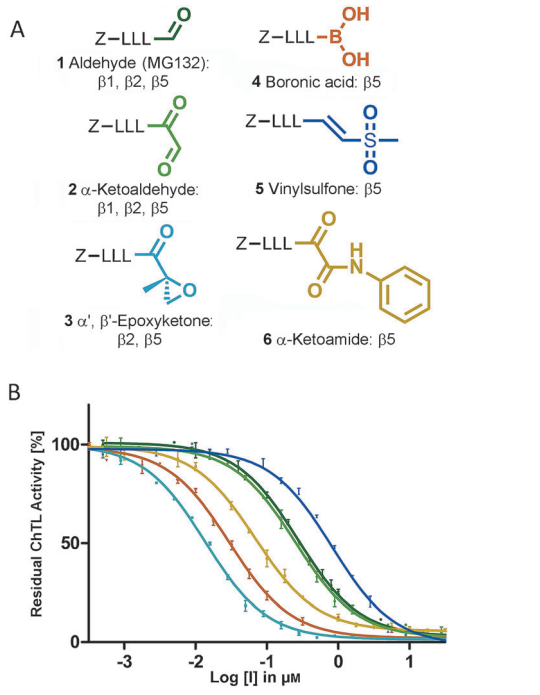

Figure 1. Analysis of head groups by in vitro experiments. A) Electrophilic warheads were coupled to a Z-Leu-Leu-Leu backbone, which led to different subuntit selectivities. B) IC_{50} measurements of the ChTL activity of the proteasome after addition of a dilution series of the proteasome inhibitors. The highly reactive epoxyketone **3** and the boronic acid **4** are the strongest inhibiting compounds, whereas the ketoamide **6** is the most potent reversible inhibitor, hereby surpassing reactive aldehydes, which are both of similar, intermediate potencies.

an α-ketoaldehyde (**2**), an α′,β′-epoxyketone (**3**), a boronic acid (**4**), and a vinylsulfone (**5**) (Figure 1 A). Apart from the commercially available compound **5**, all inhibitors were synthesized by a combination of standard peptide chemistry and distinct synthesis routes to prepare the electrophilic warhead. In the case of **3** and **4**, the functional moieties were produced separately and fused to the peptide scaffold in a convergent synthesis.[10] The aldehyde compound **1**, in contrast, was constructed on a previously generated leucinol C-terminus by oxidation using 2-iodoxybenzoic acid (IBX).[11] Compound **2** was produced from **1** by a Grignard reaction with iodoform followed by an oxidation of the resulting β-diiodoalcohol, which was subsequently hydrolyzed to yield the ketoaldehyde[12] (see the Supporting Information).

After the procurement of all the CP inhibitors, we performed IC_{50} measurements of the most affected chymotrypsin-like activity (ChTL), which is conferred by the catalytically active Thr1 nucleophile within the β5 subunit (Figure 1 B).[13] All substances inhibited the ChTL hydrolytic site with the strongest activities displayed by **3** (15 nM) and **4** (27 nM), whereas the rather unreactive vinylsulfone **5** marked the lower end of the spectrum with an IC_{50} value of 780 nM. Surprisingly, the aldehydes **1** and **2** displayed almost identical inhibitory strengths of 270 and 244 nM, respectively. Despite the entropically and enthalpically favorable contribution from the Schiff base condensation reaction, the bivalency does not endow **2** with a higher inhibitory potential. Hence, we wondered whether the reactivity of the terminal aldehyde moiety might be diminished by hydration effects, thereby considerably decreasing its electrophilicity, and altered the ketoaldehyde to an α-ketoamide (**6**, Figure 1 A).[14] We coupled **1** with phenylisocyanide and the resulting α-hydroxyamide was oxidized using IBX[15] (see the Supporting Information). Compound **6** contains an extended planar π-electron system stretching from the phenyl ring through the amide bond to the α-keto moiety; it conserves the bivalent reactivity and reversibility of **2**, but with minimal hydration of both carbonyl units (see the Supporting Information). Moreover, α-ketoamides provide the opportunity to also partially exploit the primed site of the proteasomal substrate binding channel by protruding with their C-terminal moiety towards the S1′ specificity pocket. To date, all applied peptidic CP modulators exclusively bind to the nonprimed site of the proteasomal binding channel.[16] Hence, the endoproteolytic character of the proteasome has originated the conception that compounds occupying both the nonprimed and primed substrate binding channel display enhanced selectivity and inhibitory potential. Intriguingly, the binding strength of **6** was significantly improved to as low as 70 nM and is only exceeded by the irreversible inhibitors **3** and **4.**[17]

Encouraged by these results, we set out to elucidate the molecular reasons for the vastly differing activities of the CP inhibitors and to analyze the binding profile to the Thr1O$^{\gamma}$ at the atomic level. For systematic comparison the peptidic scaffold of all compounds was kept identical, thus allowing us to solely analyze the isolated influence of the respective electrophilic head moieties. We therefore determined yeast CP:inhibitor complex structures of all compounds with final resolutions better than 3.0 Å and final R_{free} values below 25 % (Figure 2, Tables ST1 and ST2). All yeast CP crystals were soaked in a 2 mM solution of the respective inhibitor for 24 h. Data sets were recorded at the synchrotron facility of the SLS (Paul-Scherrer-Institut, Villingen, Switzerland) and data processing as well as structural refinement were performed as described previously.[18]

Inspection of the respective electron density maps illustrates that each ligand occupies at least the most favored substrate binding channel of the β5 subunit by forming identical antiparallel β-sheet structures. Interestingly, the highly reactive boronic acid **4** and the least potent vinylsulfone **5** bind quite specifically to the ChTL substrate binding channel, hence, contradicting hypotheses that a selective binding mode is determined by the reactivity of the electrophile. In contrast, the carbonyl compounds **1** and **2** are found in all active sites, whereas the epoxyketone **3** omits β1 and the ketoamide **6** displays a binding preference for the β5 subunit. These different specificities came as a surprise, as usually only the chemical nature of the amino acid backbone of the inhibitor is thought to generate subunit selectivity. It has been shown, however, that the blockage of several subunits generates a higher level of cytotoxicity against cancer cells, which can be exploited in oncological treatments. In contrast, a more subunit-specific mode of binding is desirable for the attenuation of the immunological branch of proteasomal signaling.

We thus analyzed the different binding mechanisms of the compounds to the CP. As expected, the boronic acid **4** and the

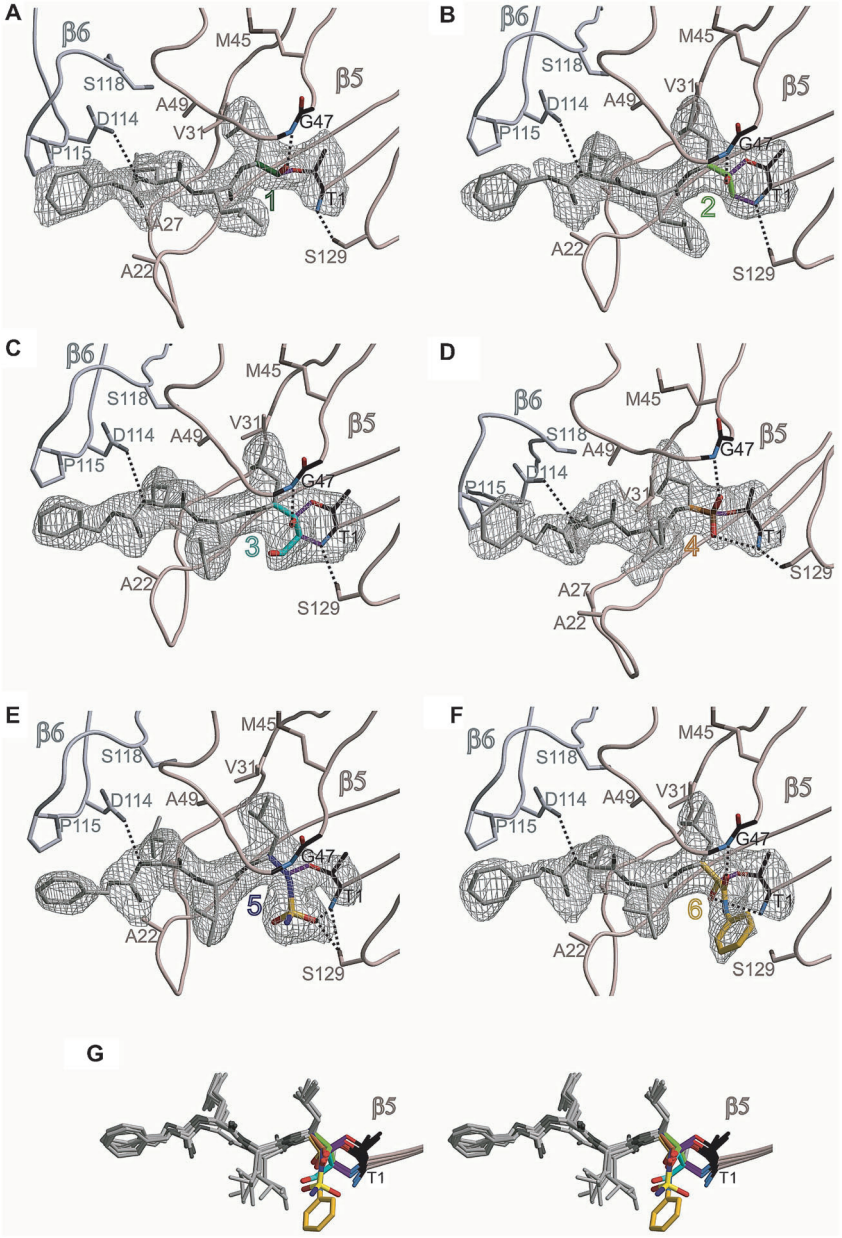

Figure 2. Crystallographic binding analysis of the electrophilic traps. A–F) The $2F_o-F_c$ omit electron density maps (gray mesh, 1σ) of the respective inhibitors bound in the β5 active site. Hydrogen bonds are indicated by dashed lines with amino acid numbering according to Löwe et al.[19] Stereoviews are depicted in Figure S2 in the Supporting Information. G) Structural superposition of all inhibitors demonstrates that the ligands intensely interact with the S1 and S3 sites, whereas the P2 side chains are solvent-exposed and thus adopt a random orientation. The coordinates and structure factors are available under the accession codes specified in Tables ST1 and ST2.

vinylsulfone **5** bind to the Thr1O$^{\gamma}$ in an irreversible single-step reaction, yielding the tetrahedral borate adduct and the ether, respectively (Figure 2 A,E). The carbonyl compounds initially form a hemiacetal or hemiketal with the Thr1O$^{\gamma}$ moiety (Figure 2 A,B,F and the Supporting Information). Both aldehydes **1** and **2** are attacked on their "*re*" face, thereby producing a hydroxy moiety that occupies the oxyanion hole formed by Gly47N.[19] Subsequent to this initial docking process, the bivalent ketoaldehyde **2** reacts—in a way that is similar, yet reversible, to the mechanism of epoxyketone **3**—through its second electrophilic carbon with the primary and N-terminal amine. This is followed by a condensation step forming a 3,6-dihydro-2*H*-1,4-oxazine ring system (Figure 2 C, Supporting Information). Although the ketoamide **6** undergoes the same coupling reaction as the aldehydes **1** and **2**, it is attacked by Thr1O$^{\gamma}$ on its "*si*" side, thus enabling a hydrogen bond between its hemiketal and the Thr1N (Figure 2 F). Unexpectedly, **6** forms only one covalent bond through its ketone moiety and occupies the oxyanion hole with its terminal amide carbonyl, thereby pointing its phenyl amide terminus into the primed side of the proteasomal substrate binding channel; this has not been observed for peptide inhibitors so far. The delocalized electron system of the *N*-phenyl-α-ketoamide moiety then creates a rigid and planar scaffold, thus providing a considerable entropic effect as well as an enthalpic stabilization by van der Waals interactions with hydrophobic patches of Ser129 and Tyr168 of subunit β5. The obtained structural results, combined with the IC_{50} measurements, illustrate that an energetically favorable bivalent binding mechanism, whose benefits are partially annihilated by hydration effects in the case of **2**, can be easily recouped by additional interactions within the primed site of the CP binding channel.

Next, our goal was to correlate these in vitro findings with in vivo effects. We therefore compared the different CP inhibitors in HeLa cell culture assays and recorded LD_{50} curves by using a standard Alamar Blue viability assay[20] following 48 h of incubation with solutions having various concentrations of the inhibitors (Figure 3 A). The ketoaldehyde **2**[21] and the vinylsulfone **5** only showed neglegible effects in these cell assays, which is in agreement with the in vitro results.

In contrast, the boronic acid **4** had the strongest impact with an LD_{50} value of 21 nM, which, however, might be caused by the well-established cross reactivity of the electrophile.[6a] Remarkably, the ketoamide **6** displayed strong toxicity (254 nM), thereby surpassing even the epoxyketone **3** (730 nM). The latter is presumably depleted by the fast hydrolysis rate of the epoxide at 37 °C in aqueous solution[22] and is now even matched by the aldehyde **1** (787 nM). This finding is also consistent with the cytotoxic properties of the FDA-approved CP inhibitors bortezomib (**7**) and carfilzomib (**8**) (structures in Figure S3),

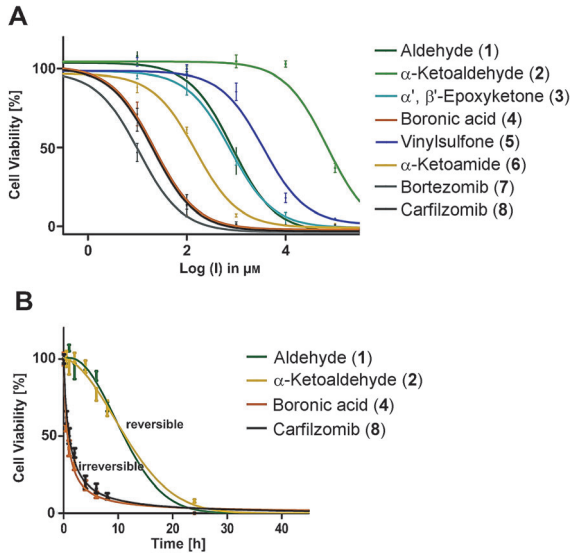

Figure 3. Cell culture experiments. A) LD_{50} curves of HeLa cells for the different compounds evaluated by Alamar Blue assays. B) Pulse chase experiments with the LD_{90} concentrations of the respective inhibitors at varying incubation periods and subsequent wash-out steps. There is an initial plateau phase for reversible inhibitors, as they require more time to take effect than the irreversible inhibitors that cannot be removed by dilution.

although their respective peptide backbones differ considerably, thereby preventing the direct comparative analysis of their electrophiles. Eventually, we characterized the kinetic behavior of the compounds by performing pulse chase experiments (Figure 3B). Therefore, HeLa cells were incubated with solutions of the inhibitors at the LD_{90} concentrations that had been measured after 48 h of incubation. The inhibitors were removed in this setup after different time intervals by a washing step and exchange of the cell culture medium. Subsequently, cells were incubated to a final incubation time of 48 h, after which their viability was again evaluated by Alamar Blue assays. Interestingly, the activity of the irreversible CP blockers **3** and **4** is characterized by an exponential degeneration of the cells, thereby suggesting that they are trapped in the cytoplasm and cannot be washed out again by dilution with fresh medium. Conversely, the initial plateau of the aldehyde **1** and the ketoamide **6** lasted for approximately 6 h due to their reversible mode of binding, thereby allowing the cells to recuperate unless they were sufficiently damaged. Hence, these compounds may be able to penetrate deeper into solid tissue, thereby making especially peptidic ketoamides promising candidates for therapies of a wider range of tumor subtypes than those targeted by bortezomib and carfilzomib.

The comparative and systematic approach that was followed in this study has, for the first time, generated a deeper understanding of the contribution of the electrophilic warheads to the in vitro and in vivo potency of applied proteasome inhibitors. Moreover, it led us to identify a promising reversible lead motif based on the ketoamide functional group, which compensates for its lack of a second strong electrophile by exploiting the primed site of the proteasomal binding channel. We conclude that the bivalency of an electrophilic trap is not necessarily required for high binding affinities and can be outbalanced by additional noncovalent interactions, which are also able to generate a similar target specificity and selectivity for malignant tumor cells. Therefore, the α-ketoamides feature an additional motif, which induces novel selectivity profiles. These properties may be exploited to expand the utility of proteasome inhibitors in chemo- and immunosuppressive therapies.

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