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Crystal Structure of Epoxomicin:20S Proteasome Reveals a Molecular Basis for Selectivity of α′,β′-Epoxyketone Proteasome Inhibitors

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In recent years, proteasome inhibitor development has received considerable interest given the critical role of the proteasome in intracellular processes such as cell cycle progression, antigen presentation, and cytokine-stimulated signal transduction.[1] We have recently shown that the α′,β′-epoxyketone peptide natural product epoxomicin potently and irreversibly inhibits the catalytic activity of the 20S proteasome.[2,3] Unlike many proteasome inhibitors, however, epoxomicin is specific for the proteasome and does not inhibit other proteases such as calpain, papain, cathepsin B, chymotrypsin, and trypsin.[3] Herein, we present the crystal structure of the epoxomicin:*S. cerevisiae* 20S proteasome complex at 2.25 Å resolution. This structure revealed an unexpected morpholino ring formation between the amino terminal threonine and the pharmacophore of epoxomicin, providing the first insights into the unique specificity of epoxomicin.

Previous biochemical and structural studies of the 20S proteasome have demonstrated that this high molecular weight proteolytic complex is composed of 28 subunits forming four stacked rings.[4−6] The two central rings each contain three catalytically active subunits, which possess an amino terminal nucleophilic threonine residue[5−7] and are thus members of the N-terminal nucleophile (Ntn) hydrolase family.[8] A number of small molecule inhibitors of the 20S proteasome have been developed for use as molecular probes of proteasome function and potential therapeutics; however, many lack specificity for the proteasome, thus compromising their utility.[9] Interestingly, we found that epoxomicin does not inhibit several nonproteasomal proteases that are targeted by other proteasome inhibitors.[3]

To address the unique specificity of epoxomicin for the proteasome, we cocrystallized this α′,β′-epoxyketone-containing natural product with the yeast *S. cerevisiae* 20S proteasome. A single proteasome crystal was soaked for 45 min with epoxomicin at a final concentration of 5 mM[10] and the structure of the *S. cerevisiae* 20S proteasome[6] was used to model the structure of the resulting cocrystal.[11] While epoxomicin displays a high degree of selectivity for inhibition of the chymotrypsin-like activity of the 20S proteasome at lower concentrations,[2] at the higher concentration used to obtain the cocrystal, structural analysis of the complex showed the tetrapeptide inhibitor covalently bound to the substrate binding pocket of all six catalytic subunits. For the sake of brevity, we present here only the epoxomicin adduct formed with the β5/Pre2 subunit, which is responsible for the chymotrypsin-like activity of the yeast 20S proteasome.

A crystal structure of the yeast 20S proteasome complexed with acetyl-Leu-Leu-norleucinal has previously been reported.[6] A comparison of that structure with the epoxomicin:20S proteasome structure communicated here reveals that both the peptide aldehyde and epoxomicin bind similarly to the catalytic subunits, completing an antiparallel β-sheet.[6] However, a striking difference is the covalent adduct formed by each inhibitor with the amino terminal threonine (Thr 1). Whereas the peptide aldehyde is attacked by the threonyl Oγ to form a hemiacetal, a well-defined electron density map of the β5/Pre2 subunit complexed with epoxomicin reveals the presence of a unique 6-atom ring (Figure 1). This morpholino derivative results from adduct formation between the α′,β′-epoxyketone pharmacophore of epoxomicin and the amino terminal threonyl Oγ and N of the β5 subunit (Figure 2). Formation of an irreversible morpholino adduct upon epoxomicin addition is consistent with the observed kinetic profile of the epoxomicin:20S proteasome interaction.[2,3]

The morpholino derivative formation is most likely a two-step process (Figure 2). First, activation of the threonyl Oγ is believed to occur by its N-terminal amino group directly[12] or via a neighboring water molecule acting as a base. The structures of the 20S proteasome alone[6] and of a related Ntn hydrolase, penicillin acylase,[13] both have a water molecule positioned to bridge the nucleophilic oxygen of the side chain and the α-amino nitrogen. It has been postulated that this water facilitates the proton transfer from the Oγ to the neutral α-amino group for the PGA catalytic mechanism.[13] Subsequent nucleophilic attack of Thr 1Oγ on the carbonyl of the epoxyketone pharmacophore would produce a hemiacetal (Figure 2) as is observed in the structure of the 20S proteasome:peptide aldehyde inhibitor complex.

The formation of the hemiacetal facilitates the second step in the formation of the morpholino adduct. In this intramolecular cyclization, the Thr 1 N opens the epoxide ring via an intramolecular displacement with consequent inversion of the C2 carbon. Activation of the epoxide may be facilitated by hydrogen bond formation between the N5 hydrogen and the oxygen of the epoxide. In addition, Ser129 of the catalytic proteasome subunit is positioned near Thr1 N and may contribute to the nucleophilic activation of Thr1 N. It is also worth noting that the nucleophilic attack by the Thr 1N occurs at C2 and not at the neighboring, less hindered C1 methylene of the epoxide. Thus, the resulting morpholino adduction formation is a 6 *Exo-Tet* ring closure, which

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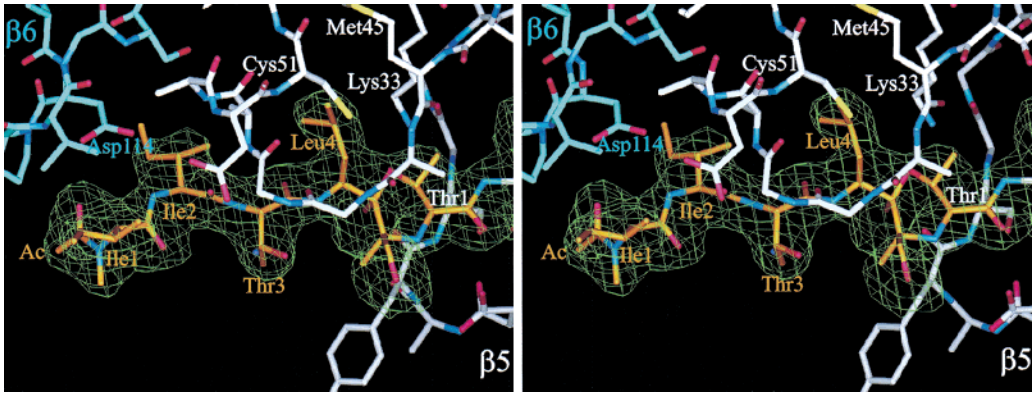

Figure 1. (a) Stereoview of the electron density map of the epoxomicin adduct at β5. The electron density was calculated with phases from the free enzyme structure and 10-fold averaged. Apart from the bound inhibitor molecule, no other structural changes were noted. Temperature factor refinement indicates full occupancy of all three inhibitor binding sites at β1, β2, and β5. Epoxomicin is covalently bound to Thr1 and the extended substrate binding site is composed of the β5 and β6 subunits.

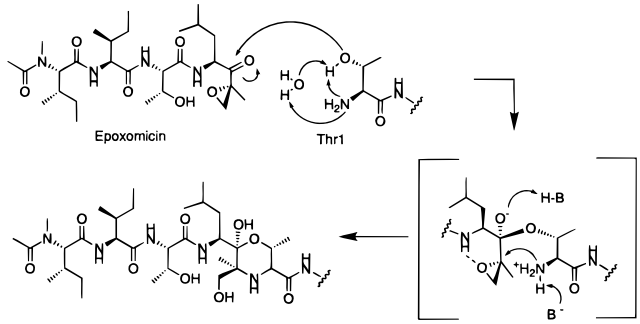

Figure 2. Scheme of the proposed morpholino derivative adduct formation mechanism. Binding of epoxomicin to the 20S proteasome results in formation of a morpholino adduct between the epoxyketone pharmacophore and the active site amino terminal Thr 1 of the β5 subunit. Nucleophilic attack by Thr1 Oγ on epoxomicin results in hemiacetal formation followed by subsequent cyclization of Thr 1 N onto the epoxide resulting in an inversion of C2 and formation of the morpholino adduct. Candidate residues for H−B and B$^-$ are the Thr1 aminoterminus, a bound water molecule, and invariant Ser129.

is favored according to Baldwin's rules[14] unlike the 7 *Endo-Tet* ring closure which would result from attack at the less hindered C1 epoxy methylene. Support for the presence of a morpholino adduct also comes from mass spectrometric analysis, which was performed after HPLC separation of the epoxomicin-bound catalytic subunits under acidic conditions where the hemiacetal bond of the morpholino ring is opened. The observed masses of these subunits confirmed the irreversible covalent adduction formation with epoxomicin (e.g., obsd 23856 and calcd 23855 for the epoxomicin:β5 subunit).

A major significance of the morpholino adduct that results from epoxomicin binding to the 20S proteasome is that it provides the structural basis for epoxomicin's unique specificity for the proteasome. Since other proteases, which are common targets for many proteasome inhibitors (e.g., peptide aldehydes, vinyl sulfones, and boronic acids), do not have an amino terminal nucleophilic residue as part of their active sites, epoxomicin cannot form the same morpholino adduct with these proteases as it does with the 20S proteasome. An exception, of course, may be the members of the Ntn family of hydrolases, since they also possess an amino terminal amino acid with a nucleophilic side chain. It remains to be tested whether epoxyketones can act as a general pharmacophore for this small hydrolase family.

An interesting observation was made when the proteasome inhibitory activities of the two C2 epimers of epoxomicin were compared. We have previously shown that while the naturally occurring *(R)* C-2 isomer potently inhibits proteasome activity, the 2(*S*) C-2 epimer is more than 100-fold less potent.[3] Spaltenstein et al.[15] also reported the same selectivity of 2*(R)* vs 2*(S)* with another peptide epoxyketone proteasome inhibitor. Structural analysis of the 2(*S*) C-2 epoxomicin epimer complexed with the 20S proteasome may shed light on this conundrum. Interestingly, Roush and colleagues have reported a 2*(S)* peptide epoxyketone inhibitor of the *T. cruzei* protease Cruzain[16] that is substantially more active than its 2*(R)* epimer against this cysteine protease. This finding is in agreement with other 2*(S)* epoxyketone containing natural products that have also been identified as cysteine protease inhibitors.[17,18] The structural determinants underlying this specificity found in certain cysteine proteases for 2*(S)* epoxyketone peptide inhibitors remain unknown and are likely different from the mechanism presented here for the epoxomicin inhibition of the proteasome.

In summary, the structure of *S. cerevisiae* 20S proteasome complexed with epoxomicin provides a framework to understand the intriguing selectivity of the α',β'-epoxyketone peptide class of proteasome inhibitors. The observed selectivity of epoxomicin for the proteasome is rationalized by the requirement for both an N-terminal amino group and side chain nucleophile for adduct formation with the epoxyketone pharmacophore. Given that proteasome inhibition is currently being evaluated for a variety of therapeutic purposes,[19,20] the need for potent and selective small molecule proteasome inhibitors is well recognized. Current efforts are focused on the synthesis of additional peptide epoxyketone proteasome inhibitors that display specificity for each of the three proteolytic activities of the proteasome.[21,22]

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Supporting Information Available: Crystallization and data collection details (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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