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# PROTEASOME INHIBITION BY THE NATURAL PRODUCTS EPOXOMICIN AND DIHYDROEPONEMYCIN: INSIGHTS INTO SPECIFICITY AND POTENCY

Kyung Bo Kim,[a] Jayhyuk Myung,[a] Ny Sin,[a] and Craig M. Crews[a,b,*]

*Departments of Molecular, Cellular, and Developmental Biology[a] and Pharmacology[b]*
*Yale University, New Haven, CT 06520-8103*

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**Abstract**: While two structurally related epoxyketone-containing antitumor natural products, epoxomicin and eponemycin, share the proteasome as a common intracellular target, they differ in their antiproliferative activity, proteasome subunit binding specificity, and rates of proteasome inhibition. As a first step towards understanding such differences and developing novel proteasome subunit-specific inhibitors, we report here the synthesis and characterization of epoxomicin/dihydroeponemycin chimerae. 

Intracellular protein degradation is a highly regulated process in which polypeptides are first targeted for degradation by conjugation to ubiquitin, a 76 amino acid polypeptide. Ubiquitinated proteins are, in turn, recognized by the 19S regulatory domain of the 26S proteasome. Through a series of ATP hydrolysis-dependent processes, targeted proteins are deubiquitinated and threaded into the core proteolytic complex, the 20S proteasome, where they are proteolyzed.[1] The ubiquitin-proteasome pathway has been implicated in a number of intracellular processes, such as antigen processing and cell cycle regulation.[1-3]

The proteasome is a member of the growing N-terminal nucleophile (Ntn) hydrolase family, whose amino-terminal side chains act as nucleophiles and free amino groups as proton acceptors.[4] Through a series of mutation studies[5-7] and proteasome-inhibitor complex structural studies,[8-11] the hydroxyl side chains of N-terminal threonines of the proteasome's three catalytic β subunit have been confirmed to serve as nucleophiles for hydrolysis. Despite our general understanding of the catalytic subunits of the 20S proteasome, the structural complexity and the multicatalytic nature of the proteasome pose a challenge to a clearer understanding of cleavage site selection by proteasome and to subunit- specific inhibitor design.

In recent years, proteasome inhibitors have been increasingly used to help define the role of the proteasome in cell biology.[12] These inhibitors include the natural product lactacystin and small cell membrane-permeable peptides with aldehyde, vinyl sulfone, boronic acid, and glyoxal functional groups.[12] While probing the modes of action of the potent antitumor compounds epoxomicin (**1**) and dihydroeponemycin (**2**), we identified the proteasome as their target using biotinylated affinity analogs of these compounds.[13-15]

Of particular interest was the finding that, despite structural similarities, epoxomicin (**1**) and dihydroeponemycin (**2**) differ in their antiproliferative activity, proteasome subunit binding specificity, and rates of proteasome inhibition. These distinct inhibitory modes of action by epoxomicin and dihydroeponemycin have led us to speculate that the structural differences between the two compounds may direct their subunit binding specificity and proteasome inhibition kinetics. Since a correlation between the structural and biochemical differences of epoxomicin and dihydroeponemycin may serve as a model system for understanding proteasome inhibitor specificity, we synthesized and characterized chimeric epoxomicin/dihydroeponemycin proteasome inhibitors in biological and biochemical assays.

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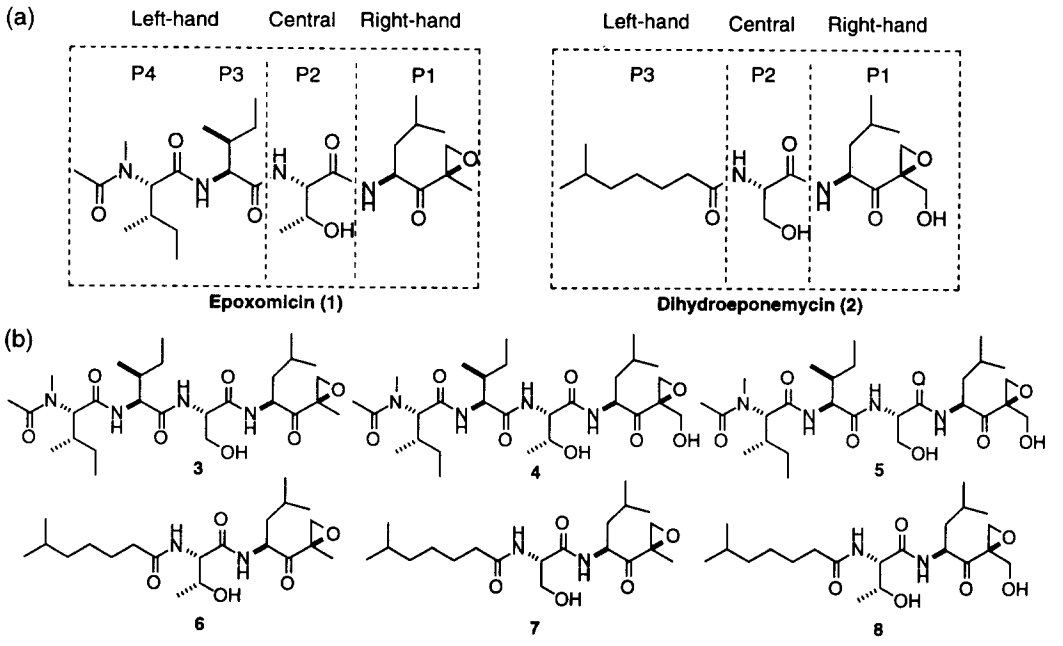


**Figure 1.** (a) Structural comparison of epoxomicin and dihydroeponemycin. (b) Structures of epoxomicin and dihydroeponemycin chimerae.

## RESULTS AND DISCUSSION

Although both epoxomicin (**1**) and dihydroeponemycin (**2**) share an α′,β′-epoxyketone pharmacophore as well as a linear peptide backbone, they possess some different structural features in their left-, central, and right-hand fragments (Figure 1a). Reasoning that these differences contribute to the unique proteasome subunit binding specificity, inhibition kinetics, and antiproliferative activities of each compound, we took a combinatorial approach to investigate the structure-activity relationship of these compounds. We first synthesized chimeric derivatives comprising the left-hand, central, and right-hand fragments of epoxomicin (**1**) and dihydroeponemycin (**2**) (Figure 1b) following our previously reported synthesis of these compounds.[15,16] The syntheses and biochemical studies of epoxomicin/dihydroeponemycin chimerae revealed important structural features of these compounds that relate to their specific subunit labeling and proteasome inhibitory activity.

***20S Proteasome Peptidase Inhibition.*** In general, as shown in Table 1, when the amino terminal left-hand *N*-acetyl-*N*-methyl-Ile-Ile fragments (**3–5**) were replaced with an isooctanoic acid moiety, the resulting *N*-isooctanoic dipeptide chimerae (**6–8**) displayed 300-500 fold decrease in $k_{obs}/[I]$ values for inhibition of the chymotrypsin-like activity. In contrast, exchange of the center or the right-hand fragments of epoxomicin (**1**) and dihydroeponemycin (**2**) had no significant effects. These results suggested that the left-hand fragment of chimeric inhibitors plays a major role in influencing the potency for inhibition of the chymotrypsin-like activity inhibition.

Given the size of the isooctanoic acid and its position at P3 of the dipeptide chimerae (**6–8**), the greatly decreased chymotrypsin-like inhibition of these chimerae relative to the tetrapeptide chimerae (**3–5**) can be attributed to decreased peptide backbone length, i.e., P4-S4 interactions. This is also supported by studies using peptide aldehyde,[17,18] vinyl sulfone,[19] and glyoxal[20] proteasome inhibitors of different lengths. In these previous

studies, tetrapeptide inhibitors invariably display greater inhibition towards the chymotrypsin-like activity compared to their truncated tripeptide counterparts.

**Table 1**. Kinetic data of chimerae for the three major proteolytic activities.[a]

| Compounds | $k_{obs}/[I]$ ($M^{-1}s^{-1}$) | | |
|---|---|---|---|
| | Chymotrypsin -like Activity | PGPH Activity | Trypsin-like Activity |
| **Epoxomicin (1)** | 20,000 ± 3,000 (0.037–0.1 μM) | 43 ± 10 (25–75 μM) | 310 ± 70 (0.25–2.5 μM) |
| **3** | 32,000 ± 3,000 (0.037–0.1 μM) | 90 ± 3 (25–50 μM) | 1100 ± 310 (0.25–1 μM) |
| **4** | 18,000 ± 1,000 (0.037–0.1 μM) | 37 ± 9 (25–75 μM) | 290 ± 50 (0.5–2.5 μM) |
| **5** | 17,000 ± 1,000 (0.037–0.1 μM) | 35 ± 6 (25–75 μM) | 470 ± 80 (0.25–1 μM) |
| **Dihydroeponemycin (2)** | 58 ± 14 (25–62.5 μM) | 175 ± 2 (15.6–62.5 μM) | 17 ± 3 (93.8–312.5 μM) |
| **6** | 25 ± 5 (20–60 μM) | 45 ± 14 (12.5–50 μM) | 4 ± 1 (125–250 μM) |
| **7** | 34 ± 6 (20–60 μM) | 57 ± 3 (12.5–50 μM) | 5 ± 1 (125–250 μM) |
| **8** | 22 ± 3 (20–60 μM) | 50 ± 5 (12.5–50 μM) | 5 ± 1 (125–250 μM) |

[a]Kinetic assays and analyses were performed as previously described.[13]

Similarly, inhibition of the trypsin-like activity is also strongly influenced by the P4-S4 interactions (Table 1). Replacement of the amino terminal left-hand fragment of epoxomicin with isooctanoic acid resulted in a greater than 75-fold decrease in $k_{obs}/[I]$ values for inhibition of the trypsin-like activity (**1** vs **6**). This is consistent with the potency for inhibition by other chimerae possessing the amino terminal *N*-acetyl-*N*-methyl-Ile-Ile, such as **4** vs **8** and **3** vs **7** (Table 1). In contrast to the left-hand fragment, the central and right-hand fragments influenced inhibition of the trypsin-like activity to a much lesser extent. These suggested that there is a minimal length of peptide backbone for the trypsin-like activity inhibition. Unlike inhibition of the chymotrypsin-like and trypsin-like activities, replacement of the amino terminal left-hand *N*-acetyl-*N*-methyl-Ile-Ile fragment with isooctanoic acid had little effect on the $k_{obs}/[I]$ values for the PGPH activity (Table 1). This is also consistent with the replacements made in all chimerae studied here. These results suggest that the subunit responsible for the PGPH activity possesses no strong preference for peptide inhibitor length.

***Proteasome Subunit Binding Specificity.*** To characterize further the structural features of epoxomicin and dihydroeponemycin that are responsible for proteasome-inhibitor recognition, we next studied the subunit binding specificity of various biotinylated chimeric compounds. Proteasome subunit binding patterns were monitored by *in vitro* labeling of murine 20S proteasome partially purified from EL4 lymphoma cells with biotinylated chimerae (Figure 2a).

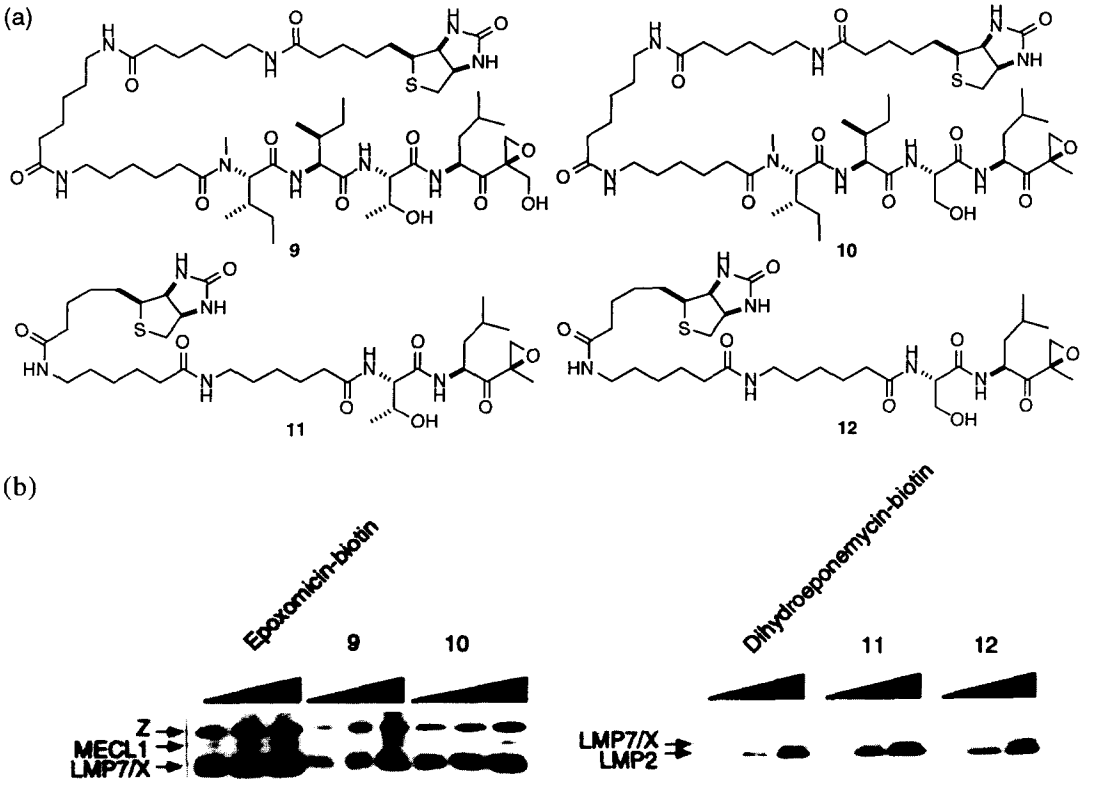


**Figure 2.** (a) Structure of biotinylated epoxomicin/dihydroeponemycin chimerae. (b) Proteasome subunit binding specificity using biotinylated chimerae. Partially purified 20S proteasome from the murine EL4 lymphoid cell line was incubated for 90 minutes with various concentrations of biotinylated chimerae before SDS-PAGE, protein immobilization onto PVDF membrane. Biotinylated proteins were detected with avidin-HRP and enhanced chemiluminescence (ECL).

Chimerae possessing left-hand *N*-acetyl-*N*-methyl-Ile-Ile fragment (**9** and **10**) displayed the same labeling pattern as biotinylated epoxomicin, which labels LMP7/X and MECL1/Z (Figure 2b). On the other hand, biotinylated chimerae lacking *N*-acetyl-*N*-methyl-Ile-Ile (**11**, **12**) display the same binding pattern as dihydroeponemycin-biotin, which binds LMP7/X and LMP2. In contrast, when the central and right-hand fragments of epoxomicin/dihydroeponemycin were also combinatorially exchanged, these changes were not found to influence the subunit binding specificity (Figure 2b). These results suggest that differences in binding specificity between epoxomicin and dihydroeponemycin can be mostly attributed to the differences at the amino terminal left-hand fragments, i.e., *N*-acetyl-*N*-methyl-Ile-Ile vs isooctanoic acid group.

***Antiproliferative Activity.*** In addition to *in vitro* kinetic and labeling studies, the chimerae were tested in cell growth assays to determine the structural motifs responsible for the differences in antiproliferative activities between epoxomicin and dihydroeponemycin. As shown in Table 2, replacement of the amino terminal left-hand fragment of epoxomicin (**1**) with isooctanoic acid (chimera **6**), resulted in the most significant change responsible for the differences in antiproliferative $IC_{50}$ values of epoxomicin (4 nM) and dihydroeponemycin (100 nM).

The importance of *N*-acetyl-*N*-methyl-Ile-Ile was also demonstrated by the ~65-fold increase in $IC_{50}$ values when chimera **3** was converted to **7**. Since antiproliferative activity is dependent on a myriad of variables, the nature of the preference for *N*-acetyl-*N*-methyl-Ile-Ile over isooctanoic acid at the P3 and P4 positions is not obvious. It is most likely due to a combination of membrane permeability, bioavailability, potency, and specificity of proteasome inhibition. It should be noted, however, that antiproliferative activities of the different chimeric inhibitors correlate with their inhibition of the chymotrypsin-like activity.

**Table 2.** Antiproliferative activity ($IC_{50}$) of chimerae.[a]

| Compounds | $IC_{50}$ (nM) |
|---|---|
| **Epoxomicin (1)** | 4 ± 1 |
| **3** | 4 ± 1 |
| **4** | 60 ± 10 |
| **5** | 140 ± 20 |
| **Dihydroeponemycin (2)** | 100 ± 10 |
| **6** | 520 ± 160 |
| **7** | 260 ± 50 |
| **8** | 850 ± 240 |

[a]Assays were performed as previously described.[13]

On the other hand, the introduction of a hydroxyl group in the epoxyketone pharmacophore contributed to a 15–35-fold increase in $IC_{50}$ values as shown by comparisons of **1** vs **4** and **3** vs **5** (Table 2). One possible explanation for this increase in $IC_{50}$ values is the decreased permeability of these chimeric inhibitors across the hydrophobic membrane bilayer. Interestingly, however, the simple removal of a hydroxyl group from the epoxyketone pharmacophore in the *N*-isooctanoic dipeptides, dihydroeponemycin **2** and chimera **7**, resulted in only a modest change in $IC_{50}$ values (**2** vs **7** and **8** vs **6**). This suggests that the long hydrophobic tail amino terminal left-hand fragment (i.e., isooctanoic acid) is the major factor determining membrane permeability of these chimerae and thus masks any decreased membrane permeability due to the presence of the hydroxyl group at the right-hand fragment.

In conclusion, this study has expanded our understanding of natural product inhibitor recognition by the 20S proteasome. We showed that the length of peptide inhibitors is important for maximal inhibition of the chymotrypsin-like and trypsin-like activities. The length of peptide inhibitors, however, was found not be important for inhibition of the PGPH activity. We also showed that the length of peptide inhibitors of dihydroeponemycin and epoxomicin determine their preferential LMP2 and LMP7 subunit labeling. Together with information obtained from previous studies, these results will facilitate the development of additional inhibitors with a high degree of subunit specificity.

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