Target Identification

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Omuralide and Vibralactone: Differences in the Proteasome-β-Lactone-γ-Lactam Binding Scaffold Alter Target Preferences**

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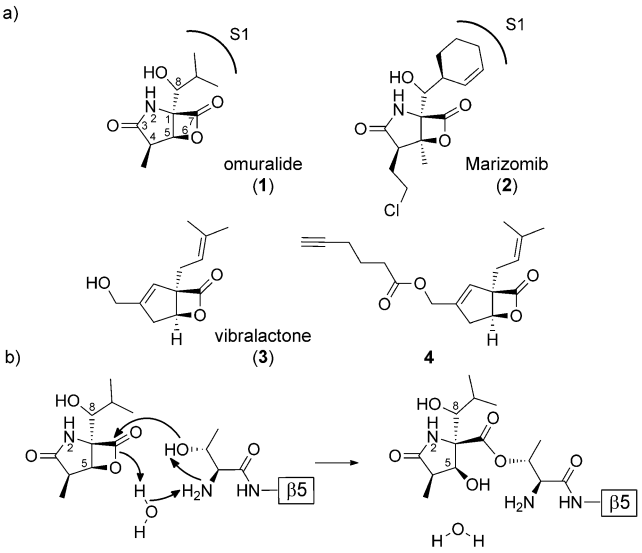
Abstract: *Despite their structural similarity, the natural products omuralide and vibralactone have different biological targets. While omuralide blocks the chymotryptic activity of the proteasome with an IC_{50} value of 47 nM, vibralactone does not have any effect at this protease up to a concentration of 1 mM. Activity-based protein profiling in HeLa cells revealed that the major targets of vibralactone are APT1 and APT2.*

The proteolytic key component of the ubiquitin-mediated degradation pathway, the 20S proteasome (core particle, CP), maintains biological homeostasis and regulates many crucial processes in the cell through the cleavage of most intracellular proteins.[1] The cylinder-shaped, multimeric architecture of the eukaryotic CP is assembled from four stacked heptameric rings, each consisting of either α- or β-type subunits, following an $\alpha_7\beta_7\beta_7\alpha_7$ stoichiometry.[2] The catalytic active sites are formed by N-terminal threonines (Thr1), which are located at subunits β1, β2, and β5 in the inner cavity of the barrel-like structure. The mechanism of peptide bond cleavage follows a universal principle[3] in which the singularity of each substrate-binding channel determines the chemical nature of the specificity (S) pockets and accommodates the ligand's side chains (P sites) with respect to their amino acid progression.[2a]

This complex hydrolytic machinery has received considerable attention from the scientific community, which in turn has led to the FDA approval of the dipeptide boronic acid, Velcade (bortezomib).[4] This blockbuster drug is used to inhibit the CP activity for the therapy of multiple-myeloma patients; however, as a result of its extremely reactive boronic acid pharmacophore, prolonged treatment is associated with toxicity and severe side effects.[5] Therefore, academic and pharmaceutical research groups have continued the search for improved inhibitors against the CP. As a result, three second-generation proteasome inhibitors, the epoxyketone ONX 0914 (PR-957), the β-lactone-γ-lactam Marizomib (salinosporamide A, NPI-0052 (**2**); Scheme 1 a), and the boronic acid delanzomib (CEP-18770; Figure S1)[6] have entered clinical trials, while the FDA-approved epoxyketone Kyprolis (PR-171, carfilzomib; Figure S1) is already available as a potential novel anticancer drug.[7]



Scheme 1. a) Chemical structures of β-lactones. b) Mode of action of β-lactones bound to the proteasomal active site.

To date, a plentitude of pathogens has been identified that disable their respective host by selective and specific inhibition of the CP.[8] Omuralide (clasto-lactacystin β-lactone (**1**); Scheme 1 a) was the first natural product described to predominantly bind to subunit β5 harboring the chymotrypsin-like (ChTL) activity[9] (Scheme 1 b). Initially isolated from

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Streptomyces sp. OM-6519,[10] the metabolite was chemically and functionally analyzed as a site-specific proteasome inhibitor.[11] After the discovery of **1**, **2** was identified from the marine bacterium *Salinospora tropica*[12] and also found to display high affinity for the ChTL active site.[13] Structural elucidation of **1** and **2** in complex with the yeast 20S proteasome provided important insights into the binding mechanism of this new set of proteasome ligands.[2a,13] Both molecules covalently react with the proteasomal active site Thr1O$^{\gamma}$ by opening the β-lactone ring and forming an ester bond (Scheme 1b).[2a,13] Cleavage of the β-lactone ring of **1** and **2** generates the hydroxy group 5-OH, which displaces the nucleophilic water molecule from the active site. The γ-lactam ring of **1** and **2** prevents free rotation of the 5-OH group about the C1/C5 bond and thus ultimately protects the ligands from intermediate hydrolysis and elimination through disturbance of the Bürgi–Dunitz trajectory.[14] Omuralide acts as a slowly reversible binder[15] which can be explained by the penetration of a water molecule at the 5-OH barrier. In contrast, Marizomib blocks the CP irreversibly, because its unique 4-chloroethyl side chain forms a tetrahydrofuran ring engaging 5-O (Figure S5).[16] This particular feature circumvents any possibility of regenerating the β-lactone once **2** is bound to the proteasome.

Vibralactone (**3**; Scheme 1a) has been identified as a natural lactone produced by the fungi *Boreostereum vibrans*.[17] Although the chemical structure of **3** is similar to that of **1**, in vitro activity tests using fluorogenic tetrapeptides demonstrated that none of the proteasomal active sites were inhibited by **3** even at concentrations as high as 1 mM (Figure S2). However, guided by the similarity in the chemical scaffold and the presence of the β-lactone ring pharmacophore in both compounds, we soaked crystals of yeast 20S proteasome with a 50 mM solution of **3** for 72 h and elucidated the structure of the complex at 2.7 Å resolution (R_{free} = 23.1%, Table S1 in the Supporting Information). The $2F_O-F_C$ electron density map displays **3** solely defined in the ChTL active site (Figure 1a), forming a covalent bond with the N-terminal β5-Thr1. The structure of the CP:**3** complex demonstrates that the entire architecture of the ligand is just as important as the functional reactive lactone group in gaining high-affinity binding to the enzyme. Due to the lack of the peptide scaffold in the β-lactones, the ligand stabilization has to be enforced through the strong binding preference within the S1 pocket as well as additional specific hydrogen bonds around the catalytic center (Figure 1b). The comparison of the crystal structures of the CP:**1**[2a] (Figure S3) and CP:**3** complexes identifies for the first time that both the γ-lactam and the 8-OH functionalities in omuralide play dominant roles for the strong binding preference of **1** towards the CP; both of these features are absent in **3** (Figure 1c): a) The 2-NH of **1** forms a strong hydrogen bond to Gly47O (3.0 Å), hereby mimicking the antiparallel β-sheet upon peptide–ligand binding (Figure S4). In the case of **3**, the γ-lactam is replaced by a cyclopent-2-ene. Though the double bond in the five-membered ring possesses reduced flexibility and together with the 3-hydroxymethyl group even mimics the amide group found in **1**, the lack of the hydrogen bond between **3** and Gly47O predominantly impairs its stabilization. b) Unlike **1**, the P1 hydroxyisobutyl residue is replaced by a prenyl group in **3**; this shifts Met45, which forms the bottom of the S1 specificity pocket, from its original position towards Ile35 by 2.5 Å (Figure 1c). Even though this structural rearrangement has been observed previously for the CP:**2** complex,[13] the loss of enthalpy is compensated by multiple hydrophobic interactions of the cyclohexenyl ring with residues of the S1 pocket (Figure S4). In contrast, the low flexibility of the double bond in the P1 site of **3** prevents any van der Waals interactions of the ligand with the enlarged S1 specificity pocket. Additionally, **3** lacks the 8-OH group which in **1** and **2** forms a hydrogen bond with Thr21N (3.0 Å). Thus, the energetic penalty caused by the structural rearrangements upon vibralactone binding cannot be regained (Figure 1c). These structural results conclude that the electrophilic lactone still requires the extensive support of a precisely

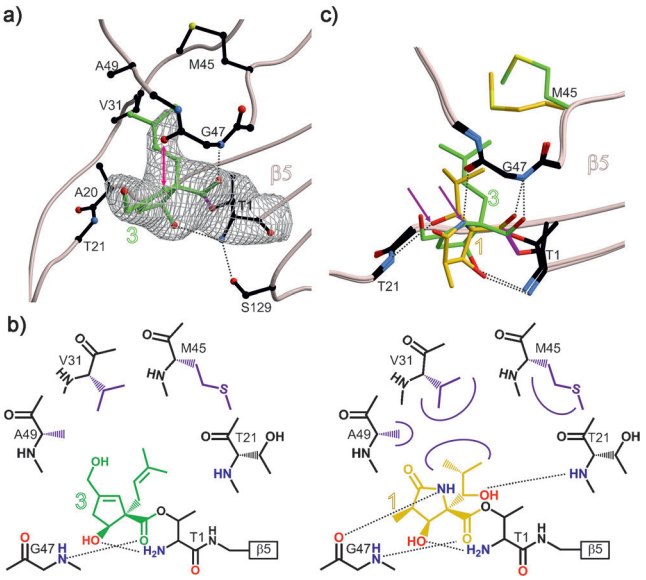

Figure 1. Binding mode and interactions of omuralide and vibralactone with the proteasome. a) Crystal structure of the proteasomal ChTL active site in complex with **3**. The backbone of the proteasomal subunit β5 is shown as a grayish pink band; the ligand is represented in green. The $2F_O-F_C$ electron density map (gray mesh) is contoured at 1σ and is displayed for **3** bound to the active site Thr1O$^{\gamma}$. Thr1 and vibralactone were both excluded prior to phase calculations, thus preventing any model bias. Protein residues forming the proteasomal S1 specificity pocket are highlighted in black and numbered according to Löwe et al.[3] The various sets of hydrogen bonds between **3** and β5 main-chain atoms and the residues Gly47O, Thr1N, and Ser129O are depicted as dashed lines. Stereo representations of this figure and the $2F_O-F_C$ omit map of CP:**1**[2a] are shown in Figure S3. b) Schematic overview of the proteasome in complex with vibralactone (green, left) and omuralide (gold, right). The S1 specificity pocket of the ChTL substrate-binding channel and their corresponding amino acids are shown in black. Hydrophobic interactions between the ligand and the S1 pocket are drawn in purple and hydrogen bonds are displayed as dashed lines. c) Structural superposition of **1** and **3** bound to the proteasome. Note: the large P1 site of vibralactone enlarges the specificity pocket by causing major structural rearrangements of Met45, hence, destabilizing the ligand. The pink arrows mark major differences in the scaffolds of **1** and **3**, which are responsible for the distinct inhibition profiles. Stereo representation of this figure is shown in Figure S4.

suited P1 anchor residue to fulfill the minimal requirements to act as a CP inhibitor.

We aimed to identify the cellular target of **3** by performing activity-based protein profiling.[18] Therefore, vibralactone modified with an alkyne tag (**4**; Scheme 1a)[19] was incubated with HeLa cells for 1 h. Subsequently, the cells were lysed and the proteome treated with rhodamine azide in order to perform click chemistry. The proteome was separated by SDS-PAGE and the labeled proteins were visualized by fluorescence scanning. Treatment of labeled proteins with rhodamine–biotin azide followed by avidin enrichment, SDS-PAGE separation, and mass spectrometric analysis of isolated fluorescent gel bands revealed the identities of the corresponding protein targets (for more detailed information, see the Supporting Information).[20] This approach allowed us to identify four targets that are covalently bound to **4**: carboxypeptidase SCPEP1, abhydrolase domain containing protein 10 (ABHD10), and predominantly the acyl-protein thioesterases 1 and 2 (APT1, APT2; Figure 2a, Table S2). When cells were incubated with **3** for 1 h prior to addition of **4**, no fluorescent bands were observed, emphasizing that the unmodified natural product and the probe share the same binding preferences. Interestingly, the same competition assay revealed that **1** also targets SCPEP1, while the classical standard CP inhibitor epoxomicin (**5**)[21] (Figure S1) exhibits no cross-reactivity with **3**. These results confirm the distinct inhibitory profiles and functionalities of the structurally similar β-lactones **1** and **3** towards the CP. When we carried out MTT assays with HeLa cells we obtained an LD_{50} value of 28 μM by **1**,[22] whereas **3** proved to completely maintain the metabolic activity (Figure 2b). These findings are in agreement with kinetic experiments which give an IC_{50} value of 47 nM for **1** towards blockage of the proteasomal ChTL activity and a lack of inhibition for **3** (Figure 2c). On the other hand, the enzymatic assays of vibralactone using recombinant APT1 and APT2 illustrate a high binding preference of the ligand with IC_{50} values of 4.3 μM for APT1 and 1.7 μM for APT2, while omuralide has no effect on these enzymes (Figure 2e). Heat denaturation of both APTs suppressed any labeling and demonstrates that the natural product binds only to the native thioesterases (Figure 2d).

Notably, **1** is uniquely expressed in bacteria and **3** is a fungal metabolite, a fact that may be related to the organism's survival strategy. *Streptomyces* lack proteasomes, whereas CP activity is vital in all eukaryotes. Hence, the alterations of the β-lactone-γ-lactam compounds at the molecular level might once more reiterate the source as well as the different target preferences of each of these compounds, perfectly optimized during evolution.[23] β-Lactone proteasome inhibitors were initially considered to be important biochemical tools;[24] however, nowadays, they are undergoing clinical studies for the treatment of several cancer types.[6a]

Most natural products targeting the proteasome challenge the classical concept of CP binding.[25] Thus, a combination of multiple techniques is necessary to identify the overall lead structure motif for streamlined binding, reactivity, and reversible[2a]/irreversible[13] CP inhibition (Figure S5). In contrast, vibralactone is the first identified natural product that

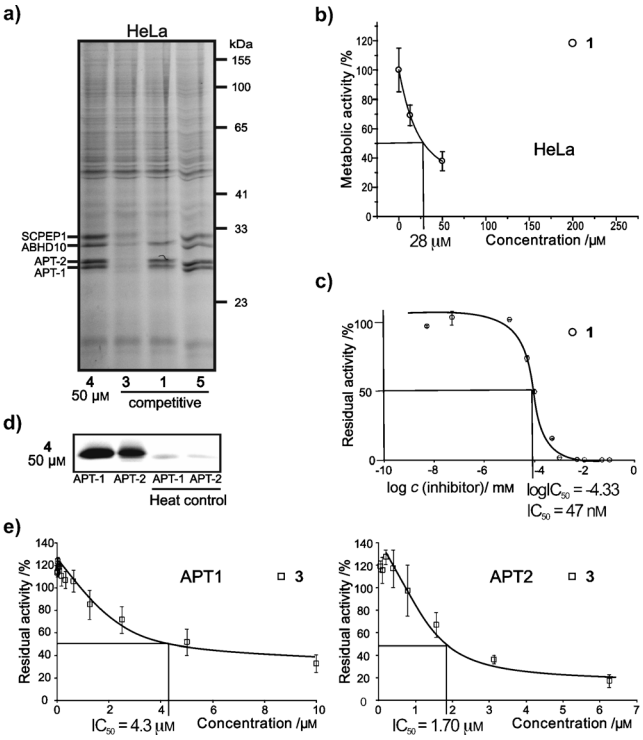

Figure 2. In vivo and in vitro characterization of omuralide and vibralactone. a) Activity-based protein profiling in HeLa cells using **4** (50 μM). SCPEP1, ABHD10, APT1, and APT2 were identified by MS analysis. Competitive targets of the natural product were determined by incubating HeLa cells with **3**, **1**, and **5** (50 μM) for 1 h prior to adding **4** (50 μM). b) Cytotoxic MTT assay with HeLa cells and omuralide. Note: Vibralactone has no effect on these cells (Figure S6). c) IC_{50} determination of **1** against the ChTL activity of yeast CP using the chromogenic substrate Suc-Leu-Leu-Val-Tyr-AMC. Note: **3** does not affect CP activity up to a concentration of 1 mM as shown in Figure S6. d) Heat denaturation of recombinant APT1 and ATP2 demonstrates that **3** only binds to the native enzymes e) Inhibition curves of vibralactone towards APT1 and APT2. Note: Omuralide did not affect APT activity up to a concentration of 500 μM (Figure S6). All results were obtained from at least three independent measurements; error bars display standard deviations from the mean.

blocks APTs. These thioesterases have received attention due to their role in the S-depalmitoylation of N- and H-Ras, which determines localization and signaling in cells.[26] It has been shown that the synthetic β-lactone palmostatin B blocks the APT1 thioesterase with an IC_{50} value of 0.67 μM and perturbs the acylation cycle of depalmitoylation thereby affecting the epidermal growth factor induced Ras activity.[27] In summary, the β-lactone scaffold in both **1** and **3** exhibits a densely functionalized matrix that facilitates promising principles and concepts for the future development of target-specific drugs. However, despite their structural similarity the natural products display distinct biological target preference.

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