Proteasome Inhibition | Hot Paper

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Indolo-Phakellins as β5-Specific Noncovalent Proteasome Inhibitors**

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Abstract: *The proteasome represents an invaluable target for the treatment of cancer and autoimmune disorders. The application of proteasome inhibitors, however, remains limited to blood cancers because their reactive headgroups and peptidic scaffolds convey unfavorable pharmacodynamic properties. Thus, the discovery of more drug-like lead structures is indispensable. In this study, we present the first structure of the proteasome in complex with an indolophakellin that exhibits a unique noncovalent binding mode unparalleled by all hitherto reported inhibitors. The natural product inspired pentacyclic alkaloid binds solely and specificially into the spacious S3 subpocket of the proteasomal β5 substrate binding channel, gaining major stabilization through halogen bonding with the protein backbone. The presented compound provides an ideal scaffold for the structure-based design of subunit-specific nonpeptidic proteasome-blockers.*

The FDA approval of bortezomib (BTZ, Velcade) and carfilzomib (CFZ, Kyprolis) confirmed the 20S proteasome (core particle; CP) as a valuable target for the treatment of oncological diseases such as multiple myeloma and mantle cell lymphoma (see Figure S1 in the Supporting Information).[1,2] Both inhibitors carry an electrophilic warhead at the C-terminal end of a peptidic backbone for covalent attachment to the catalytic Thr1 residues of the protease.[3,4] The transition from the promiscuous boronic acid warhead of BTZ to the CP-specific α′,β′-epoxyketone in CFZ represents an evolution towards better target selectivity and fewer off-target effects.[2] The next generation of proteasome blockers has to focus on selectively addressing the constitutive CP (cCP) and tissue-specific subtypes that play crucial roles in autoimmune or inflammatory diseases (immunoproteasome, iCP), as well as T cell development in the thymus (thymoproteasome, tCP).[5–8]

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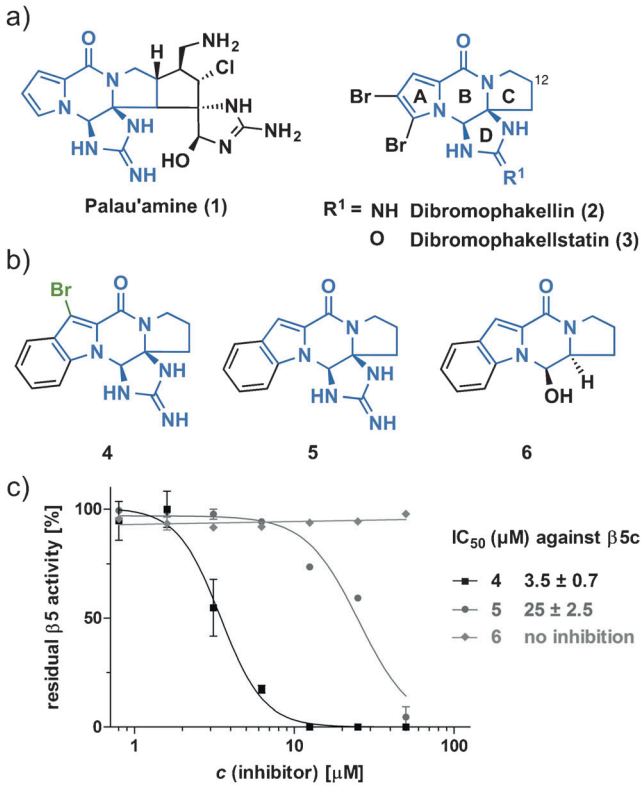
To fulfill their diverse biological tasks, the CP subtypes have different substrate cleavage preferences that are determined by the specificity pockets S1–S4 of the substrate binding channels (see Figure S2). Thus, subtype-selective CP inhibitors must exhibit finely tuned P1–P4 residues that match the corresponding pockets in order to distinguish between cCP, iCP, and tCP.[9] However, the development of novel CP ligands that precisely target the specificity pockets so far remains limited to predominantly peptide-based ligands owing to the current lack of structural knowledge concerning the mechanism of proteasome inhibition by nonpeptidic inhibitors.[10]

In our ongoing efforts to discover proteasome inhibitors with novel modes of action, we investigated compounds from the highly diverse family of biologically active pyrrole–imidazoline marine alkaloids, which differ significantly from all CP inhibitors described to date (Scheme 1). The most important member of this class of compounds is palau'amine (**1**), a cytotoxic and immunosuppressive hexacyclic bisguanidine that was isolated from the sea sponge *Stylotella agminata*.[11–13] Compound **1** was described to bind irreversibly to the CP ($IC_{50}(\beta5c) = 2.5\ \mu M$), but its scarcity and structural complexity prevents the rapid generation of more potent synthetic or semisynthetic derivatives.[14] The structurally related natural products dibromophakellstatin (**2**) and dibromophakellin (**3**) are synthetically more accessible, although these compounds display reduced CP inhibitory potency compared to **1** [$IC_{50}(\beta5c) = 25.3$ and 11.9 μM, respectively].[14,15]

We established a robust synthetic route towards derivatives of phakellins that share the same rigid cyclic framework[14,16] and revealed activity similar to **1**. The most promising candidate was the brominated indolo-phakellin compound **4**, which was prepared in an analogous manner to dibromophakellin and **5**, through an *N*-bromosuccinimide promoted addition of Boc-guanidine to the olefinic precursor **7** (Scheme 2).[16] Removal of Boc by treating **8** with 5% trifluoroacetic acid (TFA) in dichloromethane furnished the brominated indole **4** in 94% yield.

Compound **4** inhibited the β5 activity of human cCP with an IC_{50} value of 3.5 μM (Scheme 1c). Following these encouraging results, the crystal structure of the yCP:**4** complex was determined at 2.5 Å resolution ($R_{free} = 21.6\%$, PDB ID: 4RUR, see Table S1 in the Supporting Information). Surprisingly, inspection of the resulting $F_O - F_C$ electron density map revealed that **4** binds exclusively and in a well-defined manner in the S3 pocket of the chymotryptic-like active site with a novel noncovalent binding mode (Figure 1). Strikingly, **4** does not interact with either the peptide binding channel or the Thr1O^{γ} nucleophile of the β5 subunit, from which it is separated by a distance of 8.4 Å. The ligand perfectly accommodates the size and polarity of the S3 specificity



Scheme 1. Structures of pyrrole–imidazoline compounds. a) Palau'amine exhibits a complex hexacyclic structure and bears the same core pyrrole–imidazoline scaffold (blue) as phakellins and phakellstatins, although its C ring is substituted with two additional five-membered rings. Dibromophakellins (**2**) and dibromophakellstatins (**3**) are differentiated by amino or oxo substitution of the imidazoline ring. The bent pyrrole–imidazoline core structure with the ABCD tetracycle is shown in blue. b) Indolo analogues of dibromophakellin. c) IC_{50} measurements of the β5 activity of human cCP after the addition of the proteasome inhibitors over a range of concentrations. Data from three repetitions were normalized to DMSO-treated controls and are presented as relative activity with standard deviation. IC_{50} = half maximal inhibitory concentration.

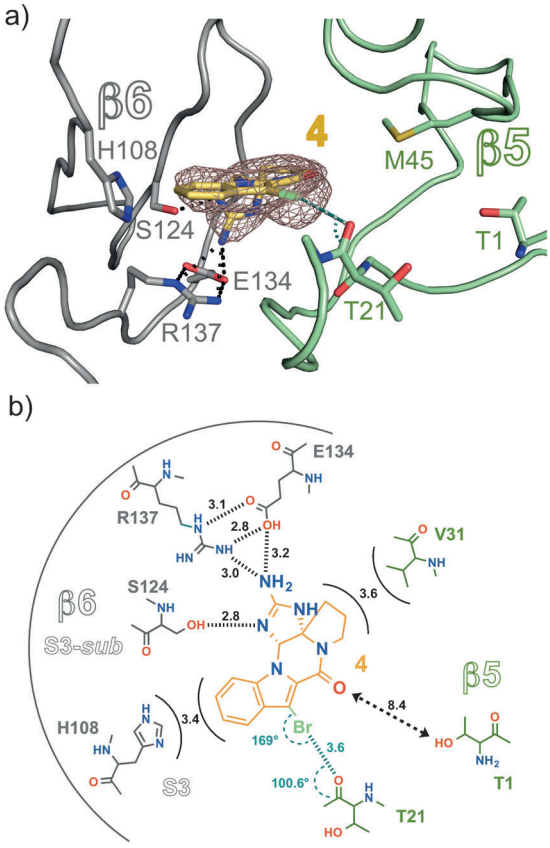
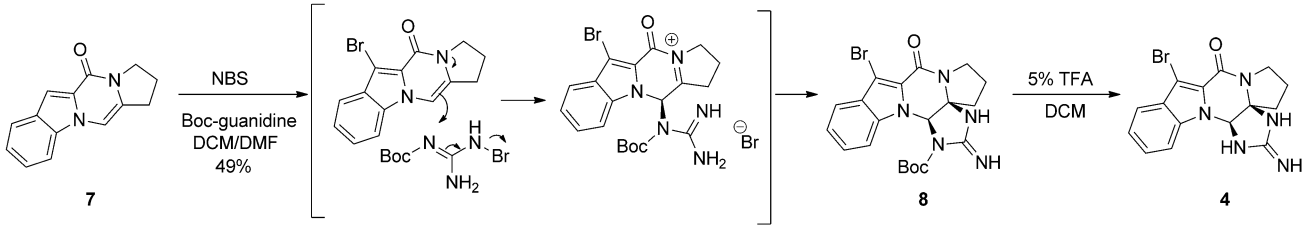


Figure 1. Crystal structure of yCP in complex with **4**. a) $2F_o-F_c$ electron density map (red mesh, 1σ) of **4** (yellow) bound to the S3-*sub* specificity pocket of the β5 active site (PDB ID 4RUR). b) A detailed overview of the molecular inteactions. The 2-aminoimidazoline group forms a network of hydrogen bonds (black dashed lines) with the side chains of S124, E134, and R137 of the β6 subunit (gray). β6-H108 and β5-V31 are involved in van der Waals interactions with **4** and the carbonyl oxygen of β5-T21 is perfectly oriented to stabilize the ligand through halogen bonding (teal) with the bromine substituent of **4** (lime green). The interaction distances are shown in Å. The distance between the ligand and the catalytically active β5-T1 is highlighted with a double headed dashed arrow (black).

pocket through tight hydrophilic interactions between the characteristic 2-aminoimidazoline moiety and S124, E134, and R137 of the adjacent subunit β6 (Figure 1 a). Additionaly, the planar tetracyclic system of **4** forms van der Waals interactions with the side chains of β6-H108 and β5-V31, which flank the ligand at the indole and C rings, respectively.

Most remarkably, the rigid inhibitor scaffold is perfectly orientated for the formation of a halogen bond between its bromine atom and the carbonyl oxygen of β5-Thr21. With a Br···O distance of 3.6 Å and a roughly collinear alignment of the acceptor/donor atoms (C–O···Br angle: 169°), the observed metrics of this **4**–Thr21CO interaction fulfill the requirements for ideal halogen bonding.[17–19] Consequently, the donor atom is able to orient its electron density into the σ-hole that results from the unoccupied σ*-orbital of the C···Br bond (Figure 1 b and Figure S3). The influence of the halogen



Scheme 2. Synthesis of indolo-phakellin **4** from precursor **7**.[14,16] Boc = tert-butoxycarbonyl.

bond for the stabilization of **4** in the cavity was evaluated through comparison with the debrominated analogue **5** (Scheme 1b).[16] Inspection of the electron density map of yCP crystals soaked with **5** revealed that the compound is not defined in the electron density map, although it has the same scaffold as **4**. In agreement with the structural observations, the inhibitory potency of **5** against purified human cCP is one order of magnitude lower than that of **4** ($IC_{50} = 25\ \mu M$, Scheme 1c).

In general, the specificity of the cytotoxic effect of proteasome inhibitors for transformed over nonmalignant cells is determined by their ability to block both the iCP and cCP β5 active sites.[20] Structural superposition of the S3-*sub* binding site of yCP with murine cCP and iCP structures revealed that the overall topology and charge of the pocket is well conserved across the two proteasome subtypes (Figure S5). The inhibitor is thus expected to bind to iCP and cCP in a comparable manner, as in the yCP:**4** complex structure, however, crystal structures of the human CP are required for detailed structure-guided ligand expansion.

Notably, **4** breaks with the hitherto accepted dogma of common CP inhibitors that are all substantially stabilized by the formation of an antiparallel β sheet within the substrate binding channel or through interaction with the S1 specificity pocket.[21] The bromoindole phakellin disturbs substrate binding by blocking the S3 specificity pocket. Superposition of the yCP:**4** complex structure with the corresponding coordinates of yCP bound to either MG-132 (PDB ID 4NNN)[22] or the FDA-approved CP inhibitor BTZ (PDB ID 2F16)[3] exemplifies the unique binding mode in comparison with peptidic proteasome inhibitors (Figure 2a,b and Figure S4a–c). Moreover, both drugs only marginally protrude into the S3 pocket and might be further improved in their affinity by taking advantage of this spacious cavity as well. Furthermore, the presented data are in line with a previous study of *N*-hydroxyureas (HUs) as reversible noncovalent CP inhibitors, which describes the optimization of a low-affinity β5 ligand ($IC_{50} = 230\ \mu M$) to an inhibitor with an IC_{50} of 0.34 μM (HU10, Figure 2c) through precise targeting of both the S1 and the S3-*sub* pockets.[23] Structural superposition of the yCP:HU10 complex (PDB ID 3SHJ)[23] with yCP:**4** reveals that the adamantyloxy moiety of HU10 closely matches the position of the 2-aminoimidazoline ring of **4** (Figure 2c and Figure S4d,e). Importantly, removal of the adamantyloxy group from HU10 resulted in complete loss of potency, thus highlighing once more the strong influence of the P3 site on ligand stabilization in the substrate binding channel.[23]

Earlier studies by Lindel and co-workers towards synthetic phakellstatin derivatives showed that the (+)-enantiomer of dibromophakellstatin exerted no cytotoxic effect against a panel of human cancer cell lines.[24,25] Only the (−)-enantiomer displayed antitumor activity, which is in accordance with the herein presented binding mode of **4**. The same enantiospecificity was observed in the case of Palau'amine, which occurs as the (−)-enantiomer in its natural form.[26]

Previous approaches towards synthetic C-ring-functionalized derivatives of dibromophakellstatin revealed that among a series of closely related compounds, only (−)-(12*R*)-dibromo-12-hydroxyphakellstatin exerted enhanced cytotoxic effects across multiple tumor cell lines compared to the natural product.[25] The yCP:**4** complex structure clearly demonstrates that larger substituents at this position clash with the protein and thus have vastly decreased cytotoxic capabilities.

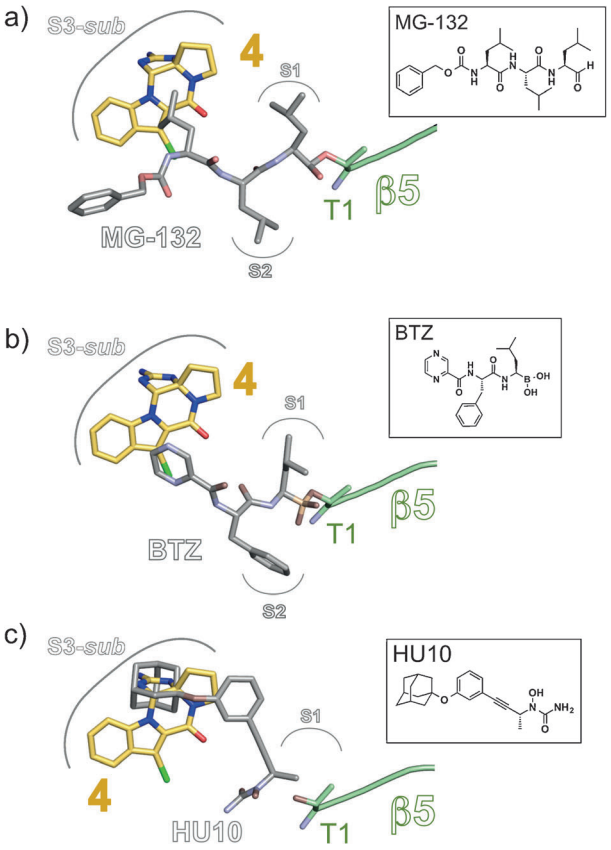
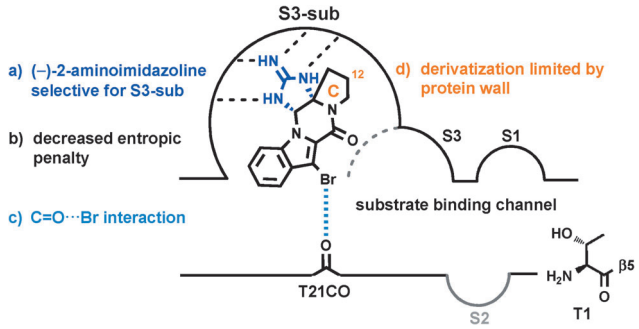


Figure 2. Structural superposition of **4** with peptidic and nonpeptidic inhibitors bound to the proteasomal β5 substrate binding channel. a/b) Superposition of **4** (yellow) with MG-132 (gray, PDB ID 4NNN)[22] or BTZ (gray, PDB ID 2F16).[3] The binding mode of **4** differs significantly from that of the peptide-based ligands. c) The adamantyloxy residue of HU10 (gray, PDB ID 3SHJ)[23] is crucial for inhibitor stabilization and superimposes well with the indispensable 2-aminoimidazoline moiety of **4**. The S1 pocket (gray arc) is indicated for orientation.

Certainly, the herein presented data for the first time provide a rational approach towards the structure activity relationship (SAR) of phakellins and phakellstatins (Scheme 3):

a) Only the (−)-enantiomer has the correct configuration for binding of the 2-aminoimidazoline into the proteasomal S3-*sub* pocket.
b) The fused pentacyclic ring system is highly rigid and minimizes the entropic penalty upon ligand binding.
c) The halogen bond of **4** to the carbonyl oxygen of T21CO is indispensable for inhibitor stabilization. Iodide-derivatives of **4** are expected to even show enhanced binding affinities.[18]
d) C ring derivatization is tolerated only to a small extent at position 12, whereas larger residues cause steric clashes with the specificity pocket.



Scheme 3. An overview of how the structural features of **4** affect its activity.

In conclusion, the crystal structure of CP in complex with **4** reveals indolo-phakellins to be a new class of proteasome inhibitors that are characterized by a noncovalent mode of action, specific targeting of the S3-*sub* pocket of the β5 active site, and a non-peptidic scaffold. The crystallographic analysis of the yCP:**4** complex significantly extends the available structural knowledge regarding proteasome inhibitors. Furthermore, the presented data provide a starting point for the structure-based design of a new generation of proteasome blockers that avoid the pharmacokinetically unfavorable reactive headgroups and peptidic scaffolds of traditional CP inhibitors.

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