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A cell-permeable inhibitor and activity-based probe for the caspase-like activity of the proteasome

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Abstract—The ubiquitin–proteasome pathway degrades the majority of proteins in mammalian cells and plays an essential role in the generation of antigenic peptides presented by major histocompatibility class I molecules. Proteasome inhibitors are of great interest as research tools and drug candidates. Most work on proteasome inhibitors has focused on the inhibition of the chymotryptic-like (β5) sites; little attention has been paid to the inhibition of two other types of active sites, the trypsin-like (β2) and the caspase-like (β1). We report here the development of the first cell-permeable and highly selective inhibitors (**4** and **5**) of the proteasome's caspase-like site. The selectivity of the compounds is directly and unambiguously established by Staudinger–Bertozzi labeling of proteasome subunits covalently modified with azide-functionalized inhibitor **5**. This labeling reveals that the caspase-like site of the immunoproteasome (β1i) is a preferred target of this compound. These compounds can be used as tools to study roles of β1 and β1i sites in generation of specific antigenic peptides and their potential role as co-targets of anti-cancer drugs.

The 26S proteasomes degrade the majority of cytosolic and nuclear proteins to oligopeptides,[1] some of which are used in the major histocompatibility complex (MHC) class I antigen presentation pathway.[2] Proteasomal protein degradation takes place inside a barrel-shaped 20S core that consists of four rings of seven subunits each. Proteolytic active sites are located on the three β subunits (β1, β2, and β5) of each of the middle two rings.[3] Each catalytic subunit has a different substrate specificity.[4] The β1 subunit cuts preferentially after acidic residues and is therefore referred to as caspase-like.[5] The β2 subunit cleaves peptide bonds after basic residues and is called trypsin-like. The β5 subunit displays a chymotrypsin-like activity and cleaves after large, hydrophobic residues. Interferon-γ induces expression of immunoproteasome, in which the catalytic β1, β2, and β5 subunits are replaced by the β1i (LMP2), β2i (MECL), and β5i (LMP2) subunits that are encoded by the MHC genome locus.[1,6] The active sites of the immunoproteasome display slightly different substrate specificities from those of the corresponding active sites of the constitutive subunits. As a result, the immunoproteasome generates more peptides amendable for MHC class I antigen presentation.[7]

To gain more insight into the contribution of the different proteasomal subunits to the process of protein degradation and antigen presentation, selective inhibitors of individual active sites are needed. Some inhibitors with selectivity for either the trypsin-like or the chymotrypsin-like subunits have been reported.[8,9] In contrast, the only cell-permeable inhibitor of the caspase-like sites[10]

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causes considerable inhibition of the chymotrypsin-like sites.[5] In addition to the chymotrypsin-like (β5) site, the caspase-like site has been recently identified as a secondary target of bortezomib (VELCADE),[11,12] a proteasome inhibitor being used for the treatment of multiple myeloma.[13] Specific inhibitors of the caspase-like site are needed to address the question whether inhibition of this site is important for bortezomib's anti-neoplastic activity and thus facilitate the development of new drugs of this class.

The most convenient and reliable way to directly measure proteasome inhibition inside living cells is by using activity-based probes. Most of the probes that report on activity of all three active sites are much weaker inhibitors of the caspase-like sites than of the chymotrypsin-like and trypsin-like sites. No specific activity-based probe for the caspase-like proteasomal subunits has been reported to date.

We considered the selective, reversible, and cell-impermeable peptide aldehyde inhibitor **1** (Fig. 1) of the caspase-like site of the proteasome to be a suitable starting point for the development of a cell-permeable inhibitor/probe **5**. Vinyl sulfones are suitable electrophilic traps to alkylate the N-terminal threonine residue, the active-site nucleophile of the proteasome.[9,14] The selectivity of peptidyl vinyl sulfones toward proteasomal catalytic residues depends on the peptide portion of the inhibitor but also on the functional group downstream of the vinyl sulfone moiety. For example, Bogyo and co-workers[14] showed that in case of GL_3-vinyl sulfones, phenolic vinyl sulfone (compound **2**; Fig. 1) was a better inhibitor of the caspase-like site than was the corresponding methyl vinyl sulfone (compound **3**; Fig. 1). Thus, a phenolic vinyl sulfone seemed to be the obvious electrophilic trap to use.

To avoid the elaborate synthesis of a suitably protected aspartic acid vinyl sulfone moiety for block coupling of the peptide vinyl sulfone building block to a peptide sequence, the P1 aspartic acid was replaced by leucine, thus generating compound **4** (Fig. 1). It is known that the caspase-like subunit also cleaves after hydrophobic branched-chain amino acids,[4,5,15] and this fact has been used in irreversible inhibitor design. An epoxyketone-based inhibitor with leucine in the P1 position can inhibit the caspase-like subunit with significant, albeit not absolute, specificity.[5,10] Furthermore, the leucine residue renders the inhibitor more apolar and thus possibly more cell-permeable.

The synthesis of vinyl sulfone **4** (described in Supporting Information) makes use of solid-phase peptide synthesis to generate the Ac-Ala-Pro-nLeu-OH oligopeptide, which was condensed in solution with phenolic leucine

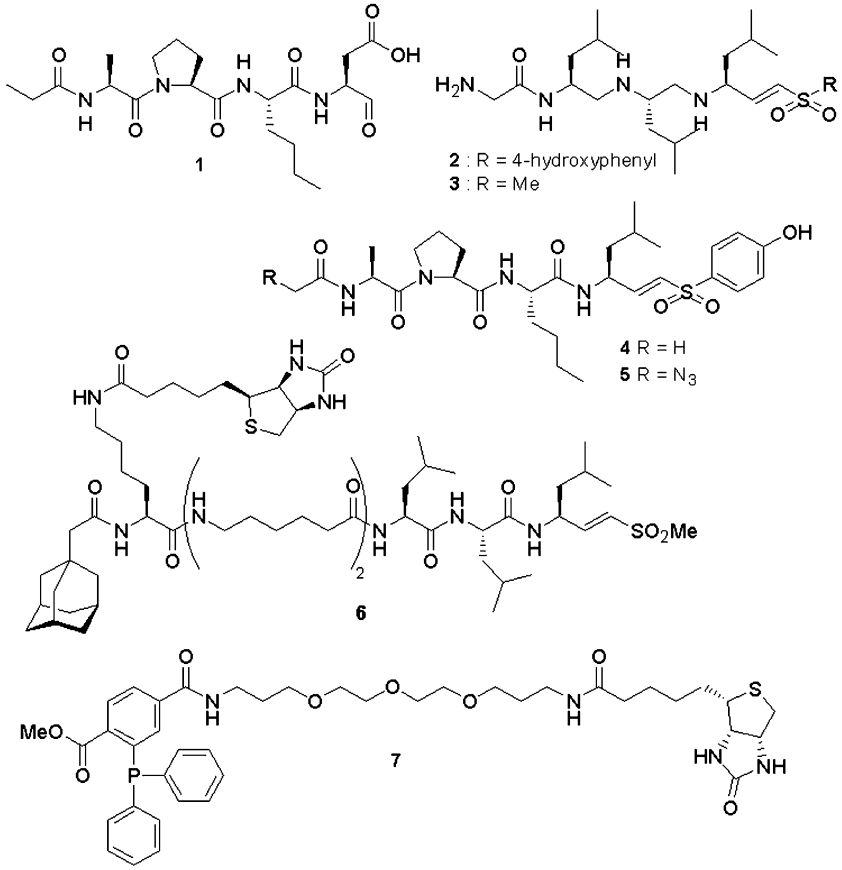

Figure 1. Proteasome inhibitors. Inhibitors **1**, **2**, **3**, and **6** are described in the literature. Compounds **4** and **5** are novel inhibitors described in this study. Compound **7** is a Staudinger–Bertozzi phosphine reagent used to biotinylate proteasome subunits covalently modified by compound **5**.

vinyl sulfone. In an initial experiment, the potency and selectivity of peptide vinyl sulfone **4** toward caspase-like activity was assessed using purified 26S proteasome from rabbit muscle[20] and fluorogenic substrates that report on either trypsin-like activity (Boc-LRR-amc), chymotrypsin-like activity (Suc-LLVY-amc), or caspase-like activity (Ac-nLPnLD-amc).[16] Compound **4** irreversibly inhibited caspase-like activity with $k_{obs}/[I] = 128 \pm 7\ M^{-1}\ s^{-1}$. Similar to compound **1**,[5] more than 90% inhibition of this site was achieved at 10–20 μM concentrations of compound **4**. No inhibition of chymotrypsin-like and trypsin-like activity was observed at concentrations of up to 500 μM. To further confirm the specificity of the compound **4**, 26S proteasomes were pre-incubated with large excess of compound **4** (50–100 μM, Fig. 2a). This resulted in a complete block of caspase-like activity, but did not affect the rate of hydrolysis by the chymotrypsin-like subunit, and activated the trypsin-like site. Similar activation of the trypsin-like activity by compound **1** has been observed.[5] Thus, compound **4** is a highly selective inhibitor of the caspase-like sites (β1) of purified proteasomes.

To convert inhibitor **4** into an activity-based probe, we decided to introduce an azide moiety on the N-terminal acetyl function to give compound **5** (Fig. 1; for preparation of compound **5**, see Supporting Information). Based on literature evidence, direct attachment of a biotin moiety to the probe was expected to influence subunit specificity and decrease cell permeability.[17] This has been circumvented before by use of an azide as latent ligation handle and by biotinylation via Staudinger–Bertozzi ligation.[18,19] Staudinger–Bertozzi ligation is a reaction of biotinylated triarylphosphine with an azido group on a biological macromolecule, which results in a selective biotinylation of the latter. In the case of proteasome, only subunits that are covalently modified by azido-inhibitor, such as compound **5**, will become biotinylated when treated with Staudinger–Bertozzi reagent.[7,19]

Assay of proteasome peptidase activities[21] in cells treated with compounds **4** (not shown) and **5** (Fig. 2b) using fluorogenic substrates[16] clearly demonstrated specific inhibition of caspase-like activity and activation of the trypsin-like site (Fig. 2b). In order to visualize the subunits modified by inhibitor **5**, the azide moieties of labeled proteasomal subunits were modified with a biotin moiety by Staudinger–Bertozzi ligation with biotinylated phosphine **7**. In short, cells were incubated for 16 h with different concentrations of **5**, harvested, and permeabilized with digitonin, and cytosol was squeezed out by centrifugation. Extracts were treated with phosphine **7** to introduce a biotin moiety, separated on SDS–PAGE, and transferred onto polyvinylydine difluoride membranes. Biotinylated proteins were stained with fluorescently labeled streptavidin (Fig. 3a). β1i is modified predominantly at lower concentrations of **5**, whereas at higher concentration both caspase-like subunits (β1 and β1i) are targeted.

Because the β1, β1i, β5, β5i proteasome subunits are not always resolved on one-dimensional SDS–PAGE, the specificities of compounds were further confirmed by two-dimensional (2D) gel electrophoresis of the same phosphane-treated cytosolic extracts.[21] 2D gels are known to separate all three catalytic subunits of consti-

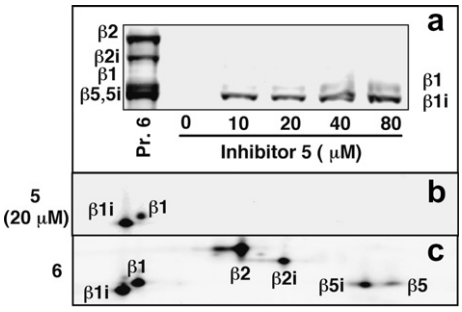

Figure 3. Two-step visualization of compound **5** targets. RPMI-8226 human multiple myeloma cells were incubated for 18 h with different concentrations of **5**. Cells extracts were prepared and treated with biotinylated phosphane **7**, followed by fractionation on SDS–PAGE (a) or 2D gel (b) and Western blotting. Membranes were incubated with streptavidin-IRDye800CW conjugate. Labeled proteins were visualized with Odyssey near-IR fluorescent imager. In panel (c) and in lane 1 of panel (a), an extract treated with 100 μM biotinylated active site probe **6**, which modifies all six catalytic subunits, was analyzed. The identities of subunits were inferred from gel migration patterns based on previous work.[17]

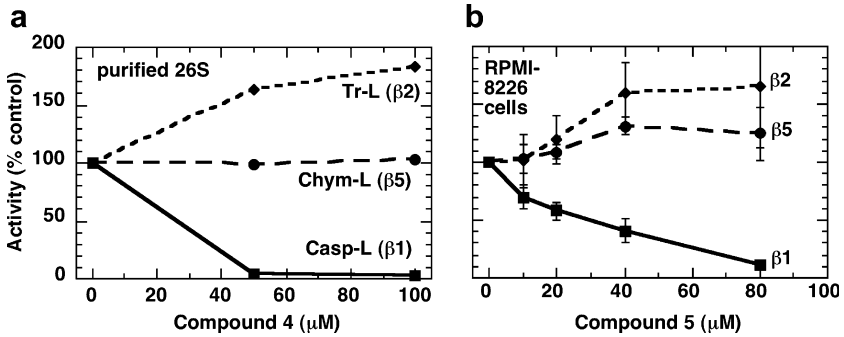

Figure 2. Selectivity of compounds **4** and **5**. (a) Purified 26S proteasomes from rabbit muscle were treated with **4** for 30 min and peptidase activities of different active sites were measured with fluorogenic peptide substrates.[16] (b) RPMI-8226 human multiple myeloma cells were incubated with **5** for 18 h, followed by measurements of proteasome peptidase activities in cell extracts. Suc-LLVY-amc was used for the chymotrypsin-like site, Ac-nLPnLD-amc for the caspase-like site, and Boc-LRR-amc (a) or Ac-RLR-amc (b) for the trypsin-like site. All substrates were at 100 μM.

tutive proteasomes from each other and from the three catalytic subunits of the immunoproteasomes.[14,17] Extracts of cells treated with inhibitor **5** were incubated with phosphine **7** and separated on a 2D gel[21] (Fig. 3). Modification of both β1 and β1i subunits was observed. The lack of labeling of the other proteasomal subunits confirms the specificity of **5** for caspase-like activity as found with peptidase assays. No other non-proteasomal spots were detected, indicating no significant inhibition of other cellular proteases.

In conclusion, we have presented here two new peptide vinyl sulfone inhibitors of the caspase-like (β1 and β1i) subunits of the proteasome. The introduction of an azide moiety did not change the inhibition profile and enabled direct visualization of caspase-like activities via Staudinger ligation followed by SDS–PAGE and Western blotting. This approach revealed that these compounds are better inhibitors of β1i sites of the immunoproteasome than of the β1 sites of normal (constitutive) proteasome. Both inhibitors presented here, as well as the Staudinger ligation protocol applied, will be of value for future research aimed at the role of the caspase-like subunit in the processing of antigens and as co-targets of anti-cancer drugs.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2007.03.092.

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21. Cells were treated with varying concentrations of inhibitors (or mock-treated) for 18 h, harvested, washed three times in ice-cold PBS, and frozen at −80 °C. After thawing, cells were re-suspended in 4 volumes of homogenization buffer (50 mM Tris–HCl, pH 7.5, 250 mM sucrose, 5 mM $MgCl_2$, 2 mM ATP, 1 mM DTT, 0.5 mM EDTA, and 0.025% digitonin). After 5 min of incubation on ice, extracts were separated from cell debris by centrifugation at 20,000*g* for 15 min (4 °C). Protein concentrations were determined by Bradford and used to normalize subsequent assays. Peptidase activities were measured with site-specific fluorogenic peptides as described,[16] using pre-treatment of extracts with epoxomicin to account for background cleavage of these peptides from non-proteasomal proteases. Another aliquot of each extract was treated with 0.5 mM phosphane **7** and analyzed on SDS–PAGE (12% Novex NuPAGE Bis–Tris gel with MOPS running buffer) or 2D gels (using Invitrogen ZOOM system with pH 3–10 isoelectric focusing strips and a 4–12% gradient NuPAGE Bis–Tris gel with MOPS buffer).