A humanized yeast proteasome identifies unique binding modes of inhibitors for the immunosubunit $\beta 5i$

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Abstract

Inhibition of the immunoproteasome subunit $\beta 5i$ alleviates autoimmune diseases in preclinical studies and represents a promising new anti-inflammatory therapy. However, the lack of structural data on the human immunoproteasome still hampers drug design. Here, we systematically determined the potency of seven α' β' epoxyketone inhibitors with varying N-caps and P3-stereochemistry for mouse/human $\beta 5c/\beta 5i$ and found pronounced differences in their subunit and species selectivity. Using X-ray crystallography, the compounds were analyzed for their modes of binding to chimeric yeast proteasomes that incorporate key parts of human $\beta 5c$, human $\beta 5i$ or mouse $\beta 5i$ and the neighboring $\beta 6$ subunit. The structural data reveal exceptional conformations for the most selective human $\beta 5i$ inhibitors and highlight subtle structural differences as the major reason for the observed species selectivity. Altogether, the presented results validate the humanized yeast proteasome as a powerful tool for structure-based development of $\beta 5i$ inhibitors with potential clinical applications.

Keywords immunoproteasome; ligand; mode of action; species selectivity
X-ray crystallography

Subject Categories Chemical Biology; Post-translational Modifications, Proteolysis & Proteomics

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Introduction

Protein breakdown by the ubiquitin-proteasome system ensures cell survival and proliferation. The 20S proteasome core particle (CP) constitutes the heart of this non-lysosomal protein degradation pathway. In mammals, three different CP types are known: the constitutive proteasome (cCP), the immunoproteasome (iCP), and the thymoproteasome (tCP) (Murata *et al*, 2007; Groettrup *et al*, 2010).

All of them are built of seven different α - and seven different β -subunits that are stacked in four heptameric rings around a central pore. Each type of CP incorporates a distinct set of catalytically active $\beta 1$, $\beta 2$ and $\beta 5$ subunits (cCP: $\beta 1c$, $\beta 2c$ and $\beta 5c$; iCP: $\beta 1i$, $\beta 2i$ and $\beta 5i$; tCP: $\beta 1i$, $\beta 2i$ and $\beta 5t$) (Groll *et al*, 1997; Unno *et al*, 2002; Murata *et al*, 2007; Huber *et al*, 2012; Harshbarger *et al*, 2015). These subunits employ the same mechanism of peptide bond hydrolysis (Huber *et al*, 2012, 2016), but display different substrate specificities. Because of its pivotal biological role, the proteasome is an attractive drug target. Blockage of the $\beta 5$ active sites causes cell cycle arrest, an effect that led to the approval of the proteasome inhibitors bortezomib, carfilzomib, and ixazomib for the treatment of multiple myeloma (Appendix Fig S1) (Huber & Groll, 2012; Muz *et al*, 2016). More recently, selective targeting of the iCP-subunit $\beta 5i$, which promotes class I antigen processing and pathogen clearance as well as the production of proinflammatory cytokines, has been shown to suppress various autoimmune diseases in mouse models (Muchamuel *et al*, 2009; Ichikawa *et al*, 2011; Basler *et al*, 2014; Ettari *et al*, 2016). Structural data on the mouse cCP and iCP in complex with ONX 0914 (formerly PR-957) (Huber *et al*, 2012), the first $\beta 5i$ selective inhibitor (Muchamuel *et al*, 2009), provided insights into its mode of binding and enabled the structure-guided design of compounds specific for $\beta 5c$ (Xin *et al*, 2016) and of ligands with improved $\beta 5i$ -selectivity (Fig 1) (de Bruin *et al*, 2014). The latter ones unify structural features of the α' , β' epoxyketone inhibitors ONX 0914 and PR-924, another $\beta 5i$ -specific compound (Parlati *et al*, 2009; Singh *et al*, 2011; Niewerth *et al*, 2014). While ONX 0914 inhibits both the mouse and the human $\beta 5i$ subunit to the same extent (Muchamuel *et al*, 2009; Huber *et al*, 2012), PR-924 was reported to be more selective for human $\beta 5i$ (Niewerth *et al*, 2014). However, structural data on the yeast 20S proteasome (yCP) in complex with PR-924 (de Bruin *et al*, 2014) could not explain the molecular reason for the enhanced $\beta 5i$ -selectivity and the species specificity of PR-924. Due to the easy purification of proteasomes from erythrocytes, cCP crystal structures from cow (Unno *et al*, 2002), mouse (Huber *et al*, 2012), and humans (Harshbarger *et al*, 2015) are available to date. By contrast, iCPs are less amenable to isolation because of their cell-type-specific and cytokine-inducible

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Figure 1. Chemical structures of α' , β' epoxyketone immunoproteasome inhibitors analyzed in this study.

The syntheses of the ligands PR-924, **9**, **14**, **16**, **17**, and **18** have been described previously (de Bruin *et al.*, 2014). All ligands share the peptide scaffold shown in the upper left corner with P1, P2 and P3 side chains and an N-cap; they are equipped with a L-Ala (stereochemistry (S)) or D-Ala (stereochemistry (R)) residue at P3 (red) and either an N-acylmorpholine or a 3-methyl-1H-indene group (green). Furthermore, compounds differ in their P2 residue (either methoxytyrosine or tryptophane; blue). Inhibitor **9**, also termed LU-015i, is endowed with an exceptional cyclohexyl residue (gray), which is associated with increased selectivity for human β 5i.

expression pattern. We therefore used mutagenesis to create yeast/human and yeast/mouse chimeric proteasomes. These mutant particles served to structurally investigate the subunit and species selectivity of PR-924 and related epoxyketone compounds. Compared to wild-type (WT) yeast, X-ray structures of β 5c/ β 6 and β 5i/ β 6 humanized yCPs visualize distinct and exceptional binding modes for PR-924 and its analogues. These results provide insights into the molecular mechanisms of these inhibitors and deliver explanations for their high selectivity toward human β 5i. Furthermore, specific guidelines for the design of human β 5i-ligands can be inferred with respect to the stereochemistry of the peptide backbone and the N-cap size. These data now set the basis for further optimization of iCP-specific ligands for future clinical applications.

Results

Immunoproteasome inhibitors

The proteasome inhibitor bortezomib, a dipeptide boronic acid (Appendix Fig S1), equally targets the subunits β 5c and β 5i (Demo *et al.*, 2007). Despite its off-target activity toward serine proteases, which causes severe side effects (Arastu-Kapur *et al.*, 2011), bortezomib was granted approval by the FDA for cancer treatment. Carfilzomib, a second-generation compound based on the natural product epoxomicin, only targets the small class of N-terminal nucleophilic hydrolases such as the proteasome. By a two-step reaction mechanism, the epoxyketone inhibitor carfilzomib

covalently and irreversibly modifies the active site Thr10^Y and Thr1N of predominantly β 5c and β 5i proteasome subunits (Groll *et al*, 2000; Demo *et al*, 2007). Screening of diverse peptide libraries (Harris *et al*, 2001; Nazif & Bogyo, 2001; Britton *et al*, 2009; Blackburn *et al*, 2010; Huber *et al*, 2015a) led to the development of subunit-selective inhibitors. The prototypes of β 5i-specific epoxyketones are ONX 0914 (Muchamuel *et al*, 2009) and PR-924 (Parlati *et al*, 2009) (Fig 1). As shown in our activity assays with genuine human as well as mouse cCP and iCP, both compounds are potent inhibitors of human β 5i ($IC_{50} \leq 0.051 \mu M$) with up to 20- and 255-fold selectivity over human β 5c, respectively (Fig 2, Appendix Table S2 and Appendix Fig S2). Notably, the superior selectivity of PR-924 is not based on enhanced h β 5i inhibition but worse h β 5c targeting compared to ONX 0914 (IC_{50} h β 5c (ONX 0914): 0.487 μM ; h β 5c (PR-924): 13.020 μM ; Fig 2B). The chemical structures of ONX 0914 and PR-924 differ in their P2 residue (methoxytyrosine versus tryptophane), in the stereochemistry of their P3-Ala residue (L versus D), and in their N-cap (morpholine versus 3-methyl-1*H*-indene; Fig 1). First, we addressed the question which of these structural differences gives rise to the improved β 5i

selectivity of PR-924, and secondly, we aimed to investigate why PR-924 is seven times less potent for mouse β 5i (IC_{50} 0.353 μM) than for human β 5i (IC_{50} 0.051 μM)—a phenomenon that is not observed for ONX 0914 (IC_{50} h β 5i: 0.024 μM versus m β 5i: 0.040 μM ; Fig 2B). Since available structural data on yeast and mouse CPs failed to provide plausible explanations for the subunit and species inhibition profiles of PR-924, we created yeast/mammalian chimeric proteasomes and analyzed their X-ray structures in complex with a set of inhibitors, including PR-924 and ONX 0914 as well as previously described derivatives of both (compounds **9**, **14**, **16**, **17** and **18**, Fig 1). For clarity reasons, compound numbering was taken over from the first report of these ligands (de Bruin *et al*, 2014).

Construction of chimeric yeast/human proteasomes

In an initial attempt, we tried to express the full-length human β 5c and β 5i as well as mouse β 5i subunits in a yeast *pre2Δ* knockout strain under the control of the *PRE2* promotor and terminator

IC₅₀ values are given in [μM] for yeast, human and mouse CPs. Errors are provided in the Appendix Table S2.

Figure 2. Inhibition of the chymotrypsin-like activities of yeast, human, and mouse proteasomes by ONX 0914, PR-924, **9, **14**, **16**, **17**, and **18**.**

A Inhibition of human β 5c and β 5i by ONX 0914 and PR-924. IC_{50} values are deduced from the fitted data.
B IC_{50} values (μM) for all compounds toward the yeast, human, and mouse β 5 subunits. For inhibition curves, see Appendix Figure S2. The ratio $IC_{50}(\beta 5c)/IC_{50}(\beta 5i)$, a measure of β 5i selectivity, is given. Large values indicate high selectivity for β 5i.
Data information: Genuine WT proteasomes were used for all inhibition assays. IC_{50} values (μM) were determined in triplicates with the fluorogenic substrate Suc-LLVY-AMC using an enzyme concentration of 13 nM. Mean standard deviations are indicated as bars and given in Appendix Table S2.

sequences (for details see Appendix Supplementary Methods). Despite the strictly conserved fold of proteasome subunits across species, this genetic setup was lethal, even after replacement of the genuine $\beta 5c/\beta 5i$ propeptide by that of $\gamma\beta 5$ (Fig 3A). Most likely the mammalian $\beta 5$ subunits are unable to adopt their correct three-dimensional structure or fail to incorporate into the yeast proteasome due to species-specific differences in the subunit interface contacts.

Next, we replaced the endogenous $\gamma\beta 5$ by various chimeric $h\beta 5i/\gamma\beta 5$ subunits that feature the $\gamma\beta 5$ propeptide, N-terminal parts of human $\beta 5i$, and C-terminal segments of $\gamma\beta 5$ (Fig 3B). Among the viable mutant yeasts, we selected that one that carried the largest fragment of $\beta 5i$ (amino acids (aa) 1–138; see also Appendix Supplementary Note). Importantly, this residue range encodes both primed and non-primed pockets of the substrate binding channel. The mutant strain suffered from a pronounced growth defect, from increased temperature sensitivity as well as significantly reduced chymotrypsin-like (ChT-L; $\beta 5$) proteasome activity (Fig 3E and Appendix Fig S3A). Crystallographic analysis (for X-ray data see Appendix Table S1) however revealed that the chimeric $\beta 5$ subunits were properly incorporated into the CP and fully matured. Despite extensive mutagenesis, superposition with the mouse iCP and WT yCP coordinates proved the preservation of the overall fold of the chimeric $\beta 5$ subunits (root-mean-square deviation (r.m.s.d.) for mouse $\beta 5i$: C^α 0.751 Å; for yeast $\gamma\beta 5$: C^α 0.209 Å; Appendix Fig S4A). Furthermore, the structures displayed Met45 in the same peculiar “open” conformation that has previously been discovered for the mouse iCP in the apo state (Huber et al, 2012) (Appendix Fig S4B). Hence, our results indicate that the reorientation of Met45 is inherent to iCPs irrespective of the species and that the molecular switch for this conformational change is encoded within the $\beta 5i$ residues 1–138.

After having addressed subunit $\beta 5i$, we extended our mutagenesis to the neighboring $\beta 6$ subunit in order to create an entire human $\beta 5i/\beta 6$ substrate binding channel for X-ray inhibitor binding studies. In total, five amino acids of $\gamma\beta 6$ that mediate important interactions with ligands bound to the $\beta 5$ active site were mutated (Fig 3C). These residues further aggravated the growth defect of the respective yeast strain (Pre7^{mut}; Fig 3E). Compared to WT yCPs, purified $h\beta 5i/h\beta 6$ chimeric proteasomes showed normal $\beta 1$ and $\beta 2$ but 90 %

reduced $\beta 5$ activity (Appendix Fig S3B). In accordance, the $\beta 5$ subunit was less sensitive to epoxyketone inhibitors (Appendix Fig S3C). X-ray data on the $h\beta 5i/h\beta 6$ chimeric proteasome however proved that the modified $\beta 6$ residues take the same place as in the human or mouse $\beta 6$ subunit (Huber et al, 2012; Harshbarger et al, 2015) and that all crucial $\beta 5$ residues including Met45 are arranged as in the natural mouse iCP (Fig 4A). In analogy to the $h\beta 5i/h\beta 6$ chimera, mouse $m\beta 5i/m\beta 6$ and human $h\beta 5c/h\beta 6$ proteasomes were constructed (aa 1–138; Fig 3B). The respective yeasts as well as the purified CPs suffered from the same defects observed for the $h\beta 5i/h\beta 6$ chimera (Fig 3E and Appendix Fig S3). Although the mutant CPs are not equivalent to endogenous WT yCP, cCP, and iCP with respect to catalytic activity, their structural congruence is striking. While in the i-chimeras, Met45 is in the open conformation, the crystal structure of the $h\beta 5c/h\beta 6$ chimera visualizes the typical $\beta 5c$ “closed” orientation of Met45 (Fig 4B–D). This structural equivalence validates the designed chimeric proteasomes for X-ray diffraction-based ligand binding studies.

Enhanced selectivity of PR-924 and its analogues for human $\beta 5i$ versus $\beta 5c$

Crystals of the $h\beta 5c/h\beta 6$ and the $h\beta 5i/h\beta 6$ chimeras were soaked with the prevalent proteasome inhibitors ONX 0914, bortezomib, and carfilzomib. These compounds were chosen, because structural data on their binding mode to the WT yCP (Groll et al, 2006; Huber et al, 2015b), the human (Harshbarger et al, 2015) and mouse cCP (Huber et al, 2012) as well as the mouse iCP (Huber et al, 2012) are available for comparison. Despite the reduced activity of the chimeric proteasomes, all ligands covalently modified the mutant $\beta 5$ active sites at high occupancy (Fig EV1A and B, Appendix Table S3). Furthermore, the coordinates revealed uniform modes of binding for all three inhibitors to WT yCP, chimeric CPs, and available WT mammalian CP structures (r.m.s.d. < 0.65 Å; Fig EV1C) (Huber et al, 2012; Harshbarger et al, 2015). For ONX 0914, we previously noticed distinct orientations of the N-cap in the mouse constitutive proteasome and the immunoproteasome crystal structures (Huber et al, 2012). Remarkably, these distinct conformations are now also observed with the humanized yeast structures (Fig EV1C).

Figure 3. Expression of mammalian $\beta 5$ proteasome subunits in yeast.

- Schematic representation of yeast and mammalian $\beta 5$ subunits and their propeptides. Secondary structure elements, helices (H) and sheets (S), are numbered. The full-length human $h\beta 5i$ (yellow) and $\beta 5c$ (red) as well as the mouse $m\beta 5i$ (green) subunits cannot substitute the endogenous yeast $\gamma\beta 5$ subunit (gray). Expression of the mammalian subunits with their natural propeptide (pp; colored) or with the $\gamma\beta 5$ propeptide (pp; gray) is lethal in a yeast *pre2Δ* knockout strain (for details, see Appendix Supplementary Methods).
- Chimeric $\beta 5$ subunits are schematically illustrated according to panel (A). The N-terminal part of the subunits, which forms the primed and non-primed substrate binding pockets and the active site surroundings, was taken from human $\beta 5i$ and the C-terminus from yeast. A construct featuring residues 1–173 from human $\beta 5i$ was still lethal. Replacement of the residues 1–139 of $\gamma\beta 5$ by the human $\beta 5i$ counterpart (aa 1–138; see Appendix Supplementary Note), however, was compatible with yeast survival. Based on this observation, analogous fragments of the human $\beta 5c$ and mouse $\beta 5i$ subunits were incorporated into the $\gamma\beta 5$ subunit to create the respective $h\beta 5c$ and $m\beta 5i$ chimeras.
- Subunit $\beta 6$ significantly contributes to the S3 and S4 pockets of the ChT-L active site. In order to mimic the entire mammalian $\beta 5/6$ substrate binding channel in yeast, we mutated five residues of the $\gamma\beta 6$ subunit as well (G89S, K90R, H98Y, T99N, and E120Q). These mutations correspond to both the human and the mouse $\beta 6$ sequence and create two mammalian segments aa 87–101 and aa 108–121 (blue; see also Appendix Supplementary Note).
- The mouse-specific point mutation V31M was inserted into the $h\beta 5i/h\beta 6$ chimera to investigate its impact on inhibitor binding.
- Serial dilutions of yeast cells were spotted on YPD plates and grown for 2 days either at 30°C or 37°C. Mutant yeasts are significantly retarded in growth and show increased temperature sensitivity.
- Surface illustration of the yCP. The $\beta 5$ and $\beta 6$ subunits are depicted in dark gray, with the residue ranges that correspond to mammalian $\beta 5$ and $\beta 6$ subunits in the chimeric proteasomes highlighted in gold and blue, respectively. The $\beta 5'$ and $\beta 6'$ subunits of the second β -ring are hidden on the back of the 20S proteasome.

Figure 3.

Figure 4. Crystal structures of chimeric human proteasomes in their apo state.

- A Superposition of the $\beta 5i/\beta 6$ active sites of genuine mouse iCP and the h $\beta 5i$ /h $\beta 6$ human/yeast chimera illustrates that Met45 adopts the same “open” conformation in both structures. Amino acid side chains important for substrate binding are depicted as sticks. The catalytic Thr1 is colored in black. Met31, the single amino acid difference between mouse and human $\beta 5i$ active sites, is highlighted in green.
- B Superposition of the genuine mouse and the human chimeric $\beta 5c/\beta 6$ active sites according to panel (A) proves the proper orientation of Met45 in the “closed” conformation.
- C Superposition of the $\beta 5c/\beta 6$ and $\beta 5i/\beta 6$ active sites of the natural mouse cCP and iCP according to panel (A). Note the distinct conformations of Met45 (Huber *et al*, 2012).
- D Superposition of the h $\beta 5c$ /h $\beta 6$ and the h $\beta 5i$ /h $\beta 6$ chimeric proteasomes confirms the distinct positions of Met45 analogously to the natural mouse subunits shown in panel (C).

Data information: This figure contains previously published coordinates (Huber *et al*, 2012): mouse cCP (PDB accession code: 3UNE) and mouse iCP (PDB accession code: 3UNH).

Next, complex structures of the chimeric human proteasomes with PR-924 and derivatives thereof were determined (Figs 5 and EV2A and B). For the constitutive h $\beta 5c$ /h $\beta 6$ chimera, we observed similar results as with WT yCP (de Bruin *et al*, 2014; and Appendix Fig S5). Compounds exclusively built of α amino acids (ONX 0914, **14**) bind in an extended manner to the h $\beta 5c$ /h $\beta 6$ chimera regardless of their type of N-cap. Ligands that are furnished with a P3- α -Ala and the 3-methyl-1*H*-indene cap (PR-924 and **18**) adopt a linear orientation as well, but those combining a P3- α -Ala amino acid and a morpholine N-cap (**16** and **17**) are bent, with the N-cap occupying a pocket outside of the natural substrate binding channel, which we termed S3* (Figs 5 and EV2A). Notably, ligands

with α stereochemistry at P3 are generally less potent for $\gamma 5$ and human $\beta 5c$ than those with natural α stereochemistry at P3 (ONX 0914 and **14**; Fig 2B).

In stark contrast, the chimeric immuno-h $\beta 5i$ /h $\beta 6$ proteasome binds all inhibitors with a P3- α amino acid—irrespective of their type of N-cap—in a kinked conformation (Figs 5 and EV2B). This observation agrees with modeling results for PR-924 (Sharma *et al*, 2012).

In summary, the compounds **14**, **16**, and **17** dock in an identical manner to both chimeras, while PR-924 and **18** significantly differ in their binding mode to h $\beta 5c$ versus h $\beta 5i$. These results also provide an explanation for the varying potencies of the ligands. As shown by our inhibition assays with endogenous human cCP and iCP, all

Figure 5. Ligand complex structures of human chimeric proteasomes reveal distinct binding modes for $\beta 5c$ and $\beta 5i$.

A Complex structures of the human $\beta 5c/\beta 6$ (left) and $\beta 5i/\beta 6$ (right) chimeric proteasomes with PR-924. The ligand binds in a linear (l) manner to the $\beta 5c/\beta 6$ substrate binding channel, but in a kinked (k) conformation to the $\beta 5i/\beta 6$ active site. The $2F_o - F_c$ electron density maps for the ligands bound to Thr1 of the chimeric $\beta 5$ active sites are shown as blue meshes contoured at 1σ . The inhibitor and Thr1 have been omitted for phasing.

B Comparison of the conformations of ONX 0914, PR-924, **14**, **16**, **17**, and **18** bound to the chimeric $\beta 5c/\beta 6$ (left) and $\beta 5i/\beta 6$ (right) chimeras. The L amino acid compounds ONX 0914 and **14** display a linear (l) binding mode to the $\beta 5c/\beta 6$ (left) and the $\beta 5i/\beta 6$ (right) chimeras, whereas the ligands **16** and **17** (P3-D-Ala and morpholine cap) adopt a kinked (k) conformation in both $\beta 5$ substrate binding channels. Remarkably, the orientation of PR-924 and its analogue **18** (P3-D-Ala and 3-methyl-1H-indene cap) in the $\beta 5c/\beta 6$ chimera (and in WT $\gamma\beta 5$; see Appendix Figure S5) significantly differs from that observed with the $\beta 5i/\beta 6$ chimera. Note that the inhibitors are rotated by about 45° compared to panel (A).

derivatives of ONX 0914 and PR-924 are highly effective human $\beta 5i$ inhibitors ($IC_{50} \leq 0.374 \mu M$, Fig 2B). However, with respect to $\beta 5c$ targeting PR-924, **9** and **18** are the worst, thereby resulting in highest selectivity ratios of ≥ 155 (Fig 2B). Obviously, the linear binding mode of ligands with non-natural P3-D stereochemistry and a 3-methyl-1H-indene cap bound to $\beta 5c$ is energetically strongly disfavored compared to the kinked orientation in the $\beta 5i$ subunit. This is remarkable, as ligands that adopt the bent conformation cannot be stabilized by Asp114 from the neighboring subunit $\beta 6$, which usually hydrogen bonds to the peptide backbone of linearly arranged inhibitors (Huber et al, 2015a).

Reducing the size of the N-cap from a 3-methyl-1H-indene to a morpholine moiety (compounds **16** and **17**) leads to bent ligand binding modes in both $\beta 5c$ and $\beta 5i$ and comparable inhibition strengths for both active sites. We thus conclude that the S3* pocket of $\beta 5c$ impedes binding of inhibitors with P3-D amino acids

and bulky N-caps such as the 3-methyl-1H-indene moiety for steric and energetic reasons. From the structural data, no obvious barrier can be inferred but hydrogen bonds at the interface of the subunits $\beta 5$ and $\beta 6$ involving Ser28 ($\gamma\beta 5$, $m\beta 5c$, $m\beta 5i$, $h\beta 5c$)/Ala28 ($h\beta 5i$), Thr30 ($\gamma\beta 5$, $m\beta 5c$, $h\beta 5c$)/Arg30 ($m\beta 5i$, $h\beta 5i$), and Glu120 ($\gamma\beta 6$)/Gln120 ($h\beta 6$, $m\beta 6$) might play crucial roles. We also determined a crystal structure of the $\beta 5i$ chimera (with entire subunit $\beta 6$ from yeast) in complex with PR-924 and found that the ligand takes the same position as in the $\beta 5i/\beta 6$ chimera. These data indicate that solely amino acid exchanges in subunit $\beta 5i$ are responsible for triggering the kinked binding mode of PR-924 (Appendix Fig S6). Taken together, the N-cap, which originally served to improve solubility and pharmacokinetic parameters of peptide inhibitors, now becomes a dominant factor for subunit selectivity of $\beta 5i$ ligands with D stereochemistry at the P3 position.

Figure 6. The impact of Met31 on ligand binding to the β5i active site.

- A** Complex structures of the mouse chimeric proteasome (left) and the hβ5i-V31M/hβ6 mutant (right) with PR-924 visualize the kinked (k) orientation of the inhibitor. The 2F_O-F_C electron density maps for the ligands bound to Thr1 of the chimeric β5 active sites are depicted according to Fig 5A. Steric hindrance of Met31 (green) with the bulky 3-methyl-1H-indene cap is indicated by a black double arrow.
- B** Superposition of inhibitors bound to the mouse chimeric proteasome (left) and the hβ5i-V31M/hβ6 mutant (right). Compounds display the same binding modes (kinked (k) or linear (l)) to the mβ5i/mβ6 active site as to the corresponding human β5i/β6 subunit (Fig 5) and to the hβ5i-V31M/hβ6 mutant. Note that the inhibitors are rotated by about 45° compared to panel (A).
- C** Superposition of chimeric mammalian and WT yeast β5 active sites bound to the P3-o-Ala compounds PR-924 and **18**. The nucleophilic Thr1 is depicted as well as the S1 pocket forming residues 31 and 45. Residue 31 impacts on the position of the 3-methyl-1H-indene cap of PR-924 and **18**. Due to steric hindrance, the N-cap is shifted in the mouse and in the hβ5i-V31M/hβ6 chimera compared to the hβ5i/hβ6 mutant proteasome crystal structures (black double arrow). Note that the structures are rotated by 90° compared to panel (B).

Structural basis for species selectivity of P3-D-Ala 3-methyl-1H-indene-capped inhibitors

As derived from our activity assays with genuine mouse and human cCP as well as iCP, no pronounced species selectivity is observed for ONX 0914 (Muchamuel *et al*, 2009; Huber *et al*, 2012) and its closest structural analogue, compound **14** (Fig 2B). By contrast, PR-924, **9**, and **18** show at least a sevenfold preference for human versus mouse $\beta 5i$ (Fig 2B). Furthermore, all three compounds discriminate strongly between h $\beta 5c$ and h $\beta 5i$ (factor ≥ 155), while this effect is less pronounced for m $\beta 5c$ and m $\beta 5i$ (factor ≤ 16 ; Fig 2B). In order to analyze the molecular reason for this species selectivity, we used the mouse m $\beta 5i$ /m $\beta 6$ chimeric proteasome. As expected, ONX 0914, bortezomib, and carfilzomib as well as PR-924, **9**, **14**, **16**, **17**, and **18** bind to the m $\beta 5i$ /m $\beta 6$ chimeric proteasome similar as has been observed for the h $\beta 5i$ /h $\beta 6$ construct (Figs 6 and EV3). As noted for the h $\beta 5i$ /h $\beta 6$ chimeric proteasomes, compounds with a non-natural D amino acid at the P3 site position the N-cap close to the P1 site. In the case of m $\beta 5i$, the N-cap approaches Met31 of the S1 pocket, which in human and mouse $\beta 5c$ as well as in human $\beta 5i$ is a Val residue (Appendix Fig S7). While the tiny Val31 is compatible with the kinked binding mode of P3-D-Ala inhibitors, the larger Met31 imposes a steric barrier to such ligands. This hindrance increases with the size of the P1 site and of the N-cap. The small and flexible N-acylmorpholine group is easily accommodated, but the

bulky and rigid 3-methyl-1H-indene cap clashes with Met31. Hence, only compounds endowed with the latter N-cap show impaired affinity for the mouse $\beta 5i$ subunit. We therefore mutated Val31 to methionine in the h $\beta 5i$ /h $\beta 6$ chimeric proteasome (Fig 3D) and determined the respective ligand complex structures (Figs 6 and EV4). In support of our hypothesis, the X-ray data revealed that the morpholine-capped compounds **16** and **17** were unaffected by Met31 (Fig EV5), while the more bulky 3-methyl-1H-indene caps of PR-924 and **18** were rotated by 180° and shifted up to 2.3 Å (Fig 6C). Notably, the N-caps of PR-924 and **18** are positioned as in the m $\beta 5i$ /m $\beta 6$ mutant.

Taken together, a combination of activity assays, yeast genetics, and X-ray crystallography elucidated how a single amino acid can significantly affect ligand binding and why compounds featuring a non-natural D-Ala at P3 and a 3-methyl-1H-indene cap are more potent and more selective for human $\beta 5i$ than for the mouse counterpart. As a result of the various crystal structures, we here provide for the first time experimental evidence that proteasome inhibitors can adopt different conformations in distinct eukaryotic CPs. This observation is of particular importance for the development of CP-type selective inhibitors and clearly limits the use of WT yeast as a model system. On the other hand, the data presented here strongly vote for yeast as an excellent tool box that can be easily genetically modified to create mammalian proteasome models for advanced structure-based drug design efforts.

Figure 7. Overview of key inhibitors and their distinct binding modes to the subunits $y\beta 5$, $h\beta 5c$, $m\beta 5i$, and $h\beta 5i$.

ONX 0914 binds in a linear mode to all active sites. Changing the stereochemistry at the P3 site from L-Ala (S) to D-Ala (R) leads to a kinked conformation in the $\beta 5$ substrate binding channels (compound **16**). Substitution of the morpholine cap of **16** by a larger 3-methyl-1H-indene moiety yields PR-924 and results in different inhibitor conformations at the $y\beta 5$ /h $\beta 5c$ and the mammalian $\beta 5i$ active sites. Hereby, the selectivity for h $\beta 5i$ over h $\beta 5c$ is drastically increased (IC₅₀ ratio h $\beta 5c$ /h $\beta 5i$: 255) compared to ONX 0914 (IC₅₀ ratio h $\beta 5c$ /h $\beta 5i$: 20) and compound **16** (IC₅₀ ratio h $\beta 5c$ /h $\beta 5i$: 5.2, Fig 2). Residue Met31 of m $\beta 5i$ sterically hinders the kinked binding mode of PR-924, thereby creating sevenfold selectivity for human $\beta 5i$.

Discussion

By systematically investigating the inhibition potencies of various epoxyketones for mouse and human $\beta 5c$ and $\beta 5i$, we noticed that the compounds tested significantly differ in their $\beta 5i$ selectivity (Fig 2). Since large amounts of human iCPs for structural analyses of all these ligands are difficult to obtain, we aimed at integrating the human ChT-L active site of cCP and iCP into the yeast 20S proteasome. Purification and high-throughput crystallization of mutant yCPs are well established, and baker's yeast in addition allows for rapid point mutagenesis to evaluate the impact of individual amino acids on ligand binding. However, all attempts to replace the endogenous $\gamma\beta 5$ by human or mouse $\beta 5$ subunits as a whole was lethal to yeast. We therefore created chimeric proteasomes that feature either the entire human $\beta 5c/\beta 6$, human $\beta 5i/\beta 6$, or mouse $\beta 5i/\beta 6$ substrate binding channel. The respective yeast strains were viable, but suffered from growth retardation and increased temperature sensitivity suggesting proteasome activity defects (Fig 3E). In fact, purified chimeric proteasomes showed WT-like $\beta 1$ and $\beta 2$ activities but were almost deprived of ChT-L activity and less sensitive to $\beta 5$ -inhibition (Appendix Fig S3B and C). Altered or disrupted inter- and intra-subunit contacts as well as impaired dynamics caused by subtle structural changes below the resolution limits might restrict reactivity of the ChT-L active site toward substrates and inhibitors. Thus, with respect to activity, the chimeric proteasomes are not equivalent to endogenous WT particles and candidate drugs have to be evaluated for their inhibition strength and subunit selectivity in enzymatic assays with genuine iCP and cCP.

However, the chimeric CPs proved to be valuable structural models. X-ray crystallographic data revealed that the mutant $\beta 5$ subunits structurally conform with the genuine mouse $\beta 5$ entities and that the orientations of Met45 correspond to those observed for the natural mouse CPs (Huber *et al*, 2012) (Fig 4A and B). Numerous inhibitor complex structures finally revealed that compounds furnished with a large 3-methyl-1*H*-indene cap and a P3- β amino acid adopt distinct conformations in the $\beta 5c$ -chimeric proteasome compared to the $\beta 5i$ mutant one (Fig 7). This observation provides an explanation for the $\beta 5i$ -selectivity of these inhibitors and validates the chimeric proteasomes for sophisticated structure-based drug development.

In a second approach, we created a mouse chimeric proteasome in order to investigate the species selectivity of PR-924 and its closest analogues **9** and **18**. Mouse and human $\beta 5i$ primary sequences differ in only 17 positions. By X-ray analysis, we found that Met31 of the mouse $\beta 5i$ subunit sterically hinders binding of inhibitors featuring a P3- β amino acid in combination with a 3-methyl-1*H*-indene cap. In contrast, the corresponding human $\beta 5i$ residue Val31 is compatible with such compounds, thereby enhancing selectivity for human $\beta 5i$ at least sevenfold. Notably, this species selectivity effect largely depends on the size of the N-cap. The substrate binding pockets and the active site residues are highly conserved among mouse and human, but residues outside the natural substrate binding channel, for example, the identified S3* pocket, are subjected to higher mutagenesis rates, thereby creating species-specific differences such as Val/Met31. Furthermore, P1 side chains larger than Phe may be incompatible with a P3- β amino acid and a large N-cap because of mutual sterical hindrance. The non-natural β stereochemistry at P3 and the associated unique binding mode render the

P3- β -Ala compounds PR-924, **9** and **18** exclusive $\beta 5i$ inhibitors (de Bruin *et al*, 2014), a fact that may reduce cytotoxicity in clinical applications (Parlati *et al*, 2009). Moreover, the β -Ala amino acid may prolong the resistance to proteases as well as improve the metabolic stability and therapeutic efficacy. However, the resistance of the murine iCP against these compounds requires the use of laboratory animals other than mice for preclinical studies. Species that encode Val31, for example, rats but also apes, might be suitable alternatives (Appendix Fig S7).

In summary, our study describes the creation of a humanized yCP variant that unifies all striking structural features of the mammalian $\beta 5i$ subunit. Proof of principle of the exceptional value of this chimeric CP for the future development of human iCP-specific inhibitors has been provided by structural analyses with PR-924 and its analogues. Certainly, these results will further support structure-guided design and optimization of CP-type-specific ligands.

Materials and Methods

Yeast strain construction

See Appendix Supplementary Methods.

Yeast growth tests

Overnight cultures of WT (WCG4a) and mutant yeast strains were grown in rich (YPD) medium at 30°C. Their optical density was determined at 600 nm, and appropriate serial dilutions in YPD were prepared. Total cell numbers of 10,000, 1,000, 100, and 10 were spotted on YPD plates and grown for several days at 30°C or 37°C to evaluate proliferation rates by colony size as well as survival rates by colony numbers.

Mammalian proteasomes

Human and mouse constitutive and immunoproteasomes were purchased from Boston Biochem (USA).

Purification of yeast proteasomes

Yeast strains were grown in 18 l YPD cultures at 30°C into early stationary phase. Cells were harvested by centrifugation for 15 min at 5,000 g and frozen at -20°C until further use. Mutant yCPs were purified according to published procedures (Gallastegui & Groll, 2012). In brief, 120 g yeast cells were solubilized in 150 ml of 50 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 7.5) and disrupted with a French press. Cell debris were removed by centrifugation for 30 min at 41,000 g (4°C). The resulting supernatant was filtered, and ammonium sulfate (saturated solution) was added to a final concentration of 30% (v/v). This solution was loaded on a Phenyl Sepharose™ 6 Fast Flow column (GE Healthcare) pre-equilibrated with 1 M ammonium sulfate in 20 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ (pH 7.5). CPs were eluted by applying a linear gradient from 1 to 0 M ammonium sulfate. Proteasome containing fractions were pooled and loaded onto a Resource Q column (GE Healthcare). Upon gradient elution (0–500 mM sodium chloride in 20 mM Tris–HCl pH 7.5), CPs

were subjected to size exclusion chromatography (Superose 6 10/300 GL (GE Healthcare), 20 mM Tris–HCl pH 7.5, and 150 mM NaCl).

Proteasome substrates and inhibitors

Peptide substrates (Bachem) and inhibitors were stored as 50–100 mM stock solutions in DMSO at -20°C . Bortezomib, carfilzomib, and ONX 0914 were purchased from Selleckchem. The chemical synthesis of PR-924 and the compounds **9**, **14**, **16**, **17**, and **18** was described previously (de Bruin *et al*, 2014).

Fluorescence-based activity assay

Proteasome activity was determined by fluorescence spectroscopy using the model substrates carboxybenzyl-Leu-Leu-Glu-7-amino-4-methylcoumarin (Z-LLE-AMC; β 1), tert-butyloxycarbonyl-Leu-Arg-Arg-AMC (Boc-LRR-AMC; β 2), and *N*-succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (Suc-LLVY-AMC; β 5). Purified yCPs (13 nM in 100 mM Tris–HCl, pH 7.5) were incubated with 200 μM substrate for 1 h at room temperature. The reactions were slowed down by diluting samples 1:10 in 20 mM Tris–HCl, pH 7.5. AMC fluorophores released by proteasomal activity were measured in triplicate with a Varian Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies) at $\lambda_{\text{exc}} = 360$ nm and $\lambda_{\text{em}} = 460$ nm.

IC₅₀ determination

Initial point measurements were carried out with 200 μM of inhibitor. Only compounds that showed significant inhibition at this concentration were further evaluated. CPs (final concentration: 13.2 nM) were mixed with serial dilutions of inhibitor or with DMSO as a control and incubated for 1 h at room temperature. After addition of the peptide substrate Suc-LLVY-AMC (final concentration of 200 μM) and incubation for 1 h at room temperature, proteolysis was slowed down by diluting the samples 1:10 in 20 mM Tris–HCl, pH 7.5. The AMC molecules released by residual proteasomal activity were measured in triplicate with a Varian Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies) at $\lambda_{\text{exc}} = 360$ nm and $\lambda_{\text{em}} = 460$ nm. Relative fluorescence units were normalized to the DMSO treated control. The calculated residual activities were plotted against the logarithm of the applied inhibitor concentration and fitted with GraphPad Prism 5. The IC₅₀ value, the ligand concentration that leads to 50% inhibition of the enzymatic activity, was deduced from the fitted data.

Note: IC₅₀ values depend on time and enzyme concentration and hence are not absolute; only relative tendencies within the same experimental settings are inferable.

Crystallization and structure determination

Mutant yCPs were crystallized as previously described for wild-type 20S proteasomes (Groll & Huber, 2005; Gallastegui & Groll, 2012). Crystals were grown at 20°C using the hanging drop vapor diffusion method. Drops contained a 1:1 mixture of protein (40 mg/ml) and reservoir solution (25 mM magnesium acetate, 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 6.8 and 9–13% (v/v) 2-methyl-2,4-pentanediol (MPD)). Crystals were cryoprotected by addition of 5 μl cryobuffer (20 mM magnesium acetate, 100 mM

MES, pH 6.8 and 30% (v/v) MPD). Inhibitor complex structures were obtained by incubating crystals in 5 μl cryobuffer supplemented with inhibitors at a final concentration of 1.5 mM for at least 8 h.

Diffraction data were collected at the beamline X06SA at the Paul Scherrer Institute, SLS, Villigen, Switzerland ($\lambda = 1.0$ Å). Evaluation of reflection intensities and data reduction was performed with the program package XDS (Kabsch, 2010). Molecular replacement was carried out with the coordinates of the yeast 20S proteasome (PDB entry code: 5CZ4; Huber *et al*, 2016) by rigid body refinements (REFMAC5; Vagin *et al*, 2004). MAIN (Turk, 2013) and COOT (Emsley *et al*, 2010) were used to build models. TLS refinements finally yielded excellent R_{crys} and R_{free} as well as r.m.s.d. bond and angle values. The coordinates, proven to have good stereochemistry from the Ramachandran plots, were deposited in the RCSB Protein Data Bank (Appendix Table S1).

Figures were prepared with PyMOL (DeLano, 2002). For the calculation of all electron density maps, the inhibitor and Thr1 have been omitted for phasing.

Data availability

Coordinates and structure factors have been deposited in the RCSB Protein Data Bank under the following accession codes (for details see Appendix Table S1):

5L52, 5L54, 5L55, 5L5A, 5LTT, 5L5B, 5L5D, 5L5E, 5L5F, 5L5H, 5L5I, 5L5J, 5L5O, 5L5P, 5L5Q, 5L5W, 5L5X, 5L5Y, 5L5Z, 5L60, 5L61, 5L62, 5L63, 5L64, 5L6B, 5L65, 5L66, 5L67, 5L68, 5L69, 5L6A, 5L6C, 5L5R, 5L5S, 5L5T, 5L5U, 5L5V

Expanded View for this article is available online.

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Author contributions

WH created yeast proteasome mutants; EMH performed yeast growth and proteasome activity/inhibition assays; GdB synthesized proteasome ligands under the supervision of HSO; EMH and MG collected and analyzed X-ray data; EMH and MG wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

References

- Arastu-Kapur S, Anderl JL, Kraus M, Parlato F, Shenk KD, Lee SJ, Muchamuel T, Bennett MK, Driessen C, Ball AJ, Kirk CJ (2011) Nonproteasomal targets of the proteasome inhibitors bortezomib and carfilzomib: a link to clinical adverse events. *Clin Cancer Res* 17: 2734–2743
- Basler M, Mundt S, Muchamuel T, Moll C, Jiang J, Groettrup M, Kirk CJ (2014) Inhibition of the immunoproteasome ameliorates experimental autoimmune encephalomyelitis. *EMBO Mol Med* 6: 226–238

- Blackburn C, Gigstad KM, Hales P, Garcia K, Jones M, Bruzzese FJ, Barrett C, Liu JX, Soucy TA, Sappal DS, Bump N, Olhava EJ, Fleming P, Dick LR, Tsu C, Sintchak MD, Blank JL (2010) Characterization of a new series of non-covalent proteasome inhibitors with exquisite potency and selectivity for the 20S beta5-subunit. *Biochem J* 430: 461–476
- Britton M, Lucas MM, Downey SL, Screen M, Pletnev AA, Verdoes M, Tokhunts RA, Amir O, Goddard AL, Pelphrey PM, Wright DL, Overkleeft HS, Kisselev AF (2009) Selective inhibitor of proteasome's caspase-like sites sensitizes cells to specific inhibition of chymotrypsin-like sites. *Chem Biol* 16: 1278–1289
- de Bruin G, Huber EM, Xin BT, van Rooden EJ, Al-Ayed K, Kim KB, Kisselev AF, Driessen C, van der Stelt M, van der Marel GA, Groll M, Overkleeft HS (2014) Structure-based design of beta1i or beta5i specific inhibitors of human immunoproteasomes. *J Med Chem* 57: 6197–6209
- DeLano WL (2002) *The PyMOL Molecular Graphics System*. San Carlos, CA, USA: DeLano Scientific
- Demo SD, Kirk CJ, Aujay MA, Buchholz TJ, Dajee M, Ho MN, Jiang J, Laidig GJ, Lewis ER, Parlati F, Shenk KD, Smyth MS, Sun CM, Vallone MK, Woo TM, Molineaux CJ, Bennett MK (2007) Antitumor activity of PR-171, a novel irreversible inhibitor of the proteasome. *Cancer Res* 67: 6383–6391
- Emsley P, Lohkamp B, Scott WG, Cowtan K (2010) Features and development of Coot. *Acta Crystallogr Sect D - Biol Crystallogr* 66: 486–501
- Ettari R, Previti S, Bitto A, Grasso S, Zappala M (2016) Immunoproteasome-selective inhibitors: a promising strategy to treat hematologic malignancies, autoimmune and inflammatory diseases. *Curr Med Chem* 23: 1217–1238
- Gallastegui N, Groll M (2012) Analysing properties of proteasome inhibitors using kinetic and X-ray crystallographic studies. *Methods Mol Biol* 832: 373–390
- Groettrup M, Kirk CJ, Basler M (2010) Proteasomes in immune cells: more than peptide producers? *Nat Rev Immunol* 10: 73–78
- Groll M, Ditzel L, Löwe J, Stock D, Bochtler M, Bartunik HD, Huber R (1997) Structure of 20S proteasome from yeast at 2.4 Å resolution. *Nature* 386: 463–471
- Groll M, Kim KB, Kairies N, Huber R, Crews CM (2000) Crystal structure of epoxomicin: 20S proteasome reveals a molecular basis for selectivity of α' , β' -epoxyketone proteasome inhibitors. *J Am Chem Soc* 122: 1237–1238
- Groll M, Huber R (2005) Purification, crystallization, and X-ray analysis of the yeast 20S proteasome. *Methods Enzymol* 398: 329–336
- Groll M, Berkens CR, Ploegh HL, Ovaa H (2006) Crystal structure of the boronic acid-based proteasome inhibitor bortezomib in complex with the yeast 20S proteasome. *Structure* 14: 451–456
- Harris JL, Alper PB, Li J, Rechsteiner M, Backes BJ (2001) Substrate specificity of the human proteasome. *Chem Biol* 8: 1131–1141
- Harshbarger W, Miller C, Diedrich C, Sacchettini J (2015) Crystal structure of the human 20S proteasome in complex with carfilzomib. *Structure* 23: 418–424
- Huber EM, Basler M, Schwab R, Heinemeyer W, Kirk CJ, Groettrup M, Groll M (2012) Immuno- and constitutive proteasome crystal structures reveal differences in substrate and inhibitor specificity. *Cell* 148: 727–738
- Huber EM, Groll M (2012) Inhibitors for the immuno- and constitutive proteasome: current and future trends in drug development. *Angew Chem Int Ed Engl* 51: 8708–8720
- Huber EM, de Bruin G, Heinemeyer W, Paniagua Soriano G, Overkleeft HS, Groll M (2015a) Systematic analyses of substrate preferences of 20S proteasomes using peptidic epoxyketone inhibitors. *J Am Chem Soc* 137: 7835–7842
- Huber EM, Heinemeyer W, Groll M (2015b) Bortezomib-resistant mutant proteasomes: structural and biochemical evaluation with carfilzomib and ONX 0914. *Structure* 23: 407–417
- Huber EM, Heinemeyer W, Li X, Arendt CS, Hochstrasser M, Groll M (2016) A unified mechanism for proteolysis and autocatalytic activation in the 20S proteasome. *Nat Commun* 7: 10900
- Ichikawa HT, Conley T, Muchamuel T, Jiang J, Lee S, Owen T, Barnard J, Nevarez S, Goldman BI, Kirk CJ, Looney RJ, Anolik JH (2011) Novel proteasome inhibitors have a beneficial effect in murine lupus via the dual inhibition of type I interferon and autoantibody secreting cells. *Arthritis Rheum* 64: 493–503
- Kabsch W (2010) XDS. *Acta Crystallogr Sect D - Biol Crystallogr* 66: 125–132
- Muchamuel T, Basler M, Aujay MA, Suzuki E, Kalim KW, Lauer C, Sylvain C, Ring ER, Shields J, Jiang J, Shwonek P, Parlati F, Demo SD, Bennett MK, Kirk CJ, Groettrup M (2009) A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. *Nat Med* 15: 781–787
- Murata S, Sasaki K, Kishimoto T, Niwa S, Hayashi H, Takahama Y, Tanaka K (2007) Regulation of CD8+ T cell development by thymus-specific proteasomes. *Science* 316: 1349–1353
- Muz B, Ghazarian RN, Ou M, Luderer MJ, Kusdono HD, Azab AK (2016) Spotlight on ixazomib: potential in the treatment of multiple myeloma. *Drug Des Devel Ther* 10: 217–226
- Nazif T, Bogyo M (2001) Global analysis of proteasomal substrate specificity using positional-scanning libraries of covalent inhibitors. *Proc Natl Acad Sci U S A* 98: 2967–2972
- Niewerth D, van Meerloo J, Jansen G, Assaraf YG, Hendrickx TC, Kirk CJ, Anderl JL, Zweegman S, Kaspers GJ, Cloos J (2014) Anti-leukemic activity and mechanisms underlying resistance to the novel immunoproteasome inhibitor PR-924. *Biochem Pharmacol* 89: 43–51
- Parlati F, Lee SJ, Aujay M, Suzuki E, Levitsky K, Lorens JB, Micklem DR, Ruurs P, Sylvain C, Lu Y, Shenk KD, Bennett MK (2009) Carfilzomib can induce tumor cell death through selective inhibition of the chymotrypsin-like activity of the proteasome. *Blood* 114: 3439–3447
- Sharma LK, Lee NR, Jang ER, Lei B, Zhan CG, Lee W, Kim KB (2012) Activity-based near-infrared fluorescent probe for LMP7: a chemical proteomics tool for the immunoproteasome in living cells. *ChemBioChem* 13: 1899–1903
- Singh AV, Bandi M, Aujay MA, Kirk CJ, Hark DE, Raju N, Chauhan D, Anderson KC (2011) PR-924, a selective inhibitor of the immunoproteasome subunit LMP-7, blocks multiple myeloma cell growth both in vitro and in vivo. *Br J Haematol* 152: 155–163
- Turk D (2013) MAIN software for density averaging, model building, structure refinement and validation. *Acta Crystallogr D Biol Crystallogr* 69: 1342–1357
- Unno M, Mizushima T, Morimoto Y, Tomisugi Y, Tanaka K, Yasuoka N, Tsukihara T (2002) The structure of the mammalian 20S proteasome at 2.75 Å resolution. *Structure* 10: 609–618
- Vagin AA, Steiner RA, Lebedev AA, Potterton L, McNicholas S, Long F, Murshudov GN (2004) REFMAC5 dictionary: organization of prior chemical knowledge and guidelines for its use. *Acta Crystallogr Sect D - Biol Crystallogr* 60: 2184–2195
- Xin BT, de Bruin G, Huber EM, Besse A, Florea BI, Filippov DV, van der Marel GA, Kisselev AF, van der Stelt M, Driessen C, Groll M, Overkleeft HS (2016) Structure-based design of beta5c selective inhibitors of human constitutive proteasomes. *J Med Chem* 59: 7177–7187