



Published in final edited form as:

ChemMedChem. 2016 October 06; 11(19): 2127–2131. doi:10.1002/cmdc.201600384.

Immunoproteasome $\beta 5i$ -Selective Dipeptidomimetic Inhibitors

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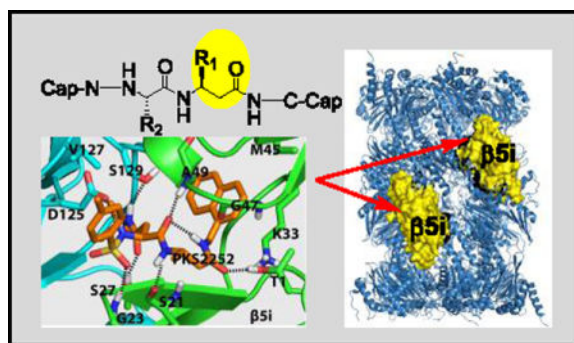
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Abstract

N,C-capped dipeptides belong to a class of noncovalent proteasome inhibitors. Here we report that insertion of a β -amino acid in N,C-capped dipeptides markedly reduces the inhibitory potency against human constitutive proteasome, yet maintains potent inhibitory activity against human immunoproteasome, thus achieving thousands-fold selectivity for $\beta 5i$ over $\beta 5c$. Structure-activity relationship studies reveal that $\beta 5c$ does not tolerate the β -amino acid based dipeptidomimetics as $\beta 5i$ does. In vitro, one such compound inhibited human T cell proliferation. Compounds of this class may have potential as therapeutics for autoimmune and inflammatory diseases with less mechanism-based cytotoxicity than agents that also inhibit the constitutive proteasome.

Entry for the Table of Contents

Class-wide selective inhibitors for immunoproteasome $\beta 5i$ over constitutive proteasome $\beta 5c$ were serendipitously identified by incorporating β -amino acids into dipeptides, of which one compound inhibited T cell proliferation.



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Supporting information for this article is given via a link at the end of the document.

Keywords

β 5i selective inhibitors; β -amino acid peptidomimetics; autoimmune; immunosuppression; T cell proliferation

The ubiquitin-proteasome system is a highly regulated protein-degradation machinery that controls many cellular functions, including removal of damaged proteins to avoid toxic gain-of-function effects, timely removal of cyclins to allow progression of the cell cycle, and inactivation of endogenous inhibitors of transcription factors to allow their nuclear translocation in the cells' response to intracellular or extracellular signals.^[1] Products of the proteasome are also the major source of antigenic oligopeptides for major histocompatibility complex (MHC) class I antigen presentation.^[2] The 20S core of eukaryotic proteasomes contains 2 copies each of 7 different α subunits and 7 different β subunits arranged in four stacked rings in $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ fashion.^[3] Of the 7 β subunits in each of the inner rings, 3 are enzymatically active: β 1 has caspase-like activity, while β 2 is tryptic and β 5 is chymotryptic. Four types of eukaryotic proteasome core particles have been identified. The constitutive proteasome (c-20S) is expressed in all types of cells. The immunoproteasome (i-20S) is constitutively expressed in immune cells at variable, cell-specific levels^[4] and in other cells when induced by cytokines such as interferon- γ . The thymoproteasome (t-20S) is expressed only in the thymus. The spermatoproteasome (sp-20S) is expressed in spermatazoa. Three of the types are distinguished by their active subunits: β 1 β 2 β 5c in c-20S, β 1 β 2 β 5i in i-20S, and β 1 β 2 β 5t in t-20S. The spermatoproteasome differs from c-20S only in α 4, giving α 4sp β 1 β 2 β 5c in sp-20S. The proteasome is a validated drug target for multiple myeloma and mantle cell lymphoma. Several classes of proteasome inhibitors have been developed.^[5] Because of the hypersensitivity of immunoglobulin-secreting plasma cells to proteasome inhibition,^[6] the first-approved proteasome inhibitor drug, bortezomib, has also been used to control allograft rejection^[7] and refractory systemic lupus erythematosus.^[8]

Given its mechanism-based toxicity, bortezomib is generally used in suboptimal doses in patients with non-malignant disease. Suppression of unwanted immune responses might be achieved with less toxicity if inhibitors were available that target i-20S but spare c-20S. Encouraging this notion, mice with combined deficiency of β 1i, β 2i and β 5i are viable, fertile and generally immune-competent.^[9] Moreover, a relatively selective β 5i inhibitor, ONX-0914 (formerly known as PR-957; Fig. 1A), has shown efficacy in animal models of rheumatoid arthritis, systemic lupus erythematosus, colitis and multiple sclerosis.^[10] However, ONX0914 is an irreversibly-acting peptide epoxyketone. The combination of limited isoform selectivity and irreversibility raises questions about possible toxicity during long-term use of such an agent. Chronic use of a similar peptide sequence with other irreversibly acting warheads, such as sulfonyl fluoride (Fig. 1B), is subject to the same concern, as is use of a compound that reacts irreversibly with Cys48 of β 5i (Fig. 1C).^[11]

To identify reversible inhibitors with a high degree of selectivity for β 5i over β 5c, we took advantage of our ongoing effort to develop inhibitors that target the *Mycobacterium tuberculosis* proteasome (Mtb20S) but spare both hu c-20S and i-20S. We observed that

Mtb20S and $\beta 5i$ both prefer bulky aromatic amino acids at the S1 pocket and both are inhibited by 1,3,4-oxathiazol-2-ones (Fig. 1D) that spare hu c-20S.^[12] N,C-capped dipeptides are a versatile class of cell-permeable compounds that can be tailored for proteasomes of different species or forms.^[13] This encouraged us to test if noncovalent N,C-capped dipeptides that we have reported to be selective for Mtb20S over both human $\beta 5i$ and $\beta 5c$ ^[13b] could be optimized to be selective for human $\beta 5i$ over $\beta 5c$. Here we report achievement of this goal with β -amino acid based N,C-capped dipeptidomimetics.

Crystal structures of N,C-capped dipeptides with yeast proteasome (Ye20S) showed the C-cap in the S1 pocket, the P1 amino acid side chain in the S2 pocket, the P2 amino acid side chain in the S3 pocket and the N-cap in the S4 pocket.^[14] For Mtb20S, both an S3 Asn side chain and an S1 naphthyl group are major determinants for potency and a diethyl amide chain of the Asn for selectivity.^[13b] We hypothesized that because $\beta 5i$ but not $\beta 5c$ was inhibited by 1,3,4-oxathiazol-2-ones, and X-ray structures of Mtb20S with 1,3,4-oxathiazol-2-ones revealed marked conformational changes, $\beta 5i$ might be more plastic than $\beta 5c$. This encouraged us to incorporate β -amino acids into the inhibitor while retaining the bulky 1-naphthalenemethyl C-cap. β -amino acids were incorporated separately at P1 or P2, or simultaneously at both P1 and P2. Synthetic routes are illustrated for mono- β amino acid dipeptidomimetic inhibitors (Scheme 1, S1) and dual- β amino acid dipeptidomimetics (Scheme S2). We confirmed all intermediates and final products by NMR and the final products by high-resolution MS.

We first introduced (L)- β -Ala into the P1 position (Table 1). C-cap was fixed as 1-naphthalenemethyl group. P2 amino acid was Asn(*t*-Bu). For the N-cap, we used substituents that varied from hydrophobic phenylpropionate (PKS2251) to polar 5-methylisoxazole-3-carboxylate (PKS2249) and a tosyl moiety (PKS2252). Commercially available boc-(L)- β -homoalanine and 1-naphthalenemethylamine were first coupled using HATU to give boc-(L)- β -Ala-NHCH₂-1-naphthyl. Boc-deprotection and coupling with Boc-Asp(OBn)-OH gave PKS2265. The *O*-benzyl protecting group of PKS2272 was removed by hydrogenolysis and the product converted to *t*-butyl amide PKS2272. Boc-deprotection of PKS2272 and subsequent reaction with a different N-cap gave the final compounds PKS2249, PKS2251, PKS2252, PKS2260 and PKS2295. PKS3054 was synthesized by coupling PKS2245 with PKS3052 (Fig. S1). Synthesis of P2-(L)- β -Gln-based dipeptidomimetics (PKS2278, PKS2279) was accomplished in a similar fashion (Fig. 1B). Boc-(L)-Ala-OSu was coupled with 1-naphthalenemethylamine and the product was mixed with TFA to remove boc protection group. The freed amine was coupled with (L)- β -Glu(OBn)-COOH, yielding PKS2266. After removal of the benzyl group by hydrogenolysis, the resulting product was converted to *t*-butyl amide PKS2271. Boc-deprotection of PKS2271 and subsequent reaction with phenylpropionate or tosyl gave PKS2278 and PKS2279, respectively. PKS2291 and PKS2292, in which both P1 and P2 are β -amino acids, were synthesized as depicted in Figure S2.

IC₅₀s against $\beta 5i$ and $\beta 5c$ were determined by a reported method (Table 1).^[13b] Although the potency of these compounds varies with the N-caps, they were all markedly selective for $\beta 5i$ over $\beta 5c$, with potency and selectivity indices ranging in tandem from high to low in the order of tosyl- (5.5 nM and 13,600-fold) > phenylpropionate (57 nM and 1,333-fold) > 5-

methylisoxazole-3-carboxylate (190 nM and 465-fold). Further changes at N-cap on this scaffold with phenylsulfonamide, Boc, or cyclopropyl sulfonamide retained selectivity without further improvement in potency. N-cap (2-(1H-indol-3-yl)-2-oxoacetyl) improved potency to 5.3 nM, but reduced the selectivity index to 57-fold. Replacing the P1- β -(L)-Ala with (L)-Ala and P2-(L)-Asn with β -(L)-Gln in compound PKS2251 and PKS2252 resulted in compounds PKS2278 and PKS2279, whose potencies were reduced by 58-fold and 2.5-fold, respectively, indicating that the sulfonamide is a better N-cap than phenylpropionate to retain potency in P2 β -amino acid-based dipeptidomimetics. Furthermore, sulfonamide reserved largely unchanged selectivity. This dataset indicates that incorporation of β -amino acids in either position of this class of inhibitors is tolerated by β 5i but not by β 5c. However, incorporation of β -amino acids in both P1 and P2 at the same time markedly reduced the potency.

To begin to understand how the binding mode of the β -amino acid based dipeptidomimetics accounts for their marked selectivity, we created a homology model of a dimer of β 5i and β 6 of hu i-20S using the crystal structure of the mouse i-20S as template.^[15] Assuming each moiety would similarly occupy S1–S4 pockets as described in Fig. 1, PKS2252 and PKS2279 were docked *in silico* in the active site (Figure 2). We found that inserting a carbon in either compound largely retains the hydrogen bond network between the backbone of the enzyme and the dipeptide ligands as shown by Blackburn et al.^[14] Additionally, the N-cap sulfonamide is predicted to form a hydrogen bond with Gly23, and the side chain of P2 N-*t*-butyl amide is predicted to make two hydrogen bonds with Ser27 of the β 5i and Ser129 of the β 6, respectively. Ser27 is replaced by Ala27 in β 5c. The one carbon extension in P1 of PKS2252 (Fig. 2A) is predicted to let the compound form a hydrogen bond between the carbonyl and the β -OH of the active site Thr1. In contrast, the one carbon extension in P2 of PKS2279 (Fig. 2B) is predicted not to lead to a hydrogen bond with the β -OH of the active site Thr1. Thus, P1- β -amino acid peptidomimetics and P2- β -amino acid peptidomimetics are likely to bind to β 5i differently. As indicated in other reports,^[15] S1 in β 5i pocket is significantly bigger than that in β 5c. Therefore, we postulate that the marked isoform selectivity displayed by the β -amino acid dipeptidomimetics may result from several factors: change of the key amino acids of the enzymes that are involved in the binding of the ligands, the enlarged S1 pocket in β 5i over β 5c and the plasticity of the β 5i that allows it to undergo conformational changes to accommodate the one carbon extension in either P1 or P2 position.^[12b]

Next, we tested the impact of PKS3054 on the function of cells a substantial proportion of whose proteasomes contain i-20S. As shown in Table 2, PKS3054 inhibited the activation of human CD4-positive T lymphocytes by tetanus toxoid (EC50 0.20 μ M); and the proliferation of T cells within PBMCs as stimulated by anti-CD3 antibody (EC50 0.57 μ M). PKS3054 was not cytotoxic against PBMCs under these conditions (Figure S1).

In summary, we have developed a series of noncovalent β -amino acid-based peptidomimetic immunoproteasome-selective inhibitors. The surprising impact on isoform selectivity of incorporating a β -amino acid into N,C-capped dipeptides suggests a subtle yet distinct structural variation at the binding sites of the i-20S and c-20S β 5 subunits. The SAR indicates that there is room to test further modifications in the interest of improving

pharmacokinetic properties while retaining potency and selectivity. PKS3054 inhibits the activation of human T cells without apparent cytotoxicity, providing an early indication that such compounds may have utility in the control of immune disorders. Additionally, a recent survey found upregulated expression of i-20S over c-20S in lymphoblastic leukemia cells and multiple myeloma cell lines,^[16] suggesting that selective inhibition of i-20S may have therapeutic potential in treatment of those malignancies. in Text Paragraph.

Experimental Section

Protocols of the biological experiments; docking studies; synthesis and compound characterization of all new compounds; Scheme S1 and S2; Figure S1; Table S1 and S2 are included in supporting information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

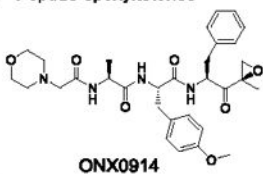
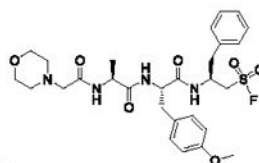
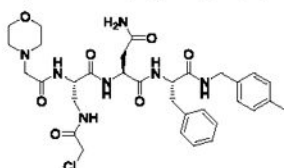
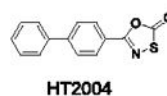
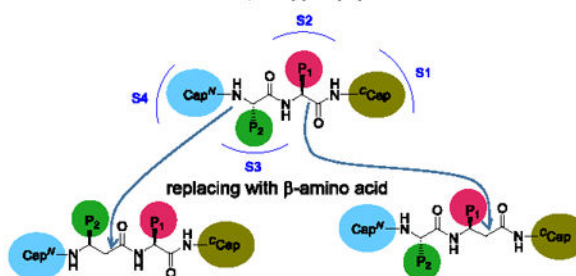
Acknowledgments

This work is supported by Alliance for Lupus Research (GL), Daedalus Fund for Innovation at Weill Cornell Medicine (GL) and by the Milstein Program in Chemical Biology and Translational Medicine (CN). We thank Ms. Rong Wang and Dr. George Sukenick at Memorial Sloan Kettering Cancer Center for assistance with high-resolution mass spectrometry. We thank Dr. J. David Warren at The Abby and Howard P. Milstein Synthetic Chemistry Core Facility at Weill Cornell Medicine for assistance. We thank Dr. Jane Salmon and Dr. Xiaoping Qing at Hospital for Special Surgery, New York, for discussions. The Department of Microbiology and Immunology is supported by the William Randolph Hearst Foundation.

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Irreversible Inhibitors**A** Peptide epoxyketones**B** Peptide sulfonyl fluorides**C** Cys48 reacting N,C-capped peptides**D** 1,3,4-oxathiazol-2-ones**Reversible Inhibitors****E** Noncovalent N,C-capped peptidomimetics**Figure 1.**

Structures of representative irreversible β 5i selective inhibitors and schematic for β -amino acid based noncovalent N,C-capped peptidomimetics. **A)** Peptide epoxyketones; **B)** Peptide sulfonyl fluoride; **C)** β 5i Cys48 reacting N,C-capped peptides; **D)** 1,3,4-oxathiazol-2-ones; **E)** Schematic for conversion of N,C-capped dipeptides to β -amino acid-based dipeptidomimetics. N- and C- caps bind to S4 and S1 pockets of the β 5, respectively. P1 binds to S2 and P2 binds to S3.

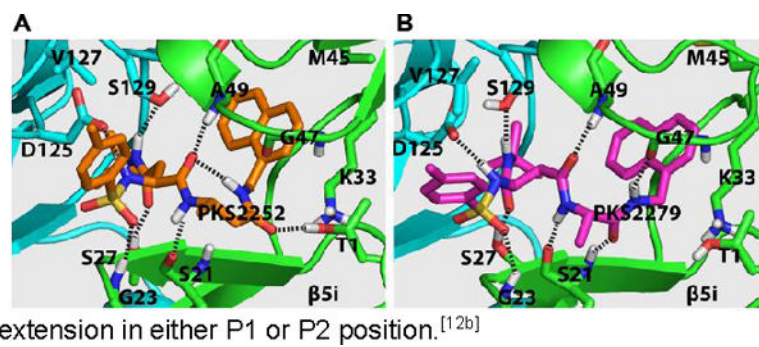
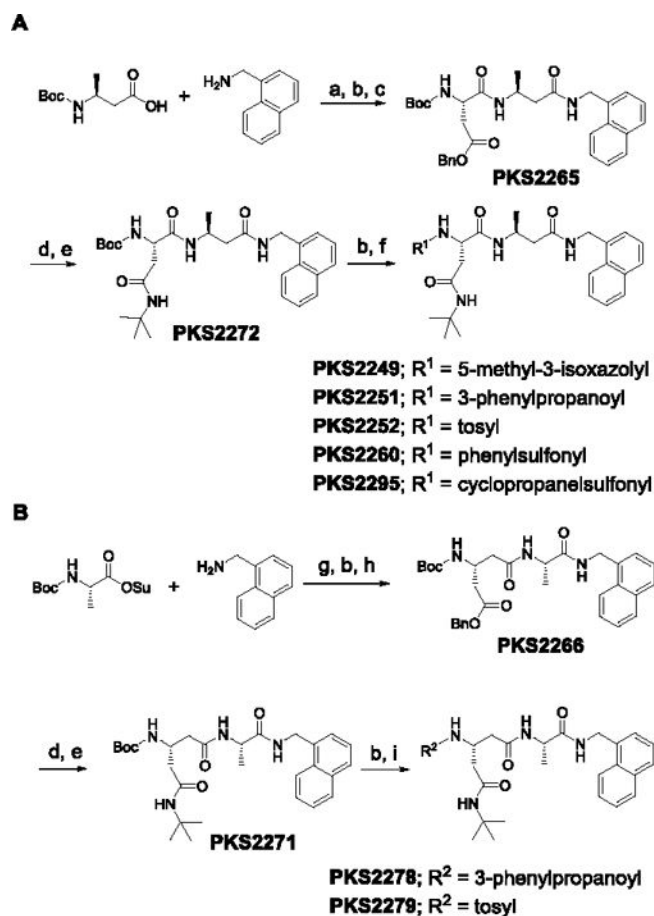


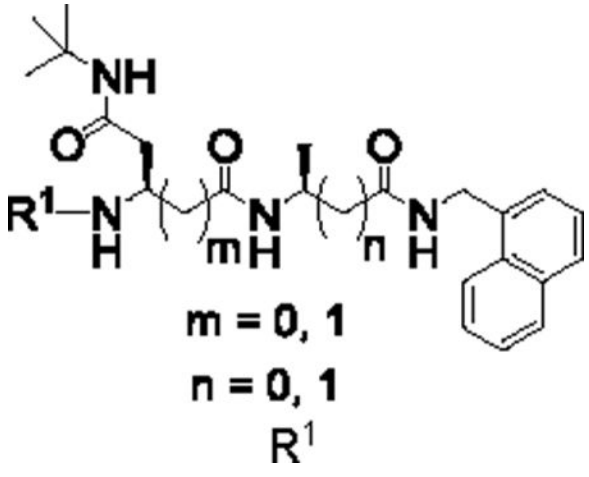
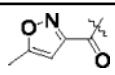
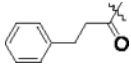
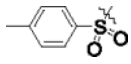
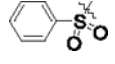


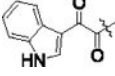
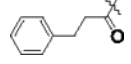
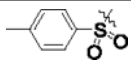
Figure 2.
The predicted binding modes of PKS2252 (A) and PKS2279 (B) in $\beta 5i\beta 6$ dimer of the human i-20S.

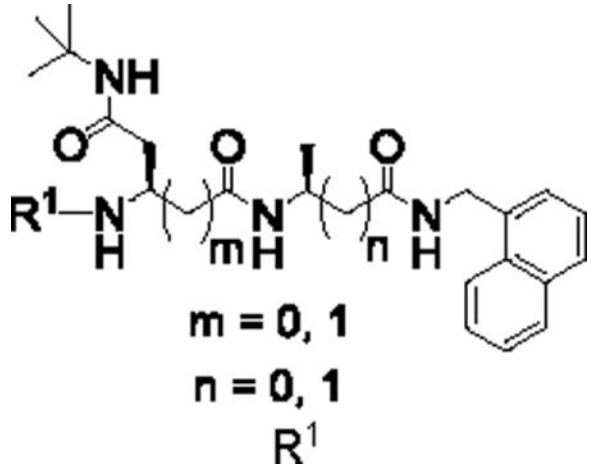
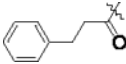
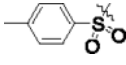
**Scheme 1.**

Synthetic route of P1-β-amino acid-based (A) and P2-β-amino acid-based (B) peptidomimetics. Reagents and conditions: a) HATU, HOAt, Hunig's base, DMF; b) TFA in DCM (20%); c) Boc-Asp(OBn)OH, HATU, HOAt, Hünig's base, DMF; d) Pd/C (10%), H₂, methanol; e) ^tBuNH₂, EDC, HOBT, Hünig's base, DMF; f) R¹COOH, HATU, HOAt, Hünig's base, DMF or R¹Cl, Et₃N, DCM; g) Et₃N, DCM, 0°C – RT; h) Boc-β-Glu(OBn)OH, HATU, HOAt, Hünig's base, DMF; i) R²COOH, HATU, HOAt, Hünig's base, DMF or R²Cl, Et₃N, DCM.

Table 1

IC50s of dipeptidomimetics against $\beta 5i$ and $\beta 5c$. Data were average of at least three independent experiments.

ID	 $m = 0, 1$ $n = 0, 1$ R^1	IC50 (μM)*		
		$\beta 5i$ (ANW)	$\beta 5c$ (LLVY)	SI ^{APP}
P1: β -AA	(m=0, n=1)			
PKS2249		0.190 \pm 0.018	88.38 \pm 5.81	465
PKS2251		0.057 \pm 0.012	76.02 \pm 25.61	1333
PKS2252		0.0055 \pm 0.0010	74.87 \pm 10.52	13600
PKS2260		0.012 \pm 0.001	13.85 \pm 3.79	1154
PKS2272		0.135 \pm 0.022	>100	>600
PKS2295		0.148 \pm 0.016	22.18 \pm 5.77	150
PKS3054		0.0053 \pm 0.0003	0.30 \pm 0.08	57
P2: β -AA	(m=1, n=0)			
PKS2278		3.35 \pm 0.64	>100	>44
PKS2279		0.014 \pm 0.002	35.3	2521
P1, P2: β -AAs	(m = 1, n = 1)			

ID	 $m = 0, 1$ $n = 0, 1$ R^1	IC50 (μM) [*]		
		$\beta 5i$ (ANW)	$\beta 5c$ (LLVY)	SI ^{app}
PKS2291		9.60 ± 0.66	>100	>11
PKS2292		>100	>100	-

AA: amino acid.

ANW: ac-ANW-AMC.

LLVY: suc-LLVY-AMC.

SI^{app}: apparent selectivity index = IC50 β 5c / IC50 β 5i.

* Data were given as mean ± SEM.

Table 2

Effects of PKS3054 in assays of adaptive immune responses

Immunological assays ^I	EC50 (μ M) [*]
Hu CD4 T cell response to tetanus toxoid	0.20 \pm 0.04
Hu PBMC response to anti-CD3	0.57 \pm 0.62

^I Assays with PBMCs from 3 healthy donors, each in triplicate.^{*} : Data were given as mean \pm SEM.

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