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Synthesis and Evaluation of Macrocyclic Peptide Aldehydes as Potent and Selective Inhibitors of the 20S Proteasome

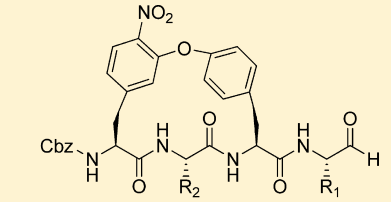
David L. Wilson,[†] Isabel Meininger,[‡,§] Zack Strater,[†,§] Stephanie Steiner,[†] Frederick Tomlin,[†] Julia Wu,[†] Haya Jamali,[†] Daniel Krappmann,[‡] and Marion G. Götz*[,†]

[†]Department of Chemistry, Whitman College, Walla Walla, Washington 99362, United States

[‡]Research Unit Cellular Signal Integration, Institute of Molecular Toxicology and Pharmacology, Helmholtz Zentrum München−German Research Center for Environmental Health, Neuherberg 85764, Germany

Supporting Information

ABSTRACT: This research explores the first design and synthesis of macrocyclic peptide aldehydes as potent inhibitors of the 20S proteasome. Two novel macrocyclic peptide aldehydes based on the ring-size of the macrocyclic natural product TMC-95 were prepared and evaluated as inhibitors of the 20S proteasome. Both compounds inhibited in the low nanomolar range and proved to be selective for the proteasome over other serine and cysteine proteases, particularly when compared to linear analogues with similar amino acid sequences. In HeLa cells, both macrocycles efficiently inhibited activation of nuclear factor-κB (NF-κB) transcription factor by blocking proteasomal degradation of the inhibitor protein IκBα after cytokine stimulation. Due to their covalent mechanism of binding these compounds represent a 1000-fold increase in inhibitory potency over previously reported noncovalently binding TMC-95 analogues. Molecular modeling of the macrocyclic peptides confirms the preference of the large S_3 pocket for large, hydrophobic residues and the ability to exploit this to improve selectivity of proteasome inhibitors.



7 $R_1 = CH_2CH(CH_3)_2$, $R_2 = CH_2CH(CH_3)_2$, 20S proteasome (CL), K_i = 241 nM

8 $R_1 = CH_2CH(CH_3)_2$, $R_2 = CH_2$-Ph(4-OMe), 20S proteasome (CL), K_i = 54.5 nM

KEYWORDS: *Proteasome, inhibitor, macrocycle, aldehyde, multiple myeloma*

The 26S proteasome is a barrel-shaped, multicatalytic protease that uses an N-terminal threonine residue to hydrolyze polyubiquitinated substrates in the ubiquitin−proteasome pathway (UPP). It consists of a 20S core particle (CP) and two 19S regulatory caps. The 20S CP of the proteasome comprises four circular heptamers consisting of α and β subunits stacked in an $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ fashion. The outer α subunits serve a regulatory function, while the two inner rings each possess three catalytic activities. These activities vary in their substrate specificity; in the human constitutive proteasome the β1 subunit has peptidyl-glutamyl hydrolyzing activity (PGPH) activity, the β2 subunit has trypsin-like (TL) activity, and the β5 subunit has chymotrypsin-like (CL) activity.[1]

The UPP is responsible for the targeted destruction of many substrates including regulatory proteins such as the cyclin-dependent kinase inhibitor $p27^{kip1}$ and the p53 tumor suppressor protein. Further, activation of transcription factor NF-κB is a prototype example for the participation of the UPP in signal transduction. Upon cell stimulation with cytokines, and many other agents, cytosolic NF-κB inhibitor proteins such as IκBα are phosphorylated by the IκB kinase (IKK) complex, and phosphorylation marks the IκBs for ubiquitin-dependent proteasomal degradation.[2] NF-κB induces expression of many genes responsible for cell proliferation and resistance to apoptosis.[3] Thus, due to the involvement of many UPP substrates in the regulation of apoptosis and the cell cycle, the proteasome has been of great interest as a therapeutic target for cancer treatment.[4,5]

In particular, the hematologic malignancy multiple myeloma (MM) has been shown to be receptive to treatment with proteasome inhibitors.[6,7] Proteasome inhibition in plasma cells results in an unfolded protein response through ER-stress activated expression of the proapoptotic signaling protein NOXA, a BH3-only member of the Bcl-2 family of proteins.[8−10] In 2012, carfilzomib became the second FDA approved proteasome inhibitor for the treatment of multiple myeloma.[11]

To date, most synthetic proteasome inhibitors consist of a short peptide with an electrophilic trap designed to covalently bind to the nucleophilic Thr1Oγ of the proteasome's catalytic β subunits. Electrophilic traps that have been reported include aldehydes, boronic acids, epoxyketones, α-ketoaldehydes, β-lactones, and vinyl sulfones.[12] Many proteasome inhibitors, including the FDA approved peptidyl boronic acid bortezomib, suffer from adverse off-target effects. In the case of bortezomib, coinhibition of serine proteases essential for neural health leads to peripheral neuropathy in patients with high dosing schedules.[13,14] For this reason, continued research toward

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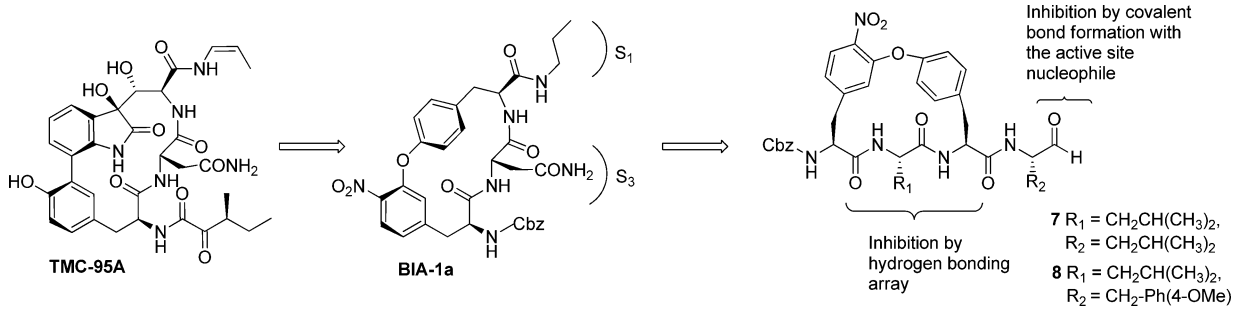

Figure 1. Inhibitor design based on the TMC-95A macrocycle.

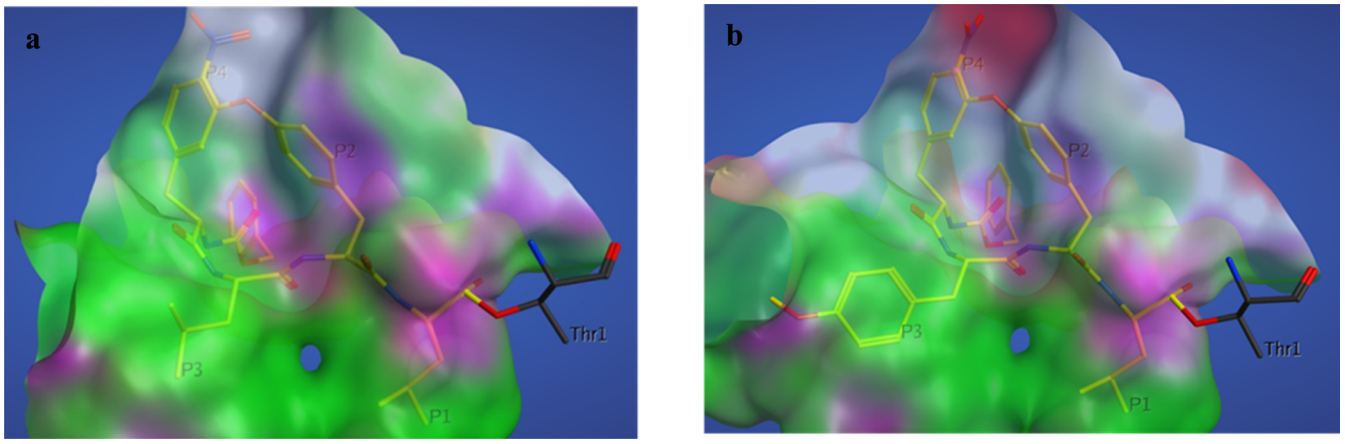

Figure 2. Minimum energy conformation of **7** (a) and **8** (b) bound to the CL active site. The molecular surface map of the binding pocket (green = nonpolar, purple = polar) shows the P_3 residue more adequately filling the S_3 pocket. Furthermore, the biaryl ether macrocycle is well accommodated by the shallow S_2 and S_4 pockets.

potent and selective inhibitors of the 20S proteasome is of great interest.

Compounds **7** and **8** (Figure 1) reported in this study represent the first macrocyclic peptide aldehydes with selectivity for the 20S proteasome. The compounds were designed to achieve the potency and reversible binding mechanism of peptide aldehydes such as MG-132 (Cbz-Leu-Leu-Leu-H, K_i = 4 nM[15]) as well as incorporate a macrocycle to increase their selectivity. The macrocyclic natural product TMC-95A (Figure 1), a noncovalent reversible inhibitor of the proteasome, is known for its potency and high selectivity. In crystallographic studies, Groll et al. found that the large, aromatic macrocycle of TMC-95A fills the S_2 and S_4 pockets of the proteasome.[16] This confers high specificity because the large, nonspecific S_2 pocket and shallow, hydrophobic S_4 pocket of the proteasome can accommodate the sterically demanding macrocycle, which most other proteases cannot.[12,13] Additionally, the macrocyclic clamp forces the inhibitor backbone into an extended β-sheet like conformation, which avoids loss of entropy upon binding.

A series of biaryl ether (BIA) analogues of TMC-95A were previously reported by Groll et al. (Figure 1). However, they show severely reduced potency, which is most likely due to their noncovalent mechanism of inhibition.[17] Li et al. have demonstrated successful inhibition of the *Plasmodium falciparum* proteasome using macrocyclic biaryl ethers.[18] A SAR by Abell et al. on macrocyclic peptide aldehyde inhibitors of ovine calpain found that the addition of an aldehyde to a peptide macrocycle can increase potency as much as ~1000-fold.[19] The results of Abell et al. combined with the proteasome's flexible S_2 and S_4 pockets suggest that a macrocyclic peptide aldehyde with a suitable peptide sequence will yield a potent and selective inhibitor of the 20S proteasome. Other desirable pharmacological properties introduced by the macrocycle include increased metabolic stability, cellular uptake, and tissue distribution.[20,21]

The size and position of the macrocycle was based on the natural product TMC-95. The inhibitors **7** and **8** incorporate a para−meta biphenyl ether linkage that results in a 17-membered macrocycle, mimicking the ring size and structure of TMC-95A while avoiding the synthetic challenges associated with the oxidized tryptophan and biaryl linkage. Previous studies by Kaiser et al. have shown that the stereospecific hydroxyl groups of TMC-95A are not essential for proteasome binding.[22]

In accordance with previously synthesized biaryl ether analogues of TMC-95A, a modified Phe at P_4 and Tyr at P_2 were used to generate the ether macrocycle.[23] Leu was selected for the P_1 position based on the peptide sequence of carfilzomib and MG-132 and is thought to form favorable interactions with Met45 in the S_1 pocket of the β5 subunit.[24] The P_3 position was varied between two hydrophobic residues, Leu and Phe(OMe). The P_3 Leu residue of **7** mimics the sequence of MG-132 and carfilzomib, while the Phe(OMe) residue of **8** was chosen for its increased hydrophobic bulk. A SAR study by Adams et al. determined that bulky, hydrophobic residues in the P_3 position can dramatically increase inhibitor potency of peptide aldehydes as much as 1000-fold when compared to inhibitors with smaller, hydrophobic residues in the P_3 position.[15] A subsequent SAR study by Momose et al.

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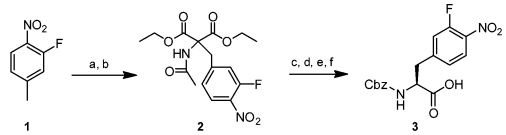
showed Phe(OMe) to be potent in the P_3 position for peptide aldehyde inhibitors of the proteasome.[25] Since the N-terminus of the inhibitor has negligible interactions with the proteasome,[16] we decided to incorporate a Cbz-group for its synthetic stability.

To further explore interactions at the P_3 position, QM/MM simulations were carried out on **7** and **8** to determine their preferred binding geometries. Using the Amber12:EHT force field and the crystal structure collected by Harshbarger et al.,[26] the minimum energy conformation of the $\beta4\beta5$ dimer bound to **7** and **8** was calculated. Each inhibitor was modeled as a hemiacetal adduct of the catalytic Thr1. As expected, EM calculations showed both inhibitors adopting almost identical geometries with the biaryl ether macrocycle extending into the shallow S_2 and S_4 pockets and the P_1 and P_3 side chains penetrating deep into the S_1 and S_3 pockets. The primary difference between **7** and **8** was the extent to which they filled the spacious S_3 pocket (Figure 2). The smaller Leu side chain of **7** did not fill the large, hydrophobic S_3 pocket as well as the Phe(OMe) side chain of **8**, suggesting that **8** would bind more tightly and have higher specificity for the proteasome than **7**. Additionally, the P_1 Leu of both inhibitors is situated approximately 3.4 Å from the Met45 residue, which is in good agreement with previous findings that Met45 is responsible for controlling the $\beta5$ subunit's specificity for hydrophobic residues.[24] We chose to specifically target the CL activity of the $\beta5$ subunit since it has been found to be most essential for proteasome function.[27] Based on both previous SAR studies and our QM/MM simulations exploring S_3/P_3 interactions, we hypothesized that **8** would be a more potent and specific inhibitor for the 20S proteasome.

The synthesis of **7** and **8** was achieved by a convergent approach (Schemes 1 and 2) of N-terminal Cbz-protected

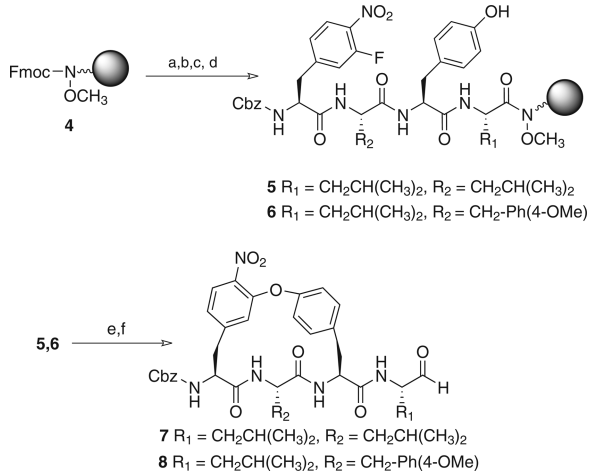
Scheme 1. Synthesis of Enantiomerically Pure Phenylalanine Analogue[a]



[a]Reagents and conditions: (a) NBS, AIBN, CCl_4, reflux, 24 h; (b) diethyl acetamidomalonate, NaH, DMF, rt, 4 h; (c) HCl, reflux, 24 h; (d) Ac_2O, $NaHCO_3$, dioxane, H_2O, rt, 12 h; (e) acylase I, pH 7.5, 37 °C, 20 h; (f) Cbz-OSu, $NaHCO_3$, dioxane, H_2O, rt, 12 h.

Phe(3-F, 4-NO_2) **3** coupled to a resin-bound C-terminal tripeptide. Compound **3** was first used by Boger et al. and Janetka et al. to form biaryl ether macrocycles with a Tyr residue in the $i + 2$ position by an intramolecular S_NAr mechanism.[28,29] In this study, the improved chemoenzymatic synthesis of **3** described by Vergne et al.[30] allowed access to the enantiomerically pure phenylalanine analogue, which was then Cbz protected. The C-terminal tripeptides were synthesized by solid phase peptide synthesis on a Weinreb amide resin using commercially available, Fmoc-protected amino acids. After coupling the modified Phe (**3**), the resin bound tetrapeptides **5** and **6** were cyclized according to the procedure developed by Boger et al.[28] The macrocycle was cleaved from the resin using lithium aluminum hydride to generate a peptide aldehyde from a Weinreb amide.[31] For proof of concept we also prepared linear analogue **9** (Cbz-Phe-Phe(4-OMe)-Phe-Leu-H) with an

Scheme 2. Solid Phase Synthesis of Peptide Aldehydes[a]



[a]Reagents and conditions: (a) (i) 20% v/v piperidine, DMF, rt, 30 min; (ii) Fmoc-Leu-OH, HATU, DIPEA, DMF, rt, 6 h; (b) (i) 20% v/v piperidine, DMF, rt, 30 min; (ii) Fmoc-Tyr-OH, HATU, DIPEA, DMF, rt, 2 h; (c) (i) 20% v/v piperidine, DMF, rt, 30 min; (ii) Fmoc-Phe(4-OMe)−OH or Fmoc-Leu-OH, HATU, DIPEA, DMF, rt, 2 h; (d) (i) 20% v/v piperidine, DMF, rt, 30 min; (ii) **3** (1.5 equiv), HATU, DIPEA, rt, 2 h. (e) K_2CO_3, $CaCO_3$, 3 Å molecular sieves, DMF, 45 °C, 4 days; (f) (i) $LiAlH_4$, THF, 0 °C, 30 min; (ii) $KHSO_4$ (sat.), K, Na tartrate (sat.), THF, rt, 40 min.

amino acid sequence similar to compound **8**. The linear tetrapeptide **9** was synthesized by solid phase approach on a Weinreb amide with Fmoc-protection chemistry (not shown in scheme).

Fluorometric kinetic enzyme inhibition assays were performed to measure K_i values for **7** and **8** for the three proteasomal activities as well as the serine protease chymotrypsin and cysteine proteases cathepsin B and m-calpain (Table 1). In addition we tested proteasome inhibitor standard MG-132 (Cbz-Leu-Leu-Leu-H), the linear analogue **9**, bortezomib and carfilzomib as a reference for **7** and **8** since reported K_i values vary depending on assay conditions. With respect to the three proteasome activities, both inhibitors as well as MG-132 showed high selectivity for the CL active site stemming from the hydrophobic P_3 residues. Comparing inhibitory potency for the CL activity, **7** (K_i = 241 nM) is less potent than MG-132 (K_i = 63.5 nM), while **8** (K_i = 54.5 nM) is as potent as MG-132 and 4-fold more potent than **7**. These data confirm our initial hypothesis that the peptide sequence of **8** could achieve improved binding compared to **7**. Linear analogue **9** showed 2-fold higher potency compared to macrocycle **8**, likely due to the less flexible design of the macrocycle. No inhibition was detected for either **7** or **8** for the PGPH activity at concentrations up to 100 μM, due to unfavorable interactions between the hydrophobic P_3 residues and the charged Lys45 residue in the $\beta1$ subunit. The TL activity was moderately inhibited by **7** but not inhibited by **8**.

Neither compound **7** or **8** inhibited the serine protease chymotrypsin, and both compounds showed similar inhibition of the clan CA cysteine protease m-calpain to MG-132 (K_i = 653 nM) with K_i = 2028 nM for **7** and K_i = 2983 nM for **8**. Inhibitor **8** is ~55-fold and analogue **9** is ~61-fold more selective for the proteasome over m-calpain, which renders these more specific than **7** and MG-132 that display 8- and 10-fold selectivity, respectively. Importantly, both macrocyclic

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Table 1. Inhibitory Potency of 7 and 8 in Comparison to 9, MG-132, bortezomib, and carfilzomib

	K_i in nM					
	20S proteasome					
inhibitor	CL-activity[a]	TL-activity[a]	PGPH-activity[a]	chymotrypsin[a]	cathepsin B[a]	m-calpain[a]
7	241 ± 62.2	8,040	N.I.[b]	N.I.[b]	3047 ± 1	2028 ± 319
8	54.5 ± 4.9	N.I.[b]	N.I.[b]	N.I.[b]	30345 ± 900	2983 ± 621
9	28.0 ± 9.9	N.T.[c]	N.T.[c]	N.I.[b]	2285 ± 1,700	1708 ± 99
MG-132	63.5 ± 21.5	N.I.[b]	3700	N.I.[b]	230 ± 206	653 ± 211
bortezomib	24.0 ± 2.8	N.T.[c]	N.T.[c]	N.I.[c]	N.I.[c]	N.I.[c]
carfilzomib	25.5 ± 12.0	N.T.[c]	N.T.[c]	N.I.[c]	N.I.[c]	N.I.[c]

[a]Assay protocols are provided in the Supporting Information. [b]No inhibition at 100 μM inhibitor concentration. [c]Not tested.

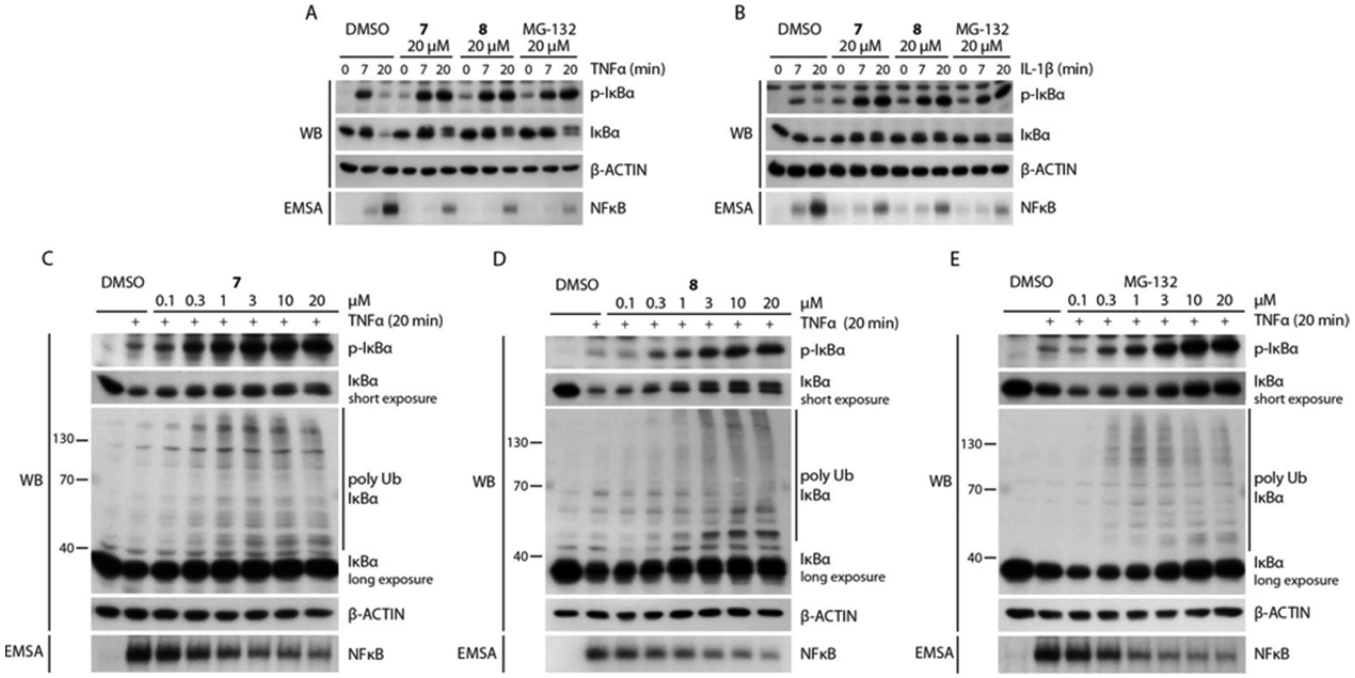

Figure 3. Macrocyclic peptides **7** and **8** inhibit proteasomal IκBα degradation and NF-κB activation in HeLa cells. (A,B) IκBα phosphorylation (S32/36) and degradation as well as NF-κB DNA binding were analyzed by Western blot and EMSA, respectively. β-ACTIN was used as loading control. (C–E) HeLa cells were incubated and analyzed as in A and B with increasing concentrations (0.1, 0.3, 1, 3, 10, and 20 μM) of compounds **7**, **8**, or MG-132 before TNFα stimulation.

aldehydes showed better selectivity for the proteasome over MG-132 as well as linear analogue **9** when compared to the clan CA protease cathepsin B suggesting that the peptide macrocycle is responsible for increased specificity of **7** and **8** over the linear peptide aldehydes. In particular, **8** was determined to be 550-fold more selective for the proteasome than for cathepsin B compared to MG-132's 4 and compound **9**'s 80-fold selectivity. As the CL active site has a preference for binding large hydrophobic residues in the peptide substrate, which correlates to the peptide sequence specificity of cysteine proteases such as calpains and lysosomal cathepsins, the increased selectivity for proteasomal inhibition of macrocycles **7** and **8** compared to the linear MG-132 and **9** is particularly advantageous. Although our assays indicate that bortezomib and carfilzomib are more potent and selective inhibitors of the 20S proteasome (bortezomib K_i = 24.0 nM, carfilzomib K_i = 25.5 nM), due to their different modes of action boronic acid and epoxyketone warheads are expected to possess higher potency and selectivity than aldehydes. Our results suggest that cyclizing warhead-containing linear analogues could further enhance specificity.

In order to assess the potency of the new macrocyclic peptides in cellular inhibition of the 26S proteasome, we evaluated the effects of **7** and **8** on NF-κB signaling and compared these to MG-132, which potently inhibits stimulus-induced NF-κB activation.[32] For this, we incubated the human cervix carcinoma cell line HeLa with compound **7** or **8** or MG-132 (20 μM each) for 2 h prior to stimulation with the pro-inflammatory cytokines TNFα or IL-1β (Figure 3A,B). Cellular effects on NF-κB signaling were monitored by determining phosphorylation and degradation of the NF-κB inhibitor IκBα by Western blot as well as NF-κB DNA binding by electrophoretic mobility shift assays (EMSA). Just like the well-characterized proteasomal inhibitor MG-132, **7** and **8** severely impaired TNFα and IL-1β induced IκBα degradation and concomitant NF-κB activation. IKKβ catalyzes phosphorylation of IκBα at serine 32/36, which marks the inhibitor for UPP. All three proteasome inhibitors promote strong accumulation of phosphorylated IκBα, providing evidence that the macrocyclic peptides **7** and **8** are acting at the level of the proteasome in the inhibition of NF-κB signaling.

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To compare the potency of **7** and **8** and MG-132 in cellular proteasome inhibition, we monitored dose-dependent effects on TNFα induced NF-κB signaling (Figure 3C–E). As evident from the inhibition of IκBα degradation and NF-κB DNA binding as well as accumulation of phosphorylated IκBα, all three proteasome inhibitors impaired NF-κB signaling at 300 nM with maximal inhibition observed at approximately 10 μM. A long exposure of IκBα in Western blot revealed an accumulation of high molecular weight IκBα adducts in **7**, **8**, and MG-132 cells. Previous results have shown that these adducts represent polyubiquitinated IκBα species,[33] and their accumulation confirms that inhibition of IκBα degradation is directly caused by inhibition of the proteasome. Thus, our results demonstrate that the macrocyclic peptides **7** and **8** are effectively inhibiting the proteasome and thus canonical NF-κB activation inside the cells.

In summary, the high potency of **7** and **8** combined with their selectivity, cellular stability, and efficacy suggest that further research into macrocyclic peptidyl inhibitors may yield synthetically facile cancer therapeutics with reduced side effects. Compared to the previously reported noncovalent macrocyclic peptide BIA-1a (Figure 1), the covalently binding macrocyclic aldehydes reported here are 1000-fold more potent.[17,23] In addition to being the first macrocyclic peptide aldehydes with specificity for the proteasome, we found that introduction of a macrocycle significantly increases selectivity for the proteasome over intracellular cysteine proteases, such as calpains and cathepsins.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmedchemlett.5b00401.

General methods; synthesis of **7**, **8**, and **9**; NMR spectra and HRMS of final compounds; in vitro kinetic assays; cell culture; Western blot and electrophoretic mobility shift assay (EMSA); QM/MM calculations (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: gotzmg@whitman.edu. Phone: (509) 527-5957.

Author Contributions

[§]These authors contributed equally to this work. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

Ac_2O, acetic anhydride; AIBN, azobis(isobutyronitrile); Cbz, carboxybenzyl; Cbz-OSu, *N*-(carboxybenzyloxy) succin-imide; CCl_4, carbon tetrachloride; DIPEA, *N*,*N*-diisopropylethylamine; DMF, dimethylformamide; ER, endoplasmic reticulum; Fmoc, fluorenylmethyloxycarbonyl; HATU, 1-[bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate; MOE, molecular operating envrionment; NBS, *N*-bromosuccinimide; O-Me, O-methyl; SAR, structure–activity relationship; THF, tetrahydrofuran

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