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Redox-Stable Cyclic Peptide Inhibitors of the SPSB2-iNOS Interaction

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Abstract

SPSB2 mediates the proteasomal degradation of iNOS. Inhibitors of SPSB2-iNOS interaction are expected to prolong iNOS lifetime and thereby enhance killing of persistent pathogens. Here, we describe the synthesis and characterization of two redox-stable cyclized peptides containing the DINNN motif required for SPSB2 binding. Both analogues bind with low nanomolar affinity to the iNOS binding site on SPSB, as determined by SPR and ^{19}F NMR, and efficiently displace full-length iNOS from binding to SPSB2 in macrophage cell lysates. These peptides provide a foundation for future development of redox-stable, potent ligands for SPSB proteins as a potential novel class of anti-infectives.

Keywords: SPSB2; iNOS; cyclic peptide; anti-infective; redox-stable

Abbreviations

SPSB2, SPRY domain of the SOCS-box protein 2; iNOS, inducible nitric oxide synthase; SPR, surface plasmon resonance; NMR, nuclear magnetic resonance; NO, nitric oxide; k_{on}, association rate; k_{off}, dissociation rate; K_D, binding constant; DTT, dithiothreitol; SPPS, solid-phase peptide synthesis; LC-MS, liquid-chromatography mass spectroscopy; GST, glutathione S-transferase; TCEP.HCl, tris(2-carboxyethyl)phosphine hydrochloride; H_2O_2, hydrogen peroxide; HPLC, high performance liquid chromatography; HCTU, O-(1H-6-chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIPEA, N,N-diisopropylethylamine; DMF, dimethylformamide; DCM, dichloromethane; HSQC, heteronuclear single quantum coherence; TFA, trifluoroacetic acid; TIPS, triisopropylsilane; DODT, 3,6-dioxa-1,8-octanedithiol; HFIP, hexafluoro-2-propanol; MeCN, acetonitrile; BMDM, bone marrow-derived macrophage; IFN-γ, interferon-gamma; LPS, lipopolysaccharide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

Introduction

SPSB2 protein plays a key role in modulating the lifetime of iNOS in macrophages during infection [1]. It acts as a negative regulator that recruits an E3 ubiquitin ligase complex that polyubiquitinates iNOS and thereby mediates its proteasomal degradation [1]. SPSB2-deficient macrophages exhibit prolonged iNOS expression, NO production, and ultimately enhanced killing of persistent pathogens such as *Leishmania major* parasites and *Mycobacterium tuberculosis*, suggesting that inhibitors of the SPSB2-iNOS interaction have potential as a new class of anti-infectives [1].

Kuang *et al*. [1] showed that a linear peptide from the disordered *N*-terminal region of iNOS, KEEKDINNNVKKT, bound to SPSB2 with a K_D of 13 nM, and that the most important residues mediating this interaction were DINNN, in particular the first, third and fifth residues of this sequence motif. In addition, structural studies by X-ray crystallography revealed that the SPRY domain of SPSB2 binds to Asp184, Asn186 and Asn188 of the DINNN sequence of the VASA peptide, the same motif as in the *N*-terminal region of iNOS (where the corresponding residues are Asp23, Asn25 and Asn27) [2]. Further binding studies by SPR experiments revealed that the linear DINNN peptide bound to SPSB2 with a K_D of 318 nM [3], suggesting that the flanking residues of the DINNN sequence in the *N*-terminal region of iNOS contributed to its low nanomolar binding affinity to SPSB2.

Recently, we established that cyclization of the linear peptide *via* a disulfide-bridge in the cyclic peptide Ac-c[CVDINNNC]-NH_2 (CP0) improved its binding to SPSB2 by approximately 70-fold, with a K_D by SPR of approximately 4 nM [3]. Comparison of the kinetic profiles of the binding to SPSB2 of DINNN linear peptide, the disulfide-bridged cyclic peptide and its reduced counterpart revealed that cyclization improved k_{on} of the peptide to SPSB2 by approximately 10-fold, presumably through preorganization of the cyclic peptide into a conformation resembling the bound state. Both ^{19}F NMR and [^{1}H,^{15}N]-HSQC NMR experiments confirmed that the cyclic peptide bound to the iNOS binding site on SPSB2. In addition, the cyclic peptide was found to be stable in human plasma and resistant to proteolysis by trypsin, α-chymotrypsin and pepsin. Not surprisingly, however, the disulfide-bridged cyclic peptide was also found to be reductively labile, with the disulfide bond being reduced completely after 15 min treatment with 1 mM DTT at 37 °C in Tris buffer, pH 7.4. While disulfide bridges can form in the cytoplasm under oxidative stress [4], for example during infection, cyclic peptide analogues that are stable to the normally reducing intracellular conditions are likely to be more effective.

There have been reports of disulfide replacements with thioethers [5-8], dicarba bridges [9,10] and diselenides [11] to generate redox-stable analogues without significantly compromising the binding affinity or activity of the parent peptide. However, it cannot be assumed *a priori* that replacement of a disulfide with a thioether will necessarily return biologically active compounds and each example needs to be investigated in a context- and sequence-specific manner. Similarly, previous work by Muttenthaler *et al*. [7] found that the head-to-tail lactam-bridge-cyclized analogue of oxytocin exhibited significant improvement in plasma stability, although little or no detectable binding or functional activity was observed, suggesting that careful design of the cyclic peptide for optimal binding to its target is crucial.

In this study, we describe the design and synthesis of a cystathionine analogue (CP1) of the disulfide-bridged cyclic peptide CP0 [3] and a lactam-bridge analogue between the *N*- and *C*-termini of a peptide incorporating the DINNN linear motif, CP2 (**Fig. 1**). Both analogues were synthesized by SPPS [6] and their identity and purity were characterized by both LC-MS and NMR studies (**Fig. S1-S6**). The effect of replacing the disulfide bond with a thioether bridge or a lactam-bridge on the binding of the analogues to SPSB2 was determined by both SPR and ^{19}F NMR experiments. Their ability to inhibit the interaction of full-length iNOS with SPSB2 in macrophage lysates was also determined. To determine the stability of these analogues, redox assays were carried out in both reducing and oxidizing environments.

Materials and Methods

Synthesis of cyclic peptide analogues CP1 and CP2

The cystathionine analogue CP1 was synthesized on-resin using an automated Peptide Synthesizer 3 (PS3^{TM}) *via* Fmoc chemistry essentially as described by Dekan *et al*. [6] and Yap *et al*. [3]. 0.3 mmol (3 equiv) of the first residue Fmoc-Cys(S-Stbu)-OH was coupled to 0.1 mmol Rink Amide AM Resin (0.47 mmol·g^{-1} loading) for 50 min per coupling cycle under the activation of 0.3 mmol (3 equiv) HCTU and 0.6 mmol (6 equiv) DIPEA in DMF. Subsequent residues were assembled on resin using similar conditions as above (with double coupling used to couple the second Asn to the first Asn as well as the third Asn to the second Asn). The *tert*-butyldimethylsilyl (OTBDMS) derivative of the homoserine (Hse) residue [6] at the *N*-terminus was converted to its Cl-homoalanine analogue with 10 equiv triphenylphosphine dichloride in DCM for 6 h, while the *tert*-butylthio side chain of Cys residue at the *C*-terminus was reduced with 10% (w/v) DTT and 2.5% (v/v) DIPEA in DMF overnight. The resin was washed 6 times with DMF, followed by 3 times with DMF:H_2O (3:2) prior to cyclization in 0.1

M $NaHCO_3$ in DMF:H_2O (3:2) for 72 h. After washing with DMF:H_2O extensively (to remove NaCl), followed by washing 3 times with DMF, the Fmoc protecting group at the *N*-terminus was removed with 20% (v/v) piperidine in DMF for 30 min. *N*-acetylation was then carried out with 10 equiv acetic anhydride and 15 equiv DIPEA in DMF for 3 h. The crude cyclic peptide was cleaved from the resin with a solution containing 92.5% (v/v) TFA and scavengers containing 2.5% (v/v) TIPS, 2.5% (v/v) dimethoxybenzene and 2.5% (v/v) DODT. After cleavage, the cyclic peptide was precipitated with cold diethyl ether twice and dissolved in a 1:1 water:acetonitrile (H_2O:MeCN) mixture prior to lyophilisation. All reactions were carried out at room temperature.

Synthesis of analogue CP2 was carried out according to **Scheme S1**. 0.1 mmol (1 equiv) of the first residue Fmoc-β-alanine-OH was loaded to 0.2 mmol of pre-swelled 2-chlorotrityl resin (1.2 $mmol \cdot g^{-1}$ loading) for 1 h in 5 equiv DIPEA in DCM. The resin was further reacted with 0.8 $mL \cdot g^{-1}$ resin of methanol for 30 min to cap remaining uncoupled resin. After washing the resin 3 times each with DCM and DMF, the resin was subjected to SPPS, in which subsequent amino acid residues were assembled on the resin under the activation of 0.3 mmol (3 equiv) HCTU and 0.6 mmol (6 equiv) DIPEA in DMF. Double coupling was used to couple the second Asn to the first Asn as well as the third Asn to the second Asn. The Fmoc protecting group at the *N*-terminus was removed with 20% (v/v) piperidine in DMF for 30 min. The resin was then washed 3 times each with DMF, methanol and diethyl ether and dried under vacuum. Cleavage of the side chain-protected linear peptide from the resin was carried out with 30% (v/v) HFIP in DCM for 30 min twice and the cleaved peptide was pooled and dried under a stream of nitrogen (N_2) gas. The cleaved peptide was redissolved in H_2O:MeCN (1:9) and lyophilized. Cyclization was carried out in solution at a concentration of 0.5 $mg \cdot mL^{-1}$ overnight with 3 equiv of HCTU and 6 equiv of DIPEA in DMF. The crude cyclic peptide was dried under vacuum and the side chain protecting groups were cleaved from the cyclic peptide with a cleavage cocktail solution containing 95% (v/v) TFA and scavengers 2.5% (v/v) TIPS and 2.5% (v/v) DODT. After cleavage, the cyclic peptide was precipitated with cold diethyl ether twice and dissolved in a H_2O:MeCN (1:1) mixture prior to lyophilisation. All reactions were carried out at room temperature.

The crude peptides CP1 and CP2 were purified by reverse-phase HPLC on a Phenomenex® Luna C18 (2) column (100 Å, 5µm, 100 x 10 mm) using a gradient of 20 –50% B (A: 99.9% H_2O, 0.1% TFA; B: 80% MeCN, 19.9% H_20, 0.1% TFA) over 30-60 min. The purity and molecular mass of the synthesized cyclic peptides were confirmed with LC-MS on a Shimadzu

LCMS2020 instrument, incorporating a Phenomenex® Luna C8 column (100 Å, 3μm, 100 x 2 mm) using a linear gradient of 100% H_2O (0.05% TFA) for 4 min, followed by 0-60% MeCN (0.05% TFA) in water over 10 min at a flow rate of 0.2 $mL \cdot min^{-1}$.

SPSB2 protein expression and purification

The expression and purification of SPSB2 were carried out as described previously [3]. The protein concentration was determined *via* absorbance at 280 nm with a NanoDrop® spectrophotometer. 5-F-Trp labeled SPSB2 (5-F-Trp SPSB2) was prepared as described by Leung *et al*. [12].

NMR spectroscopy

To determine NMR assignments for both CP1 and CP2**,** the cyclic peptides were dissolved in water at pH 4.8 with 10% ^2H_2O. All NMR experiments were recorded on a Bruker Avance 600 MHz spectrometer, equipped with a 5 mm TCI cryoprobe. Homonuclear 2D spectra were recorded at 10 and 25 °C for CP1 and CP2, respectively, where the peaks are best resolved (**Fig. S7-S8**). A 2D total correlation spectroscopy (TOCSY) spectrum was acquired using the DIPSI-2 pulse sequence [13] with excitation sculpting for water suppression [14], a spin-lock time of 70 ms and a relaxation delay of 1.3 s. 2D rotating frame nuclear Overhauser effect (ROESY) spectra [15,16] with WATERGATE [17,18] for water suppression were collected with a 300 ms mixing time and relaxation delay of 3 s. 2D double quantum filtered correlation spectroscopy (DQF-COSY) spectra [19] was acquired with a relaxation delay of 1.3 s. For carbon and nitrogen chemical shift assignments, [^{1}H,^{13}C]-HSQC (256 x 2048) and [^{1}H,^{15}N]-SOFAST HMQC (64 x 2048) spectra [20] were acquired. For all these spectra, a sine-bell squared window function was used for processing. All spectra were processed using Bruker TopSpin (version 3.2) and analyzed using CcpNmr-Analysis (version 2.1.5). ^{1}H chemical shifts were referenced to 3-trimethylsilyl-1-propanesulfonic acid sodium sulfate (DSS), while ^{13}C and ^{15}N chemical shifts were referenced indirectly through their gyromagnetic ratios [21]. The chemical shift assignments are summarized in **Fig. S3** and **Fig. S6** and accessible at the BioMagResBank (BMRB) website (CP1, BMRB ID: 26676; CP2, BMRB ID: 26677). ^{19}F NMR experiments of 50 μM 5-F-Trp SPSB2 in 50 mM sodium phosphate buffer, pH 7.4, and 50 mM NaCl were recorded at 30 °C in the presence and absence of 75 μM peptide. ^{19}F NMR chemical shifts were referenced against 0.005% trifluoroethanol. All spectra were processed using an exponential window function with line broadening of 40 Hz.

Surface plasmon resonance

SPR experiments were carried out at 25 °C on a Biacore™ T200. Immobilization was carried out *via* standard amine coupling on a CM5 sensor chip as described in Yap *et al*. [3]. The binding of all cyclic peptides, CP0, CP1, $CP1_{ox}$ and CP2 was investigated in 10 mM HEPES, 150 mM NaCl, 3 mM EDTA and 0.05% (v/v) Tween 20, pH 7.4. The cyclic peptides were injected onto the surface with a contact time of 60-100 s, flow rate of 100 μL·min^{-1} and dissociation time of 600 s. The double-referenced sensorgrams were fitted to a steady state 1:1 binding model with Biacore T200 Evaluation software (version 2.0) and K_D, k_{on}, and k_{off}, were estimated. Differences between the K_D of each analogue were determined using one-way ANOVA and Tukey's multiple comparison test in GraphPad Prism (version 6.05). Cyclic peptide concentrations were determined using a Direct Detect® spectrometer.

Redox stability assays

For reduction assays, cyclic peptides CP0 (synthesized according to method described by Yap *et al*. [3]), CP1 and CP2 were incubated in 0.1 M ammonium bicarbonate pH 7.4 at 37 °C at a final peptide concentration of 0.5 mg·mL^{-1} for 30 min in the presence of 5 mM TCEP.HCl. At time zero and 30 min, 30 μL samples were collected and 5 μL aliquots were injected and analyzed immediately by LC-MS. For oxidation experiments, both CP1 and CP2 were incubated in 0.1 M ammonium bicarbonate, pH 7.4, at 37 °C at a final peptide concentration of 0.5 mg·mL^{-1} for 5 h in the presence of 10 mM H_2O_2. At time zero, 20 min, 1 h, 3 h and 5 h, 30 μL samples were collected and 5 μL aliquots were injected and analyzed immediately by LC-MS. In both experiments, LC-MS analyses were carried out on a Shimadzu LCMS2020 instrument incorporating a Phenomenex® Luna C8 column (100 Å, 3μm, 100 x 2 mm) and using a linear gradient of 100% H_2O (0.05% TFA) for 4 min, followed by 0-60% MeCN (0.05% TFA) in water over 10 min at a flow rate of 0.2 mL·min^{-1}.

Cell lysate assays

Murine BMDM cells were derived by culture of whole bone marrow of C57BL/6 mice in Dulbecco's modified Eagle's medium supplemented with 100 U·mL^{-1} penicillin, 0.1 mg·mL^{-1} streptomycin, 10% (v/v) fetal bovine serum and 20% (v/v) L-cell conditioned medium as a source of macrophage colony stimulating factor [22]. Cells were cultured for 5 days prior to re-plating and incubation with 10 ng·mL^{-1} murine IFN-γ and 20 ng·mL^{-1} *Salmonella minnesota*-derived LPS overnight at 37 °C. Cells were lysed in kinase assay lysis buffer [23] containing Roche's cOmplete Protease Inhibitor Cocktail and 1 mM phenylmethylsulfonyl fluoride on ice for 30 min. The cell lysates were incubated with 2.5 μg·mL^{-1} recombinantly expressed GST (as

a negative control), or GST-tagged SPSB2-SPRY domain, in the absence and presence of 10 μM cyclic peptide for 2 h. The SPSB2-iNOS protein complexes were recovered from the cell lysate *via* incubation with glutathione Sepharose 4B beads (GE Healthcare) for 1 h. Proteins were separated by SDS-PAGE under reducing conditions and electrophoretically transferred to nitrocellulose membranes (Millipore). Membranes were blocked overnight in 10% (w/v) skim milk and incubated with mouse monoclonal anti-iNOS or anti-GST primary antibodies (BD Biosciences) for 2 h. Antibody binding was visualized with peroxidase-conjugated sheep anti–mouse Ig antibody (GE Healthcare) and the ECL system (GE Healthcare).

Results and Discussion

Two redox-stable cyclic peptides containing the DINNN motif required for SPSB2 binding were designed and synthesized, one with a thioether-bridge, CP1, and the other with a lactam bridge between the *N*- and *C*-termini of the DINNN linear motif, CP2. In the design of CP2, different numbers of β-Ala and L-Ala residues were incorporated between the *N*- and *C*-termini of the DINNN sequence in order to determine the effect of different linker lengths on the overall conformation of the cyclic peptide models. Models were generated with the Biopolymer module of SYBYL®-X (version 2.0, Tripos) and energy minimized with restraints using Maestro (version 10.3, Schrödinger). This study showed that the cyclic peptide sequence c[ADINNNβA] could be accommodated within the binding site with a backbone root mean square deviation (RMSD) of 0.22 Å to the crystal structure of the DINNN linear peptide bound to SPSB2 and with no violation of dihedral angles in the Ramachandran plot [24] (**Fig. S9**). Substitution of the Ala residue of c[ADINNNβA] with other amino acid residues did not cause significant changes in the overall conformation of the models, with the backbone RMSD between the models and the crystal structure of SPSB2-bound DINNN linear peptide remaining in the range 0.22-0.34 Å. Furthermore, rigid docking of these models with SPSB2 using Glide SP (version 5.8, Schrödinger) suggested no steric clashes between the iNOS binding site of SPSB2 and the side chains of 19 other common amino acid residues replacing Ala in c[ADINNNβA], indicating that the nature of this residue is not essential. To enable easier quantification of this analogue for further studies and to improve its overall hydrophobicity, an analogue with Trp was selected for synthesis. The cyclic peptide c[WDINNNβA] (CP2) (**Fig. 1B**) was selected over analogues containing Tyr and Phe in place of Trp because CP2 had an additional hydrogen bond between the Trp indole NH and the side chain amide of the second Asn of the cyclic peptide c[WDINNNβA] (**Fig. S10**). CP2 was

therefore synthesized by SPPS and its identity and purity were confirmed by LC-MS and NMR.

The binding of cyclic peptide analogues CP1 and CP2 to SPSB2 was investigated by SPR and ^{19}F NMR. SPR experiments on CP1 and CP2 (**Fig. 2)** revealed a ~5-fold ($p<0.05$) and ~3-fold ($p>0.05$) decrease in binding affinity to SPSB2 in CP1 ($K_D \approx 31$ nM) and CP2 ($K_D \approx 21$ nM), respectively, compared to CP0 ($K_D \approx 6$ nM). ^{19}F NMR experiments were carried out on 5-F-Trp SPSB2 as described by Leung *et al*. [12]. The ^{19}F resonance corresponding to Trp207, a key residue in the iNOS binding site of SPSB2, is expected to be shifted significantly downfield when the peptide is bound in the correct pose in the iNOS binding site. Consistent with our previous observations [3], addition of CP0 caused a significant downfield shift of the ^{19}F resonance from Trp207 (**Fig. 3A**). Smaller shifts were also observed in three other ^{19}F resonances (at -43 to -45 ppm), as observed by Leung *et al*. [12]. Addition of 1.5 molar excess of CP1 or CP2 to the protein caused a significant 2.4 ppm downfield shift of the ^{19}F resonance from Trp207, implying that these analogues also bind to the iNOS binding site of SPSB2. Minor shifts were also observed in the three ^{19}F resonances at -43 to -45 ppm, although the extent of the shift of the most downfield resonance is smaller than the corresponding shift in the presence of CP0. Intriguingly, the Trp207 peak of SPSB2 in the presence of CP2 is 0.2 ppm further downfield and broader than in the presence of CP0 or CP1, suggesting that there may be some conformational averaging in the vicinity of Trp207 in the complex between CP2- and 5-F-Trp-SPSB2. A slightly faster k_{off} as measured by SPR, for the interaction between CP2 and SPSB2 ($k_{off} \approx 0.04$ s^{-1}) compared to CP0- or CP1-SPSB2 ($k_{off} \approx 0.02$ s^{-1}) (**Table 1**) supports this notion, although further work would be required to confirm this correlation.

The ability of the cyclic peptide analogues to compete with full-length iNOS for binding to SPSB2 in a more physiological setting was also investigated. A GST-based affinity purification assay was carried out on murine BMDM cell lysates. GST-SPSB2-SPRY domain, with and without the cyclic peptide analogues, was added to the cell lysates containing endogenous iNOS (induced in response to LPS and IFN-γ) and recovered using glutathione-Sepharose beads. Western blotting with anti-iNOS antibodies indicated that both CP1 and CP2 (at 10 μM peptide concentration) were able to compete with full-length iNOS for binding to the SPSB2 protein (**Fig. 3B**), as was observed for the disulfide-bridged cyclic peptide CP0, although the extent of inhibition is slightly stronger for CP1 than CP2. Similar inhibition was also observed for the 13-residue linear peptide from the disordered *N*-terminal region of iNOS ($K_D \approx 13$ nM) at 1-10 μM peptide concentration [1].

The stabilities of the cyclic peptide analogues CP1 and CP2 in both reducing and oxidizing environments were also investigated. When both CP1 and CP2 were incubated in 0.1 M ammonium bicarbonate buffer, pH 7.4, and 5 mM TCEP.HCl at 37 °C for 30 min, no significant changes were observed in the concentrations of the cyclic peptide in the reactions mixture (as estimated from the $\log_{10}$ peak area of the HPLC peak at 214 nm) after 30 min in reducing buffer, indicating that both analogues were resistant to reduction under these conditions (**Fig. 4A**). In contrast, the disulfide-bridged cyclic peptide Ac-c[CVDINNNC]-NH_2 (CP0) was completely reduced to Ac-CVDINNNC-NH_2 ($CP0_{red}$) under the same conditions and a peak with a mass increase of +2 Da from 933 to 935 Da was observed in the LC-MS (**Fig. S11**), consistent with the previous observation by Yap *et al.* [3]. While it was expected that both CP1 and CP2 would be reductively stable, it was possible that CP1 might be susceptible to oxidation since it is known that thioether bridges can be oxidized to sulfoxides [5,6]. Thus, both CP1 and CP2 were incubated in 0.1 M ammonium bicarbonate buffer, pH 7.4, at 37 °C for 5 h in the presence of 10 mM H_2O_2, a level that has been reported during acute oxidative bursts in phagocytes [25]. More than 50% of an oxidized peak ($CP1_{ox}$) with an additional mass of +16 Da relative to CP1 was observed after 5 h of incubation in the oxidizing buffer (**Fig. S12**), while no oxidation of CP2 was observed in the same environment (**Fig. 4B**), indicating that CP2 is more stable to oxidation than CP1 under these conditions. To assess the ability of oxidized CP1 to bind to SPSB2, $CP1_{ox}$ was purified and the binding to SPSP2 was measured. SPR revealed a ~2-fold drop in $CP1_{ox}$ binding affinity to SPSB2 ($p<0.01$) compared to CP1, with measured K_D of 68 nM (**Fig. 2D**), and ^{19}F NMR experiments confirmed its binding to the iNOS binding site on the SPSB2 protein (**Fig. 3A**).

Conclusions

In summary, we have generated two cyclic peptide analogues that are reduction-resistant, with CP2 being more oxidatively stable than CP1. Both cyclic peptide analogues, as well as the oxidized product of the cystathionine analogue, were able to bind to the iNOS binding site of SPSB2 with reasonably strong affinities, with the K_D of the most potent analogue, CP2 (21 nM) being comparable to that reported for the 13-residue iNOS linear peptide. Both CP1 and CP2 were also able to compete with full-length iNOS for binding to SPSB2 in macrophage cell lysates. As it is important to know if these cyclic peptide analogues also have similar effects in whole cells, our current efforts are focused on their delivery to the cytoplasm of macrophages. This study thus provides a foundation for the future development of a redox-stable, potent drug candidate targeting SPSB proteins as a potential novel class of anti-infectives.

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Author Contributions

BKY, EWWL, JRH and SEN designed and performed the experiments, and analyzed the data. BKY and RSN wrote the manuscript with input from JBB, MJS, DKC and PET.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's website:

Scheme S1. Synthesis of lactam-bridge cyclic peptide CP2

Fig. S1. LC-MS chromatogram and 1D ^{1}H NMR spectrum of purified CP1

Fig. S2. 2D NMR spectra of CP1

Fig. S3. ^{1}H, ^{13}C and ^{15}N chemical shifts of CP1

Fig. S4. LC-MS chromatogram and 1D ^{1}H NMR spectrum of purified CP2

Fig. S5. 2D NMR spectra of CP2

Fig. S6. ^{1}H, ^{13}C and ^{15}N chemical shifts of CP2

Fig. S7. Temperature-dependence experiments of CP1

Fig. S8. Temperature-dependence experiments of CP2

Fig. S9. Rigid docking studies of *in silico* models of c[ADINNNβA] and its analogues on the iNOS binding site of SPSB2

Fig. S10. *In silico* models of three aromatic analogues of c[ADINNNβA]

Fig. S11. LC-MS profiles of CP0 at zero time and 30 min post-incubation in TCEP.HCl-containing reaction mixture

Fig. S12. LC-MS profiles of CP1 at zero time and 5 h post-incubation in H_2O_2-containing reaction mixture

Table

Table 1. The binding kinetics (K_D, k_{on} and k_{off}) ± standard error of the mean (s.e.m.) of the cyclic peptide CP0-CP2 estimated from the reference-subtracted SPR sensorgrams fitted to a steady state 1:1 interaction model.

Cyclic Peptide	K_D (nM)	k_{on} (x 10^6 $M^{-1}{\cdot}s^{-1}$)	k_{off} (s^{-1})
CP0	6 ± 2	3.37 ± 0.54	0.020 ± 0.003
CP1	31 ± 7	1.07 ± 0.62	0.024 ± 0.008
CP2	21 ± 6	2.63 ± 1.16	0.043 ± 0.001

Figure Legends

Fig. 1. Chemical structures (upper panel) and *in silico* models (bottom panel) of cyclic peptide analogues. (A) CP1, the cystathionine analogue of Ac-c[CVDINNNC]-NH_2 and (B) CP2, the lactam-bridged cyclized peptide c[WDINNNβA]. The linker joining the *N*- and *C*-termini of DINNN linear peptide is highlighted in purple.

Fig. 2. Cyclic peptide analogues CP1, CP1$_{ox}$ and CP2 bind to the iNOS binding site of SPSB2 with low nanomolar affinities. (A) The binding constant, K_D, of CP0, CP1, CP1$_{ox}$ and CP2 measured from double-referenced subtracted SPR sensorgrams fitted to a steady-state 1:1 interaction model from three independent runs (n=3), indicated as mean ± standard error of the mean. The statistical significance difference between the calculated K_D of each cyclic peptide as determined using one-way ANOVA and Tukey's multiple comparison test in GraphPad Prism (version 6.05) was represented by * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$. Representative SPR sensorgrams of immobilized SPSB2 exposed to increasing concentrations of (B) CP0, (C) CP1, (D) CP1$_{ox}$ and (E) CP2 in 10 mM HEPES, 150 mM NaCl, 3 mM EDTA and 0.05% Tween 20, pH 7.4 at 25 °C. The data were fitted using a single-site binding model.

Fig. 3. CP1 and CP2 bind to the iNOS binding site of SPSB2 and are able to compete with full-length iNOS for binding to SPSB2 in macrophage cell lysate. (A) ^{19}F NMR spectra of 5-F-Trp SPSB2 in the absence (most upper panel) and presence of 1.5-fold cyclic peptide Ac-c[CVDINNNC]-NH_2 (CP0), its cystathionine analogue, CP1, and its oxidized product, CP1$_{ox}$, and lactam-bridged cyclized peptide analogue, CP2, in 50 mM sodium phosphate buffer, pH 7.4, and 50 mM NaCl at 30 °C. The resonance of the Trp residue closest to the iNOS peptide binding site, Trp207, is labeled. (B) Cell lysates from LPS/IFN-γ-stimulated BMDM were incubated with GST (as a negative control) or GST-SPSB2-SPRY domain in the presence (+) or absence of cyclic peptides CP0-CP2. GST-fusion proteins were recovered by affinity purification (AP) using glutathione-Sepharose and the interaction with iNOS was analyzed by Western blotting with either anti-iNOS antibody (upper panel) or anti-GST antibody (lower panel).

Fig. 4. Redox stability of cyclic peptide analogues. (A) Amount of cyclic peptide remaining based on the $\log_{10}$ peak area determined by HPLC after treatment with 5 mM TCEP.HCl in degassed 0.1 M ammonium bicarbonate buffer, pH 7.4 at 37 °C (B) % cyclic peptides

remaining after treatment with 10 mM H_2O_2 in 0.1 M ammonium bicarbonate buffer, pH 7.4 at 37 °C.

Fig. 1

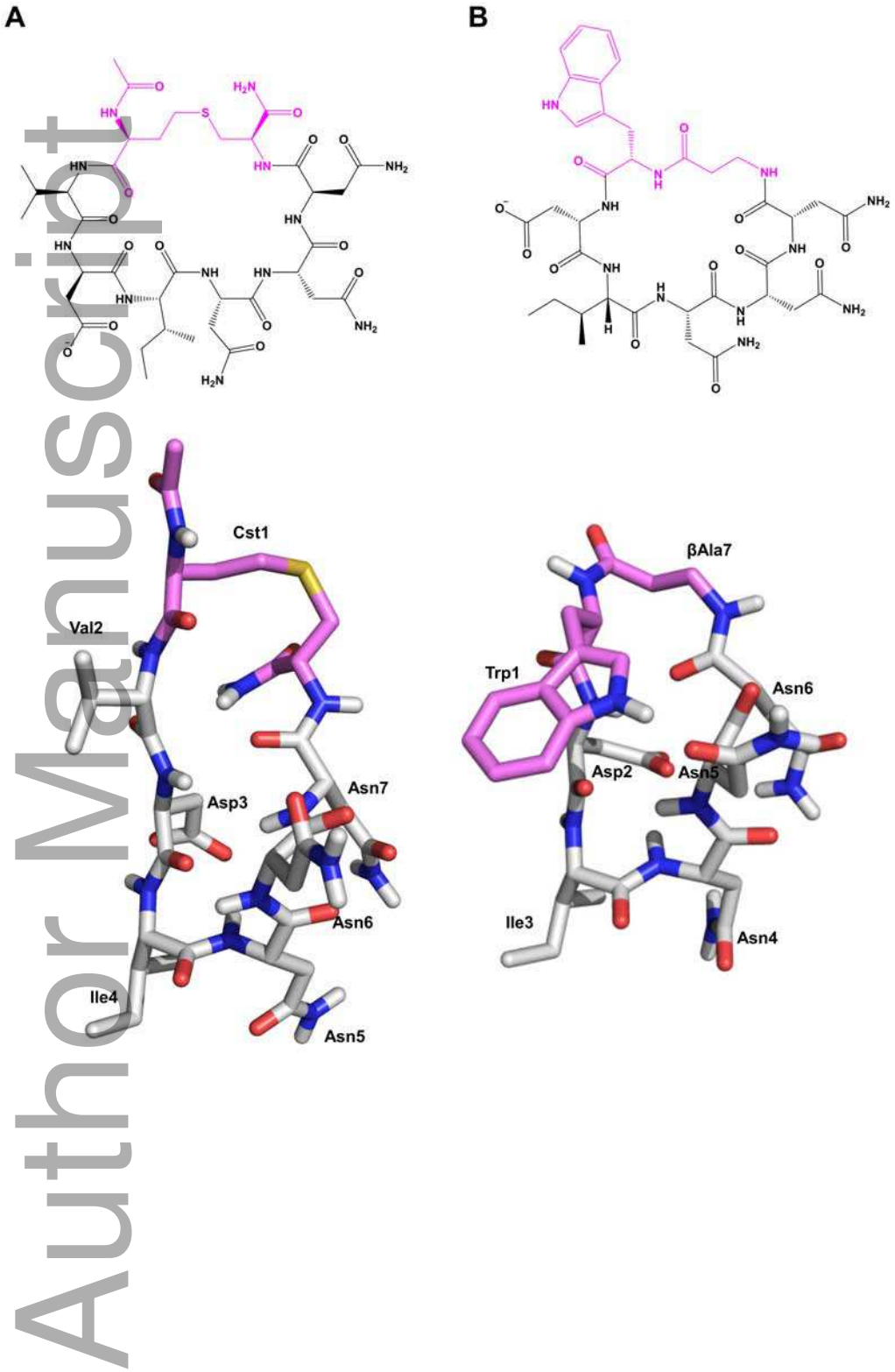

Fig. 2

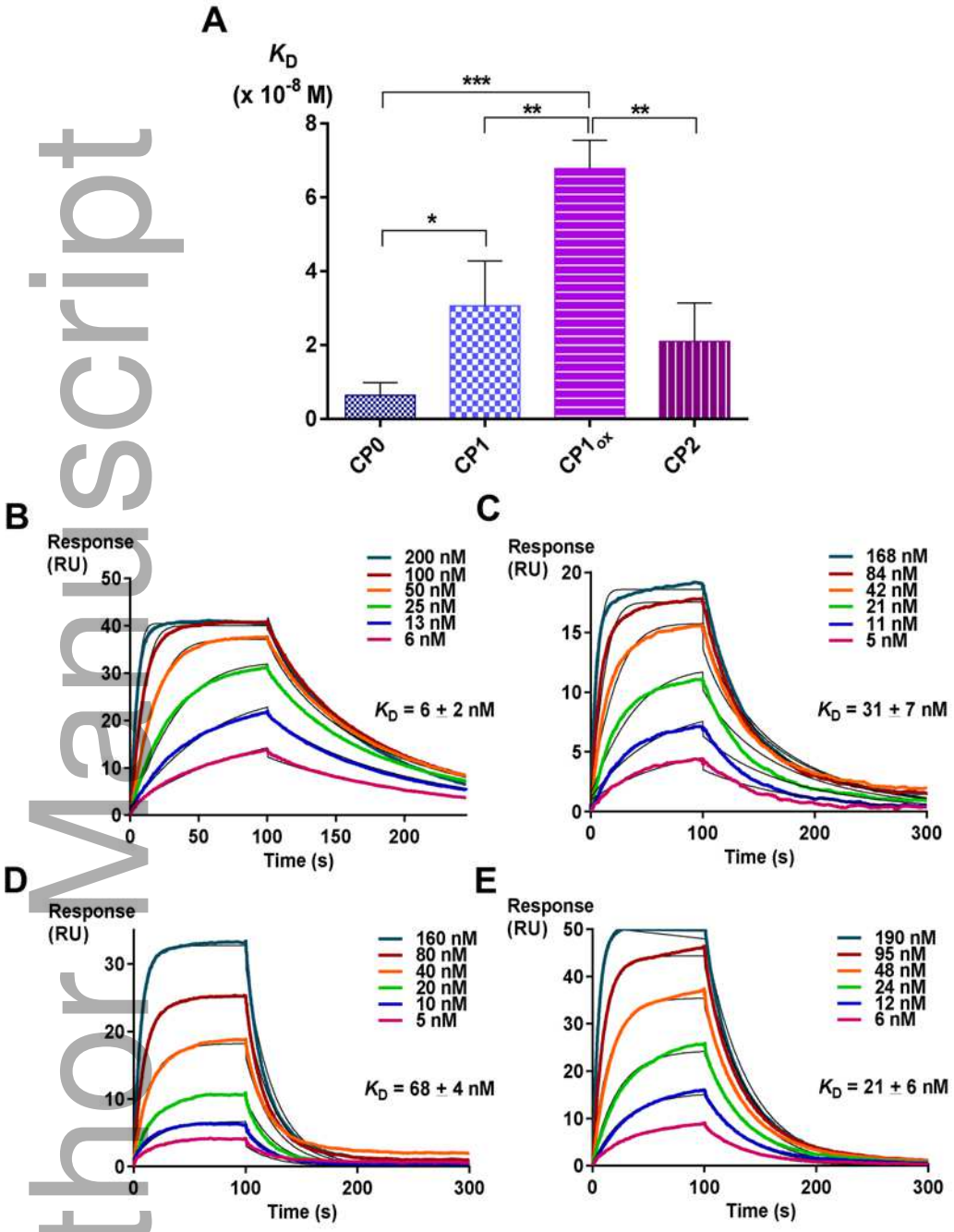

Fig. 3

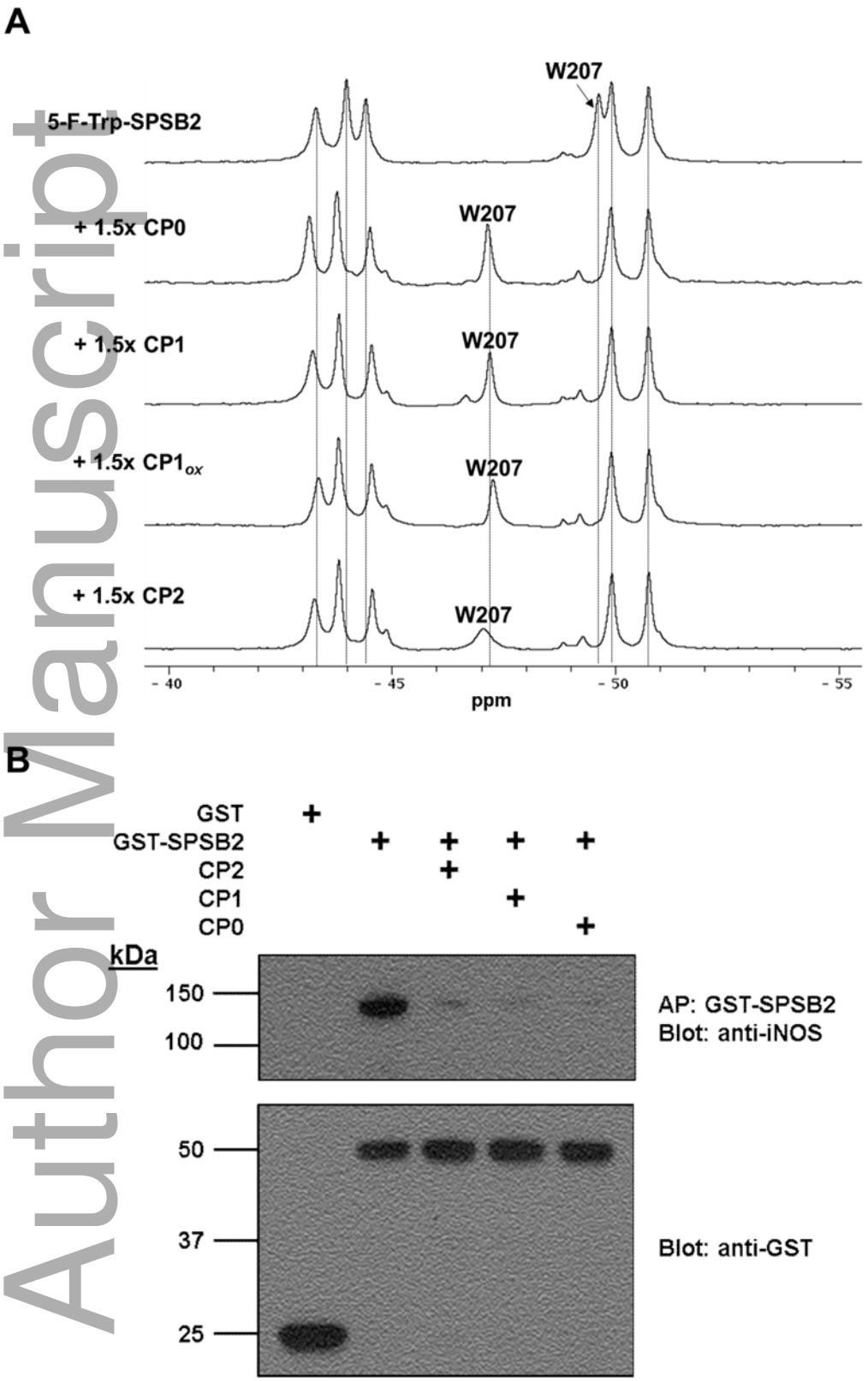

Fig. 4

A

Cyclic Peptide	Log_{10}(Peak Area)	
	0 h	30 min
CP0	6.48	0
$CP0_{red}$	0	6.36
CP1	6.41	6.34
CP2	7.19	7.16

B