Supporting Information

Inhibiting HIV Fusion with a β-Peptide Foldamer

Olen M. Stephens[†], Sunghwan Kim[§], Brett D. Welch[§], Michael Hodsdon[#], Michael S. Kay[§], and Alanna Schepartz*[†,‡]

Departments of [†]Chemistry and [‡]Molecular, Cellular and Developmental Biology, Yale University, New Haven, Connecticut 06520-8107, [#]Department of Laboratory Medicine, Yale School of Medicine, New Haven, CT 06510 and [§]Department of Biochemistry, University of Utah School of Medicine, MREB 211, 50 N. Medical Drive, Salt Lake City, UT 84132, USA.

Contents

I. **General Information**

II. **β-peptide preparation**

III. **Fluorescence polarization assays**

IV. **NMR Spectroscopy of βWWI-1**

I. General Information. Fmoc-protected α-amino acids, PYBOP®, HOBt, and Wang resin were purchased from Novabiochem (San Diego, CA). Dimethylformamide (DMF), N-methyl-2-pyrrolidone (NMP), N-methyl morpholine (NMM), trifluoroacetic acid (TFA), and piperidine were purchased from American Bioanalytical (Natick, MA). CD_3OH (99.5% d_3) was obtained from Cambridge Isotopes (Andover, MA). Hard shell 384-well microplates (black wells) were used for fluorescence polarization experiments (MJ Research, Waltham, MA). All other reagents were purchased from Sigma-Aldrich. Mass spectra were acquired with an Applied Biosystems Voyager-DE Pro MALDI-TOF mass spectrometer (Foster City, CA). Reverse-phase HPLC was performed using a Rainin Dynamax HPLC and Vydac analytical (C18, 300 Å, 5 μm, 4.6 mm x 150 mm) or semi-preparative (C18, 300 Å, 5 μm, 10 mm x 250 mm) columns, using water/acetonitrile gradients with 0.1% TFA. β^3-peptides, IZN17, and $C14^{Flu}$ were synthesized using a Symphony/Multiplex peptide

synthesizer (Protein Technologies, Tuscon, AZ). Fluorescence polarization experiments were performed with an Analyst AD (Molecular Devices, Sunnyvale CA) spectrofluorimeter. Analytical ultracentrifugation was performed using a Beckman XLI instrument (Beckman, Fullerton, CA). Amino acid analyses were performed by the Keck Foundation Biotechnology Resource Laboratory at Yale University School of Medicine.

II. β-peptide preparation

A. *β-peptide synthesis*. Fmoc-protected β^3-amino acids were prepared following methods described by Seebach.[1] β-peptides were synthesized on a 25 μmole scale using standard Fmoc chemistry and Wang resin loaded with β^3-homoglutamic acid as described.[2] One cycle of peptide elongation consisted of the following steps: First, the loaded resin was washed with N-methyl-2-pyrrolidone (NMP) (3 x 30 sec) and the terminal Fmoc protecting group removed with 20% piperidine/DMF (1 x 2 min, 2 x 8 min). The deprotected resin was then washed with NMP (6 x 30 s) and treated for 60 min with a cocktail containing 3 eq of the appropriate β^3-amino acid, 3 eq PYBOP®, 3 eq HOBt, and 8 eq diisopropylethylamine (DIEA). The coupled resin was then washed once with NMP (1 x 30 s), unreacted amino groups acetylated upon treatment with 5% v/v acetic anhydride and 5% v/v NMM in NMP (20 min), and the capped resin washed with NMP (2 x 30 s). These steps were repeated until the β-peptide sequence was complete. Once the final Fmoc protecting group had been removed, the resin was washed with NMP (8 x 30 s) and methylene chloride (8 x 30 s) dried 20 min under N_2, and then treated for 90 min with a cleavage cocktail composed of 1% v/v water, 1% v/v phenol, and 3% v/v triisopropylsilane in trifluoroacetic acid (TFA). The cleaved resin was washed once with the cleavage cocktail (1 x 30 s) and the cleaved β-peptide collected, concentrated by rotary evaporation and reconstituted in H_2O/CH_3CN (1:1).

B. *β-peptide purification and analysis*. The success of each synthesis was assessed first by HPLC and MALDI-TOF analysis of the crude reaction mixture. β-peptides were then purified to homogeneity by reverse-phase HPLC. The identities and purities of purified β-peptides were assessed by analytical HPLC and mass spectrometry (Table S1). Following purification, β-peptides were lyophilized, kept at $^-20$ °C, and reconstituted PBC (1 mM each phosphoric, boric, and citric acids,

adjusted to pH = 7.0 with NaOH) for CD or PBS buffer for FP or cell-cell fusion assays immediately prior to use.

C. *Synthesis of IZN17 and C14^{Flu}*. IZN17 (IKKEIEAIKKEQEAIKKKIEAIEKLLQLTVWGIKQLQARIL) was synthesized on a 25 μmole scale using standard Fmoc chemistry and Wang resin as described previously,[3] and was acetylated on the N-terminus and amidated on the C-terminus. C14^{Flu} was generated by reaction of a HPLC-purified sample of C14[4], extended at the C-terminus with a single cysteine residue, (succinimide-MTWMEWDREINNYTC-amide) with a 25-fold molar excess of 5-iodoacetamidofluorescein (Molecular Probes) in a 1:9 mixture of dimethylformamide:phosphate-buffered saline (DMF:PBS). Labeling reactions were incubated with rotation for 2 h at RT in the dark. The crude fluorescein-labeled peptide was purified by reverse-phase HPLC and characterized by MALDI TOF mass spectrometry and amino acid analysis (Table S1).

Table S1. Mass spectrometry data for β- and α-peptides used in this study.

Peptide	**Calculated Mass**	**Mass Observed**
βWWI-1	1427.7	1429.9
βWWI-1^{Flu}	1905.3	1904.7
βWWI-2	1427.7	1427.7
βWWI-2^{Flu}	1905.3	1905.7
βWWI-3	1427.7	1427.2
βWWI-3^{Flu}	1905.3	1902.8
βWWI-4	1427.7	1428.3
βWWI-4^{Flu}	1905.3	1903.4
βWAI-1	1312.6	1311.2
βWAI-1^{Flu}	1788.1	1788.7
βAWI-1	1312.6	1314.2
βAWI-1^{Flu}	1788.1	1791.0

βWWA-1	1385.7	1388.0
βWWA-1^{Flu}	1863.3	1865.3
IZN17	4854.9	4855.3
C14^{Flu}	2478.6	2482.0

D. *Preparation of fluorescein-labeled βWWI variants*. Each β-peptide was labeled at the N-terminus using Fluorescein-5-EX, succinimidyl ester (Molecular Probes). Labeling reactions were performed with the peptides on a Wang resin using manual operations on the Symphony/Multiplex peptide synthesizer (Protein Technologies, Tuscon, AZ). Following synthesis of the Fmoc-protected β-peptide, Fmoc protecting group removed with 20% piperidine/DMF (1 x 2 min, 2 x 8 min). The deprotected resin was then washed with NMP (6 x 30 s) and treated for 60 min with a cocktail containing 5 mg of the Fluorescein-5-EX, succinimidyl ester, 3 eq PYBOP®, 3 eq HOBt, and 8 eq DIEA. This full-length peptide, capped with the fluorescein dye was washed on the resin with NMP (8 x 30 s) and methylene chloride (8 x 30 s) dried 20 min under N_2, and then treated for 90 min with a cleavage cocktail composed of 1% v/v water, 1% v/v phenol and 3% v/v triisopropylsilane in (TFA). The cleaved resin was washed once with the cleavage cocktail (1 x 30 s) and the cleaved β-peptide collected, concentrated by rotary evaporation and reconstituted in H_2O/CH_3CN (1:1). The labeled product was isolated by HPLC purification and identity verified by MALDI-TOF MS.

III. Fluorescence polarization assays

A. *Direct FP assays.* Fluorescence polarization experiments were performed at 22 °C in 384-well plates (MJ Research, Waltham, MA). For direct binding measurements, serial dilutions of IZN17 were made in PBS buffer, pH 7.2, and an aliquot of fluorescently labeled peptide (**C14^{Flu}**, **βWWI-1-4**, or **βWAI-1**) was added to a final concentration of 25 nM, to a total volume of 12 μL. The binding reaction was incubated for 30 min at RT. Thirty minutes was a sufficient length of time for the

binding reaction to reach equilibrium, as judged by an absence of change in observed polarization values after 1 h and 90 min. The equilibrium dissociation constant of a β-peptide•IZN17 complex (L•P) may be determined by fitting the fluorescence polarization (FP) data to the equation $F = F_L + ((F_{LP} - F_L)/(2[L]_T))*([L]_T + [P]_T + K_d - (([L]_T + [P]_T + K_d)^2 - 4[L]_T[P]_T))^{0.5}$, where K_d = the equilibrium dissociation constant of the L•P complex; F_L = fluorescence polarization of free ligand L; F_{LP} = the observed fluorescence polarization of the L•P complex; $[L]_T$ = total concentration of ligand L; and $[P]_T$ = total concentration of protein P. In the cases of β**WWI-1-4**, the F_L was fixed at 95; for β**WAI-1**, the F_L was fixed at 45; for β**AWI-1** and β**WWA-1**, the F_L was not fixed. Data illustrating the binding of β**WWI-1^{Flu}**, β**WAI-1^{Flu}**, β**AWI-1^{Flu}**, and β**WWA-1^{Flu}** to IZN17 are shown in **Figure S1**.

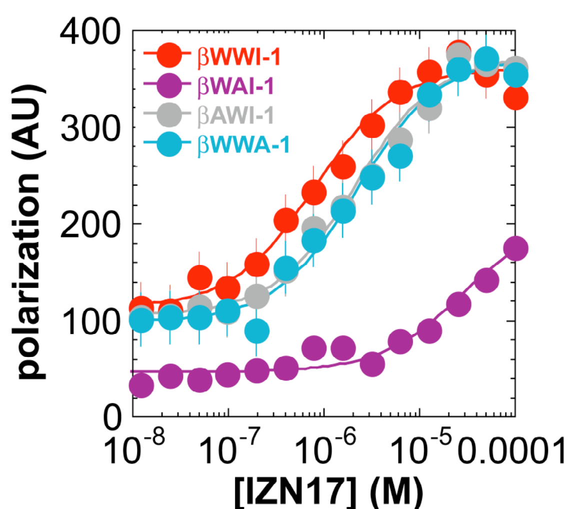

Figure S1. Fluorescence polarization analysis of the binding of IZN17 by β**WWI-1^{Flu}**, β**WAI-1^{Flu}**, β**AWI-1^{Flu}**, and β**WWA-1^{Flu}**. **WWI-1** binds IZN17 with significantly higher affinity (K_d = 0.75 ± 0.1) than does β**WAI-1^{Flu}** (K_d >20 μM), β**AWI-1^{Flu}** (K_d = 2.4 ± 1.6 μM) or, β**WWA-1^{Flu}** (K_d 1.5 ± 0.1 μM). Each point represents the average of at least three determinations; the error bar shown represents that standard error.

B. *Competition FP assays* For competition experiments, serial dilutions of β**WWI-1-4** or β**WAI-1** were incubated with 30 μM IZN17 and 25 nM **C14^{Flu}** for 30 min at RT, in a total volume of 30 μL. The concentration at which inhibition is half its maximum value (the IC_{50}) was calculated from a FP competition assay in which IZN17 (P) can alternatively complex with labeled ligand L (**C14^{Flu}**) or unlabeled inhibitor I (β-peptides), but not both, using the equation $F = F_L + ((F_{LP} - F_L)/(1 + (IC_{50}/[I])^{-n}))$ Where [I] = [β-peptide] and n = Hill coefficient.

C. *FP analysis of calmodulin/carbonic anhydrase affinity*. Selectivity was explored by incubating β**WWI-1**$^{\mathbf{Flu}}$ at 25 nM with serial dilutions of calmodulin or carbonic anhydrase in direct binding measurements. Experiments were performed identically to those for binding to IZN17 and are illustrated in **Figure S2**. The fluorescence data was fit to the same equation as used to determine the K_d for IZN17.

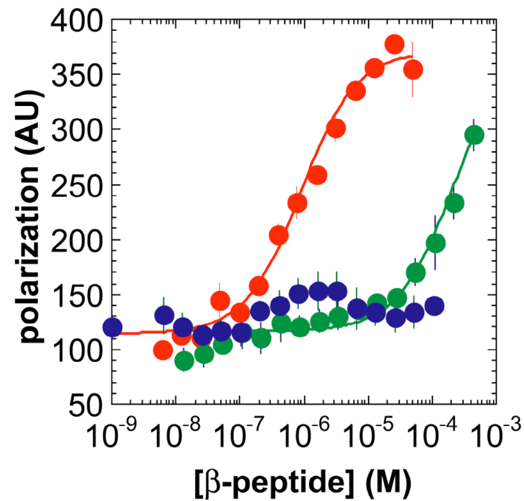

Figure S2. Affinity of β**WWI-1**$^{\mathbf{Flu}}$ for calmodulin (blue) and carbonic Anhydrase (green) in comparison to IZN17 (red). β**WWI-1** binds IZN17 with significantly higher affinity (K_d = 0.75 $\pm$ 0.1) than it binds carbonic anhydrase II ($K_d \geq$ 115 μM) or calmodulin ($K_d \geq$ 100 μM).

V. Cell-cell fusion (syncitia) assays. Inhibition of cell-cell fusion (syncitia formation) was assayed as described previously.[5] HeLa cells that express CD4 and a *tat* inducible β-gal gene (M. Emerman, National Institutes of Health AIDS Reagent Program) were co-cultured with HXB2 Env-expressing CHO cells (gift of M. Krieger) that express HIV-1 *env*, *tat*, and *rev* in the presence of varying concentrations of β-peptides. In the absence of inhibitors, these cells fuse and form syncitia[6] that express β-galactosidase and can be detected by staining with 5-bromo-4-chloro-3-indoyl-β-D-galactoside.[5] Syncitia were manually counted and the EC_{50} were determined by fitting the data for each β-peptide to the equation $S = k/(1 + ([I]/IC_{50}))$, where S = # of syncitia; k = a scaling constant; $[I]$ = [β-peptide]. In the absence of added β–peptide, we observed an average of S = 69.5 syncytia per well. We note that β–peptides β**WWI-1-4** were toxic at concentrations greater than 50 μM. Although potencies similar to those reported in the syncytia assay were observed in a viral infectivity assay, high toxicity precluded an accurate IC_{50} determination.

IV. NMR Spectroscopy of βWWI-1

A. *Sample preparation and data acquisition.* β**WWI-1** was dissolved in CD_3OH to ~3 mM for NMR analysis. 100 μM DSS (2,2-dimethyl-2-silapentane-5-sulfonate sodium salt) was used as a reference standard. All data

were acquired at 12 °C on an 800 MHz Varian Inova NMR spectrometer (Varian, Palo Alto, CA) with a 5 mm triple resonance (HCN) probe equipped with triple axis (XYZ) pulsed magnetic field gradients. All pulse sequences were part of the Varian Biopack user library. Homonuclear two-dimensional (2D) NMR spectra were acquired with spectral widths of 8000 Hz in both dimensions, a 3 s recycle delay between successive scans, and acquisition times of 0.256 and 0.032 s along F2 and F1, respectively. For the z-filtered[7] 2D TOCSY[8] NMR experiment, isotropic mixing was applied for 100 ms using an ~11 kHz DIPSI-[9] subsequence. Similarly, spin-locking during the 2D NOESY NMR experiments were achieved using a 5 kHz continuous radiofrequency field applied during the NOE mixing period. The solvent resonance was suppressed in both experiments using WET[10,11] subsequences containing 5 ms selective sinc pulses. Acquisition required a total of 15 and 52 hours due to signal averaging of 32 and 128 scans for the TOCSY and NOESY experiments, respectively. Assignments were aided by the collection of natural abundance ^{1}H, ^{13}C-HSQC NMR spectra with a 2 second recycle delay between scans, and acquisition times of 0.205 and 0.022 seconds for the F2 and F1 dimensions, respectively. Total acquisition time required for each HSQC (one for aliphatic-region carbons, one for aromatic-region carbons) was 20.3 hours due to signal averaging of 64 scans. All spectra were processed using NMRpipe[12] and analyzed using the Sparky[13] software package. Unambiguously-identified NOEs observed between sequentially non-adjacent residues are shown as solid arrows in **Figure S3**. Additional NOEs consistent with 14-helical structure may be present but were obscured by resonance overlap. Additionally, a few NOEs were not observed due to the proximity of their chemical shifts to solvent resonances.

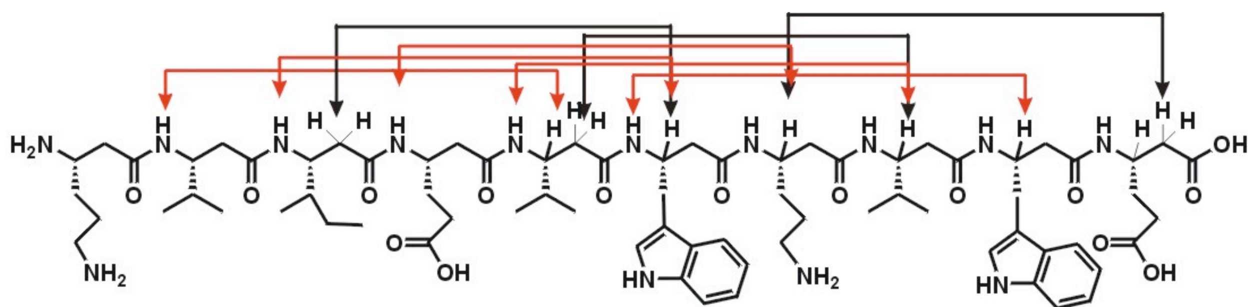

Figure S3. Unambiguous $C_N(i) \rightarrow C_\beta(i+3)$ NOEs (red) and $C_\alpha(i) \rightarrow C_\beta(i+3)$ (black) observed for β**WWI-1** in CD_3OH.

References cited

(1) Seebach, D.; Overhand, M.; Kuhnle, F.N.M.; Martinoni, B.; Oberer, L.; Hommel, U.; Widmer, H. *Helv. Chim. Acta* 1996, *79*, 913.

(2) Hart, S.A.; Bahadoor, A.B.F.; Matthews, E.E.; Qiu, X.J.; Schepartz, A. *J. Am. Chem. Soc.* 2003, *125*, 4022.

(3) Aimoto, S. *Curr. Org. Chem.* 2001, *5*, 45.

(4) Sia, S.K.; Carr, P.A.; Cochran, A.G.; Malashkevich, V.N.; Kim, P.S. *Proc. Natl. Acad. Sci. USA* 2002, *99*, 14664.

(5) Chan, D.C.; Chutkowski, C.T.; Kim, P.S. *Proc. Natl. Acad. Sci. USA* 1998, *95*, 15613.

(6) Kimpton, J.; Emerman, M. *J. Virol.* 1992, *66*, 2232.

(7) Rance, M. *J. Magn. Reson.* 1987, *74*, 557.

(8) Braunschweiler, L.; Ernst, R.R. *J. Magn. Reson.* 1983, *53*, 521.

(9) Shaka, A.J.; Lee, C.J.; Pines, A. *J. Magn. Reson.* 1988, *77*, 274.

(10) Smallcombe, S.H.; Patt, S.L.; Keifer, P.A. *J. Magn. Reson. A* 1995, *117*, 295.

(11) Ogg, R.J.; Kingsley, R.B.; Taylor, J.S. *J. Magn. Reson. B* 1994, *104*, 1.

(12) Delaglio, F.G.; Vuister, G.W.; Zhu, G.; Pfeifer, J.; Bax, A. *J. Biomol. NMR* 1995, *6*, 277.

(13) Kneller, D.G.; Goddard, T.D. *SPARKY 3*, University of California, San Francisco: 1997.