Supporting Information

Identification of a β-Peptide HIV Fusion Inhibitor with Improved Potency in Live Cells

Arjel D. Bautista,[a] Olen M. Stephens,[a] Ligong Wang,[c] Robert A. Domaoal,[c] Karen S. Anderson,[c] and Alanna Schepartz [a,b,*]

[a]Department of Chemistry, Yale University, New Haven, CT 06520, USA

[b]Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT 06520, USA

[c]Department of Pharmacology, Yale University School of Medicine, New Haven, CT 06510, USA

Contents

I. General Information. Fmoc-protected α-amino acids, PYBOP®, HOBt, and Wang resin were purchased from Novabiochem (San Diego, CA). Dimethylformamide (DMF), N-methylmorpholine (NMM), trifluoroacetic acid (TFA), and piperidine were purchased from American Bioanalytical (Natick, MA). N,N-diisopropylethylamine (DIEA) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) were purchased from Sigma-Aldrich. Fmoc-(S)-3-amino-4-(3-trifluoromethylphenyl)-butyric acid, Fmoc-(S)-3-amino-4-(3-pyridyl)-butyric acid, Fmoc-(S)-3-amino-4-(4-iodophenyl)-butyric acid, and Fmoc-(S)-3-amino-4-(2-naphthyl)-butyric acid were purchased from Anaspec, Inc. (San Jose, CA). Fmoc-L-3-fluorophenylalanine and Fmoc-L-2-chlorophenylalanine were purchased from Peptech Corporation (Burlington, MA). Hard shell 384-well microplates (black wells) were used for fluorescence polarization experiments (MJ Research, Waltham, MA). 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 Varian HPLC and Vydac analytical (C8,

300 Å, 5 μm, 4.6 mm x 150 mm) or semi-preparative (C8, 300 Å, 5 μm, 10 mm x 250 mm) columns, using water/acetonitrile gradients with 0.1% TFA. β-peptides were synthesized using a MARS Microwave Assisted Reaction System (CEM Corporation, Matthews, NC). Fluorescein-labeled β^3-peptides and IQN17 were synthesized using a Symphony/Multiplex peptide synthesizer (Protein Technologies, Tucson, AZ). Fluorescence polarization experiments were performed with an Analyst AD (Molecular Devices, Sunnyvale, CA) spectrofluorimeter. Amino acid analyses were performed by the Keck Foundation Biotechnology Resource Laboratory at the Yale University School of Medicine.

II. β-peptide preparation

A. □□□□□□□□□□□□□□□□□□□□ *of unlabeled β-peptides*. Fmoc-protected β^3-amino acids (with the exception of Fmoc-(S)-3-amino-4-(3-trifluoromethylphenyl)-butyric acid, Fmoc-(S)-3-amino-4-(3-pyridyl)-butyric acid, Fmoc-(S)-3-amino-4-(4-iodophenyl)-butyric acid, and Fmoc-(S)-3-amino-4-(2-naphthyl)-butyric acid) were prepared according to methods described by Seebach[1]. β-peptides were synthesized on a 40 μmole scale in a CEM MARS microwave reactor, using standard Fmoc chemistry and Wang resin loaded with β^3-homoglutamic acid as described[2]. Microwave irradiation was conducted at a maximum power of 400 W and monitored via fiber optic temperature sensor. Reactions were agitated by magnetic stirring during irradiation. One cycle of peptide elongation consisted of the following steps: First, the loaded resin was washed manually with dimethylformamide (DMF) and the terminal Fmoc group removed with 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) / 2% piperidine / DMF (50% power at 400 W maximum, 70 ºC, ramp 2 minutes, hold 4 minutes). The deprotected resin was then washed with DMF and treated with a cocktail containing 3 eq PYBOP®, 3 eq HOBt, and 8 eq diisopropylethylamine (DIEA) (50% power at 400 W maximum, 60 ºC, ramp 2 minutes, hold 6 minutes). The coupled resin was then washed extensively with DMF. These steps were repeated until the β-peptide sequence was complete. Following removal of the final Fmoc protecting group, the resin was washed alternately with DMF and methylene chloride for a total of 16 washes, dried 20 min under N_2, and treated for 90 min with a

cleavage cocktail of 2% v/v water, 2% v/v phenol, and 2% v/v triisopropylsilane in trifluoroacetic acid (TFA). The cleaved resin was washed once with the cleavage cocktail 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 by 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 confirmed by analytical HPLC and mass spectrometry (Table S1). Following purification, β-peptides were lyophilized and stored at –20 ºC.

C. *Synthesis of IQN17*. IQN17 (RMKQIEDKIEEIESKQKKIENEIARIKKLLQLTVWGIKQ-LQARIL) 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.

D. *Preparation of fluorescein-labeled βWWI variants.* Fluorescein-labeled β-peptides were synthesized on a 12.5 μmole scale on Wang resin, using manual operations on the Symphony/Multiplex peptide synthesizer. One cycle of peptide elongation consisted of the following steps: First, the loaded resin was washed with dimethylformamide (DMF) (3 x 30 sec) and the terminal Fmoc protecting group removed with 20% piperidine / DMF (1 x 8 min), 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) / 2% piperidine / DMF (1 x 8 min) and a second treatment with 20% piperidine/DMF (1 x 8 min). The deprotected resin was then washed with DMF (6 x 30 sec) and treated for 20 minutes with a cocktail containing 2 eq of the appropriate β^3-amino acid, 2 eq PYBOP®, 2 eq HOBt, and 5 eq diisopropyethylamine (DIEA). The resin was washed once with DMF (1 x 30 sec) and treated a second time with fresh coupling solution (Note: For all variable positions X_6, the coupling reaction was allowed to proceed for 60 minutes per round). The coupled resin was washed with DMF (1 x 30 sec), unreacted amino groups acetylated upon treatment with 5% v/v acetic anhydride and 5% v/v NMM in DMF (20 min), and the capped resin washed with DMF (2 x 30 sec). Following synthesis of the completed peptide, the Fmoc protecting group was removed as above and

the deprotected resin washed with DMF (6 x 30 sec). The resin was then treated for 60 min with a cocktail containing 4.5 mg of 6-(fluorescein-5-(and-6)-carboxamido) hexanoic acid, succinimidyl ester (mixed isomers) (Invitrogen Corporation, Carlsbad, CA), 3 eq PYBOP®, 3 eq HOBt, and 8 eq DIEA. The labeled peptide was then washed on the resin with DMF (8 x 30 sec), and methylene chloride (8 x 30 sec), dried 20 min under N_2, and treated for 90 min with a cleavage cocktail composed of 2% v/v water, 2% v/v phenol and 2% triisopropylsilane in TFA. The cleaved resin was washed once with the cleavage cocktail (1 x 30 sec) and the cleaved β-peptide collected, concentrated by rotary evaporation and reconstituted in H_2O / CH_3CN (1:1). The labeled product was purified by HPLC and identity verified by MALDI-TOF MS (Table S1).

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

Peptide	**Calculated Mass**	**Observed Mass**
βWWI-1	1427.8	1427.7
$\beta WWI\text{-}1^{Flu}$	1901.3	1898.3
βWXI-a	1456.7	1457.6
$\beta WXI\text{-}a^{Flu}$	1930.2	1930.1
βWXI-b	1389.7	1393.3
$\beta WXI\text{-}b^{Flu}$	1863.2	1864.0
βWXI-c	1514.6	1515.8
$\beta WXI\text{-}c^{Flu}$	1988.1	1985.8
βWXI-d	1438.8	1440.9
$\beta WXI\text{-}d^{Flu}$	1912.3	1912.5
βWXI-e	1406.7	1405.8
$\beta WXI\text{-}e^{Flu}$	1880.2	1878.4
βWXI-f	1423.2	1424.6
$\beta WXI\text{-}f^{Flu}$	1896.7	1896.3

III. Fluorescence polarization assays. Fluorescence polarization experiments were performed at room temperature in 384-well plates. For direct binding measurements, serial dilutions of IQN17 were made in PBS buffer, pH 7.2, and an aliquot of fluorescently labeled peptide was added to a final concentration of 25 nM, to a total volume of 30 μL. The binding reaction was incubated at 30 min at room temperature. 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 1h and 90 min. The equilibrium dissociation constant of a β-peptide•IQN17 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.

IV. MTT assay. The compounds were tested in a drug susceptibility assay for determining their effectiveness against the wild-type IIIB strain of HIV-1[4-6] by use of MT-2 human T-cells[7,8] at a multiplicity of infection (MOI) of 0.1. The EC_{50} values were obtained as the dose required to achieve 50% protection of the infected cells by the MTT colorimetric method. LD_{50} for inhibition of MT-2 cell growth by 50% was obtained simultaneously[9, 10] (Figure S1).

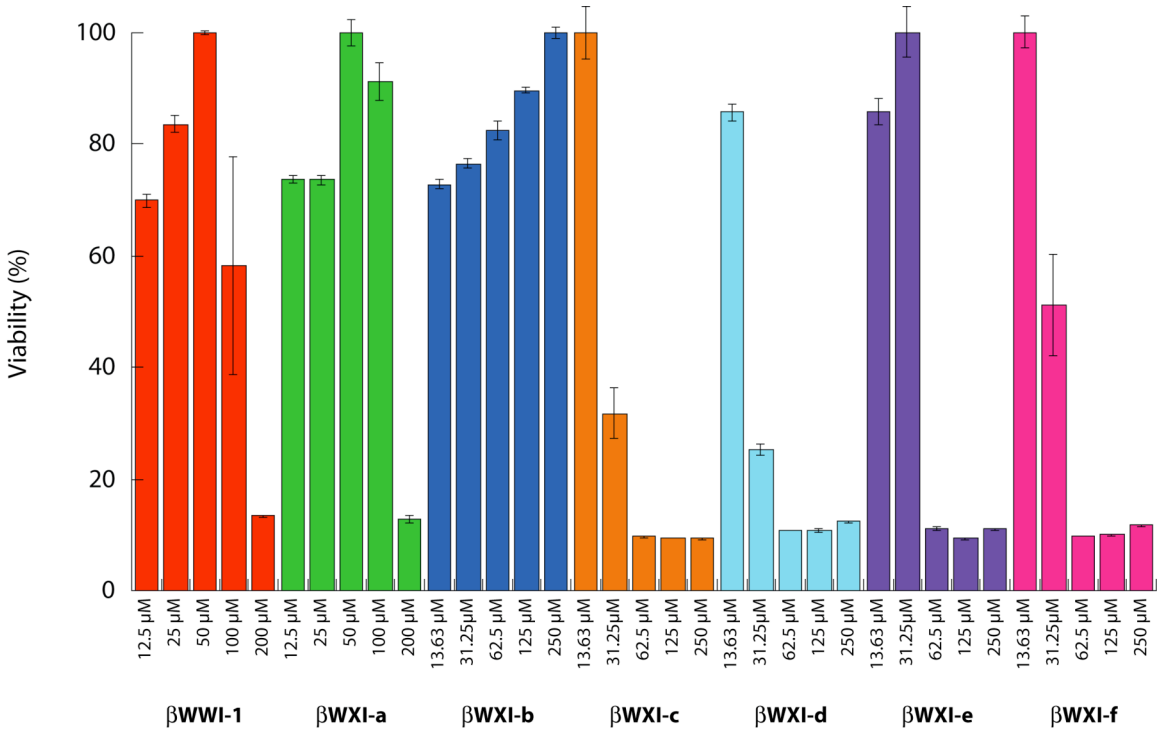

Figure S1. Graph illustrating survival of uninfected MT-2 cells in the presence of the indicated β-peptides. Reported LD_{50} values represent the concentration required to achieve 50% survival. Viability was measured with an MTT colorimetric assay[9,10] as described in the text.

References

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. Popovic, M.; Read-Connole, E.; Gallo, R. *Lancet* **1984**, *324*, 1472.

5. Popovic, M.; Sarngadharan, M. G.; Read, E.; Gallo, R. C. *Science* **1984**, *224*, 497.

6. Ratner, L.; Haseltine, W.; Patarca, R.; Livak, K. J.; Starcich, B.; Josephs, S. F.; Doran, E. R.; Rafalski, J. A.; Whitehorn, E. A.; Baumeister, K.; Ivanoff, L.; Petteway, S. R.; Pearson, M. L.; Lautenberger, J. A.; Papas, T. S.; Ghrayeb, J.; Chang, N. T.; Gallo, R. C.; Wong-Staal, F. *Nature* **1985**, *313*, 277.

7. Harada, S.; Koyanagi, Y.; Yamamoto, N. *Science* **1985**, *229*, 563.

8. Haertle, T.; Carrera, C. J.; Wasson, D. B.; Sowers, L. C.; Richman, D. D.; Carson, D. A. *J. Biol. Chem.* **1988**, *263*, 5870.

9. Lin, T-S.; Luo, M-Z.; Liu, M-C; Pai, S. B.; Dutschman, G. E.; Cheng, Y-C. *Biochem, Pharmacol.* **1994**, *47*, 171.

10. Ray, A. S.; Yang, Z.; Chu, C. K.; Anderson, K. S. *Antimicrob, Agents Chemother.* **2002**, *46*, 887.