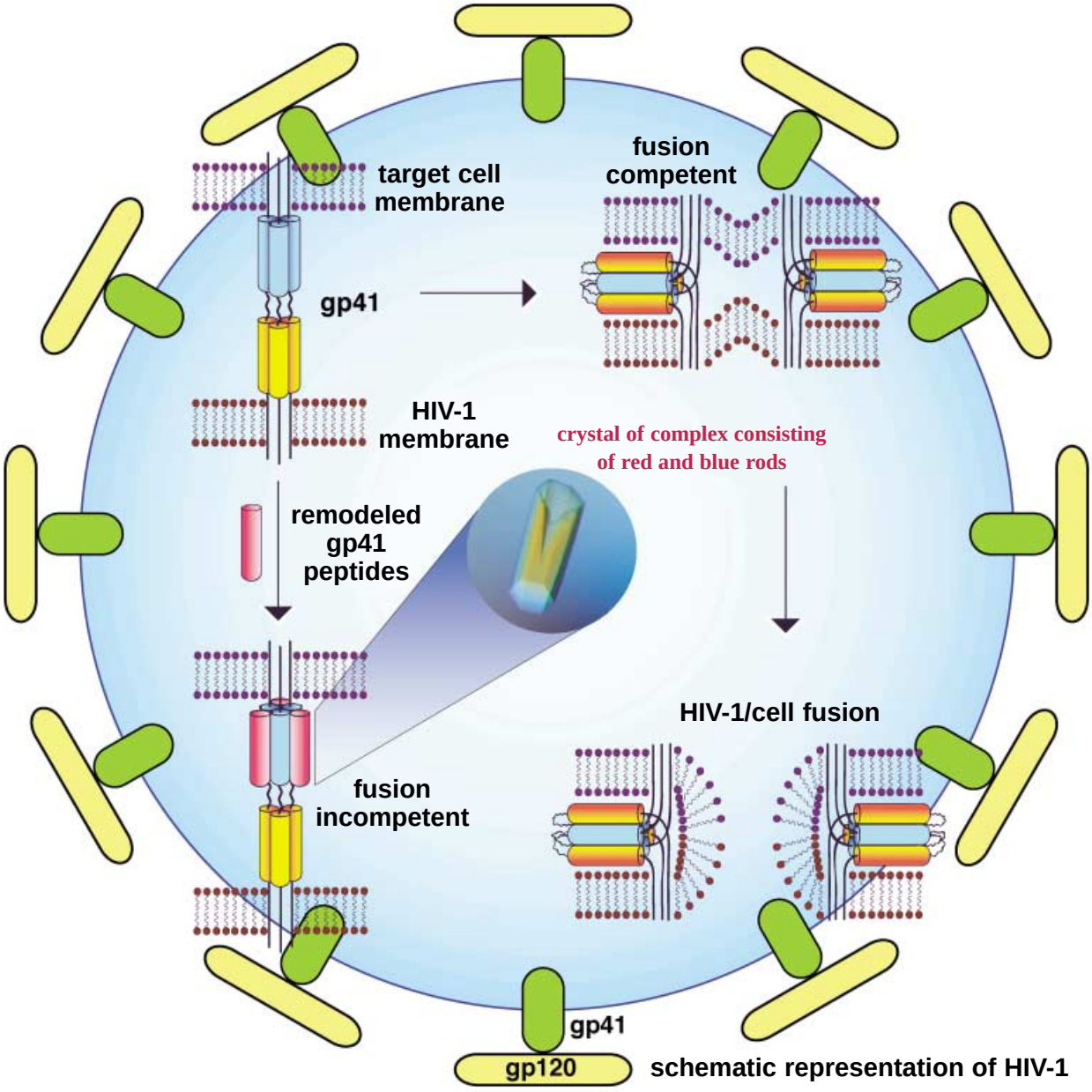
Promising anti-HIV-1 peptide was developed



Fusion of HIV-1 with target cells is mediated by gp41 insertion into the cell membrane, followed by six-helix bundle formation. Artificially remodeled pg41 peptides are likely to inhibit the formation of the six-helix bundle, and exhibit extremely high anti-HIV-1 activity and water solubility.

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Remodeling of gp41-C34 Peptide Leads to Highly Effective Inhibitors of the Fusion of HIV-1 with Target Cells**

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The disclosure of a dynamic supramolecular mechanism involved in the fusion of HIV-1 to cells[1] provides insights into inhibitors for blocking HIV entry into host cells. Among them, molecules targeting events involved in the formation of fusogenic trimeric hairpin structures in the envelope glycoprotein (gp41) are potential candidates for anti-HIV-1 drugs.[2] The trimeric hairpin is formed as a bundle of six α helices, consisting of inner three-stranded coiled coils formed by the N-terminal helical region (N region) of gp41 and three outer α helices. These latter α helices, which are derived from the C-terminal membrane-proximal region (C region) of gp41,[3] are packed antiparallel to the inner strand.

The packing of the C regions onto the inner strand brings the viral and cell membranes into close proximity, thereby prompting membrane fusion. C peptides derived from these C regions are known to prevent formation of the fusogenic structure and inhibit HIV-1 infection[4] (Figure 1A). Among several C peptides, DP178 (T20, pentafuside)[4a] was initially subjected to clinical trials to meet with, unfortunately, the emergence of DP178-resistant HIV-1 strains.[4d,5] Compared to DP178, C34[3b,4c] and T649[4d] exhibit both potent anti-HIV activity as well as reduced susceptibility to the evolution of resistant viruses. However, C34 is less soluble in aqueous media than DP178, which might prevent C34 from being used in clinical cases. We report herein the development of highly soluble C34 variants (referred to as SC peptides) that exhibit amplified anti-HIV-1 activity.

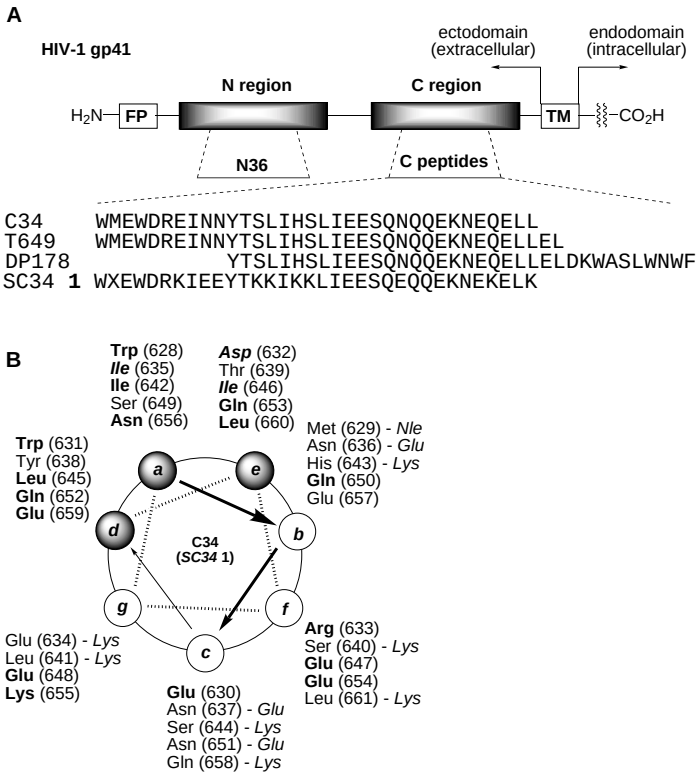

Figure 1. A) Schematic representation of HIV-1 gp41 and sequences of synthetic C peptides. Amino acids are represented by one-letter codes. Underlined letters in SC34 **1** show substituted residues (X in SC34 denotes Nle: **1a** or Met: **1b**). Listed peptides have an acetylated N terminus and a C-terminal amide. FP: fusion peptide; TM: transmembrane domain. B) Helical-wheel representation of C34 and SC34. Residues at each position are symbolized according to their conservation between HIV-1 (NL4-3 or HXB2) and SIV (Mac239):[3b] bold: identity; bold–italic: conservative change; plain: nonconservative change; italic: amino acid replacements in SC34. Underlined Glu and Lys were changed to Lys and to Glu, respectively, in SC34EK **2**. For SC35EK **3** only Glu residues are situated in both *b* and *c* positions and only Lys residues in both *f* and *g* positions. Shaded spheres (*a*, *d*, and *e*) represent important sites for interaction with the N region (or N36). Residues are numbered starting at the first amino acid of the NL4-3 gp160.

Based on a helical-wheel diagram depicting the interaction of the inner coiled-coil strand formed by an N-region peptide (N36) with C34 helices[3b] (Figure 1B), we modeled C34 according to the following concepts: firstly, maintenance of amino acid residues critical for interaction with the inner strand formed by N36 (*a*, *d*, and *e* positions); and secondly, replacement of only nonconserved residues located at solvent-accessible sites in the six-helix bundle (*b*, *c*, *f*, and *g* positions) by Glu or Lys. These should be arranged so that glutamate/lysine ion pairs can be formed between i and $i+4$ positions along the C34 sequence. We expected that such replacements with Glu or Lys could enhance solubility as well as α-helicity through intrahelical salt bridges,[6] which could stabilize the six-helix bundle crucial for anti-HIV activity.[4c,7] Peptides SC34(Nle) (Nle = norleucine) **1a** and SC34(Met) **1b,** designed according to these principles, are summarized in Figure 1. These peptides were synthesized by standard solid-phase methods (see Supporting Information). In **1a** Met was replaced with Nle to avoid oxidation of the Met residue. The

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solubility of **1a** in H_2O increased by more than 1000-fold over that of C34. The hydrophilicities of the designed molecules **1**–**3** relative to that of C34 were also ascertained by reversed-phase HPLC analyses (Table 1).

The inhibitory effects of **1** were determined in the multinuclear activation of the galactosidase indicator assay (MAGI assay).[8] The anti-HIV-1 activities of SC peptides **1** were ten times higher than that of DP178. Furthermore, **1** exhibited strong anti-HIV-1 effects comparable to those of the parent C34 (Table 1). We also evaluated whether **1a** was active against the NL4-3_{SIM} HIV-1 variant, which is resistant to DP178.[4d] Although DP178 showed anti-HIV activity against the wild-type HIV clone NL4-3, its anti-HIV effect decreased sevenfold against NL4-3_{SIM}. In contrast, **1a** effectively inhibited the NL4-3_{SIM} strain as well as the NL4-3 strain. These results encouraged us to develop further C peptides based on SC34 derivatives.

Among the eight *i* to $i+4$ Glu-Lys (or Lys-Glu) intrahelical salt bridges (shown as thick and thin lines, respectively, in Table 1) introduced in the designed SC34 **1**, two pairings had Lys-Glu orientations ordered in the N to C terminus. This was due to an initial working hypothesis that conserved residues should not be substituted (Glu 647, 648 are conserved residues). However, helix dipole interactions should be favorable when anionic Glu residues close to the N terminus and cationic Lys residues close to the C terminus are involved, since the positive pole of the α-helix dipole is near the N terminus and the negative pole is near the C terminus. Therefore, we speculated that reversal of the two Lys-Glu arrangements would further stabilize helix formation for SC34 based on expected interactions of these charged groups with the helix dipole. Additionally, three possible *i* and $i+3$ Lys-Glu intrahelical interactions (represented by dotted lines in the formulas in Table 1) could possibly stabilize the helix.

These reversed derivatives (referred as SC34EK **2**) were also prepared by standard methods (see Supporting Information). MAGI analysis showed that the anti-HIV-1 activity of both SC34EK(Nle) **2a** and SC34EK(Met) **2b** was higher than that of **1**. Of note, **2a** was three times more potent than either C34 or SC34s **1**. From these results we concluded that conserved residues in the solvent-accessible face (*b*, *c*, *f*, and *g*) of SC34s (C34) could be changed unless such replacements interfered with α-helix formation. Accordingly, we synthesized five-repeats of Z-EE-ZZ-KK (SC35EK **3**), in which Z residues interact with the inner strand, and both E and K residues stabilize α-helix conformation (Z corresponds to *a*, *d*, and *e* residues in Figure 1 B). Synthetic **3** was shown by MAGI analysis to exhibit high anti-HIV-1 activity comparable to that of **2**. We observed that peptides **1**–**3** were not cytotoxic up to 10 μM in MAGI cells.

Circular dichroism (CD) analyses of SC peptides **1a**, **2a**, and **3** in the absence of N36 indicate that introduction of *i* to $i+4$ Glu-Lys (or Lys-Glu) pairs with resultant possible intrahelical salt bridges remarkably increases the helicity of SC peptides in the order **1a** < **2a** < **3**. By contrast, the parent C34 has an almost featureless spectrum. CD spectra of equimolar mixtures of N36 and SC peptides **1a**, **2a**, or **3** are typical of α-helical conformations, similar to that observed for the N36/C34 complex. Stabilities of N36/SC peptide complexes were assessed by monitoring the changes in $[\theta]_{222}$ as a function of temperature. The apparent melting temperatures (T_m) of the N36/SC peptide complexes are higher than T_m of the N36/C34 complex (Table 1). Analytical ultracentrifugation sedimentation of the N36/SC peptide (**1a**, **2a**, or **3**) complexes, over a fourfold range of peptide concentrations, indicated that each N36/SC peptide forms a six-helix complex consisting of three molecules each of N36 and SC peptide. The stability of the N36/SC peptide complex correlates to some extent with its anti-HIV activity.[4c,7] Therefore the design of

Table 1. Anti-HIV-1 activity and physicochemical properties of the tested peptides.

Peptide[a,b]		EC_{50}[c] [nM]	T_m[d] [°C]	M_W (calcd)[e] [kD]	RT[f] [min]
C34 **Ac-WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL-NH_2**		0.68 ± 0.12	57.2	–[g]	28.0
SC34 **1** **Ac-WXEWDRKIEEYTKKIKKLIEESQEQQEKNEKELK-NH_2**	**1a** (X = Nle)	0.64 ± 0.20	65.2	26.1 (25.9)	18.7
	1b (X = Met)	0.73 ± 0.12	–[g]	–[g]	18.9
SC34EK **2** **Ac-WXEWDRKIEEYTKKIEELIKKSQEQQEKNEKELK-NH_2**	**2a** (X = Nle)	0.23 ± 0.11	75.2	25.1 (25.9)	19.5
	2b (X = Met)	0.42 ± 0.06	–[g]	–[g]	19.5
SC35EK **3** **Ac-WEEWDKKIEEYTKKIEELIKKSEEQQKKNEEELKK-NH_2**		0.39 ± 0.11	77.2	26.8 (26.3)	19.5
DP178		6.7 ± 2.2			

[a] Incorporated Glu(E) and Lys(K) residues are underlined. [b] Thick and thin lines represent *i* to $i+4$ Glu-Lys and Lys-Glu arrangements, respectively. A dotted line shows an *i* to $i+3$ arrangement modified with conversion of **1** to **2**. [c] Anti-HIV activity determined by three independent MAGI assays. EC_{50}: concentration of peptide which blocks HIV-1 replication by 50 %. [d] Thermal melting temperature of 40 μM C-peptide/N36 complex. [e] M_W: molecular mass of C-peptide/N36 complex determined by sedimentation equilibrium; calcd: calculated molecular mass of the complex consisting of three molecules each of C peptide and N36. [f] Retention time in reversed-phase HPLC analysis with linear gradient of MeCN (30–50 % over 30 min) in 0.1 % aqueous trifluoroacetic acid. [g] Not measured.

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highly helical C peptides based on C34 proved to be a useful guide for developing highly active fusion inhibitors.

In this work we have developed soluble and highly active inhibitors of the fusion of HIV-1 with target cells based on the remodeling of a parent C34 pharmacophore by introducing *i* to *i* + 4 Glu-Lys pairs into the solvent-accessible surface of the six-helix bundle. Effects on anti-HIV-1 activity of amino acid changes in the inner-strand contact surface have been well documented.[4e,7b] However, little attention has been paid to the effects of substitution in the outer surface of the six-helix bundle.[7a] The α-helical SC peptides reported herein can be divided functionally into two α-helical surfaces: surfaces that interact with the inner strand where Z residues (*a*, *d*, and *e* in Figure 1 B) are located, and surfaces that are responsible for α-helix formation, which are formed by both Glu and Lys residues. Reportedly, the latter is needed for appropriate positioning of Z residues through α-helix formation. Therefore, other approaches for presenting Z functionality potentially could be used as alternatives to replacement with Glu-Lys pairs.[9] A Z-EE-ZZ-KK repeated peptide such as **3** could serve as a scaffold to explore the interactive surfaces responsible for anti-HIV-1 activity. Finally, we have elucidated the structures of N36/SC peptide complexes using X-ray analysis in order to finetune the Z residues.

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[1] D. C. Chan, P. S. Kim, *Cell* **1998**, *93*, 681 – 684.

[2] Review: D. M. Eckert, P. S. Kim, *Annu. Rev. Biochem.* **2001**, *70*, 777 – 810.

[3] a) M. Lu, S. C. Blacklow, P. S. Kim, *Nat. Struct. Biol.* **1995**, *2*, 1075 – 1082; b) D. C. Chan, D. Fass, J. M. Berger, P. S. Kim, *Cell* **1997**, *89*, 263 – 273; c) W. Weissenhorn, A. Dessen, S. C. Harrison, J. J. Skehel, D. C. Wiley, *Nature* **1997**, *387*, 426 – 430; d) K. Tan, J.-H. Liu, J.-H. Wang, S. Shen, M. Lu, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 12303 – 12308.

[4] a) C. T. Wild, T. K. Greenwell, T. J. Matthews, *AIDS Res. Hum. Retroviruses* **1993**, *9*, 1051 – 1053; b) S. Jiang, K. Lin, N. Strick, A. R. Neurath, *Biochem. Biophys. Res. Commun.* **1993**, *195*, 533 – 538; c) D. C. Chan, C. T. Chutkowski, P. S. Kim, *Proc. Natl. Acad. Sci. USA* **1998**, *95*, 15613 – 15617; d) L. T. Rimsky, D. C. Shugars, T. J. Matthews, *J. Virol.* **1998**, *72*, 986 – 993; e) M. Ferrer, T. M. Kapoor, T. Strassmaier, W. Weissenhorn, J. J. Skehel, D. Oprian, S. L. Schreiber, D. C. Wiley, S. C. Harrison, *Nat. Struct. Biol.* **1999**, *6*, 953 – 959; f) S. Jiang, A. K. Debnath, *Biochem. Biophys. Res. Commun.* **2000**, *269*, 641 – 646.

[5] J. M. Kilby, S. Hopkins, T. M. Venetta, B. DiMassimo, G. A. Cloud, J. Y. Lee, L. Alldredge, E. Hunter, D. Lambert, D. Bolognesi, T. J. Matthews, M. R. Johnson, M. A. Nowak, G. M. Shaw, M. S. Saag, *Nat. Med.* **1998**, *4*, 1302 – 1307.

[6] a) S. Marqusee, R. L. Baldwin, *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 8898 – 8902; b) P. Burkhard, M. Meier, A. Lustig, *Protein Sci.* **2000**, *9*, 2294 – 2301.

[7] a) J. K. Judice, J. Y. K. Tom, W. Huang, T. Wrin, J. Vennari, C. J. Petropoulos, R. S. McDowell, *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 13426 – 13430; b) W. Shu, J. Liu, H. Ji, L. Radigen, S. Jiang, M. Lu, *Biochemistry* **2000**, *39*, 1634 – 1642.

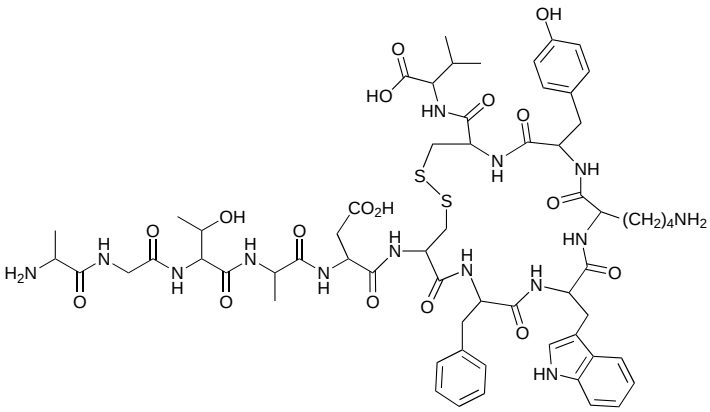
[8] a) J. Kimpton, M. Emerman, *J. Virol.* **1992**, *66*, 2232 – 2239; b) E. Kodama, S. Kohgo, K. Kitano, H. Machida, S. Shigeta, M. Matsuoka, H. Ohrui, H. Mitsuya, *Antimicrob. Agents Chemother.* **2001**, *45*, 1539 – 1546.

[9] J. T. Ernst, O. Kutzki, A. K. Debnath, S. Jiang, H. Lu, A. D. Hamilton, *Angew. Chem.* **2002**, *114*, 288 – 291; *Angew. Chem. Int. Ed.* **2002**, *41*, 278 – 281.

Structure–Function Analysis of Urotensin II and Its Use in the Construction of a Ligand–Receptor Working Model

William A. Kinney,* Harold R. Almond, Jr., Jenson Qi, Charles E. Smith, Rosemary J. Santulli, Lawrence de Garavilla, Patricia Andrade-Gordon, Daniel S. Cho, Anita M. Everson, Mark A. Feinstein, Perry A. Leung, and Bruce E. Maryanoff

Urotensin II (U II) is a cysteine-linked cyclic peptide with potent vasoactive properties. Originally, this substance was isolated from the urophysis (a caudal neurosecretory organ) of the goby fish (*Gillichthys mirabilis*) as a 12-mer, AG-TADCFWKYCV (Scheme 1),[1] but it has now been identified



Scheme 1. U II peptide from the goby fish (**1**).

in all classes of vertebrates. The composition of U II ranges from 11 amino acids in humans to 14 amino acids in mice, always with the conserved cysteine-linked macrocycle, CFWKYC. Recently, the U II receptor was identified[2] as a G-protein-coupled receptor (GPCR) previously known as the GPR14 orphan receptor,[3] which is expressed predominantly in cardiovascular tissues.

Goby U II (**1**) possesses powerful vasoconstrictor activity in fish, mammals, and humans.[4,5] Moreover, it appears to be the most potent vasoconstrictor known,[6] causing concentration-dependent contraction of isolated arterial rings of rats and humans with an EC_{50} value of less than 1 nM, which means it is about ten times more potent than endothelin 1. However, the

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