EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 38, No. 1, 18-26, February 2006

Structure-activity relationships of anti-HIV-1 peptides with disulfide linkage between D- and L-cysteine at positions *i* and *i+3*, respectively, derived from HIV-1 gp41 C-peptide

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Accepted 6 December 2005

Abbreviations: *C,* L-cysteine; *dC,* D-cysteine; HGD, hydrogen domain; HPD, hydrophobic domain; IFD, interface domain; MRE, mean residue ellipticity; MuLV, murine leukemia virus

Abstract

The constrained α-helical structure of a C-peptide is useful for enhancing anti-HIV-1 activity. The *i* and *i+3* positions in an α-helical structure are located close together, therefore D-Cys (*dC*) and L-Cys (*C*) were introduced at the positions, respectively, to make a *dC-C* disulfide bond in 28mer C-peptides. Accordingly, this study tested whether a *dC-C* disulfide bond would increase the α-helicity and anti-HIV-1 activity of peptides. A C-peptide can be divided into three domains, the N-terminal hydrophobic domain (HPD), middle interface domain (IFD), and C-terminal hydrogen domain (HGD), based on the binding property with an N-peptide. In general, the *dC-C* modifications in HPD enhanced the anti-HIV-1 activity, while those in IFD and HGD resulted in no or much less activity. The modified peptides with no activity clearly showed much less α-helicity than the native peptides, while those with higher activity showed an almost similar or slightly increased α-helicity. Therefore, the present results suggest that the introduction of a *dC-C* bridge in the N-terminal hydrophobic domain of a C-peptide may be useful for enhancing the anti-HIV-1 activity.

Keywords: anti-HIV agents; HIV-1; HIV envelope protein gp41; receptors, HIV; structure-activity relationship; viral fusion proteins

Introduction

Cellular receptor binding is the first step in viral infection. Many different cell surface proteins can serve as receptors for entry of viruses (Lee *et al.*, 1999; Song *et al.*, 2005). The human immunodeficiency virus type 1 (HIV-1) infects host cells by fusion between the viral and cellular membranes. The HIV-1 envelope glycoprotein, gp160, is first cleaved into gp120 and gp41. Gp120 then binds to the host receptors, CD4 (Sattentau and Moore, 1991), and one of the chemokine receptors (Choe *et al.*, 1996; Doranz *et al.*, 1996; Feng *et al.*, 1996; Lee *et al.*, 1999), and the bindings induce membrane fusion between the virus and the host mediated by the membrane protein, gp41 (Chan and Kim, 1998).

The gp41 ectodomain has two heptad-repeat α-helical regions (Figure 1A), and the N- and C-terminal haptad-repeat regions of gp41 interact to form a six-helix bundle in which three C-peptide molecules pack into hydrophobic grooves and/or pockets (Chan *et al.*, 1997; Weissenhorn *et al.*, 1997). The process to form a six-helix bundle structure is essential for the process of HIV-1-mediated membrane fusion (Melikyan *et al.*, 2000).

It has been reported that the N- and C-peptides (Figure 1A) derived from the N- and C-terminal heptad-repeat regions, respectively, have potent inhibitory activities as regards HIV-1 infection (Wild *et al.*, 1994; Chan *et al.*, 1998; Eckert and Kim, 2001a). These peptides are considered to interfere with the six-helix bundle formation of gp41 by binding to the corresponding regions (Eckert and Kim, 2001b).

An increased α-helicity has been reported to enhance the anti-HIV-1 activity of peptides (Judice *et al.*, 1997; Jin *et al.*, 2000). Cyclization via an amide linkage between the side chains of glutamic acid or aspartic acid and lysine at positions *i* and *i+3* or *i+7* can enhance α-helicity (Judice *et al.*, 1997; Rivier *et al.*, 1998), yet these methods require complicated

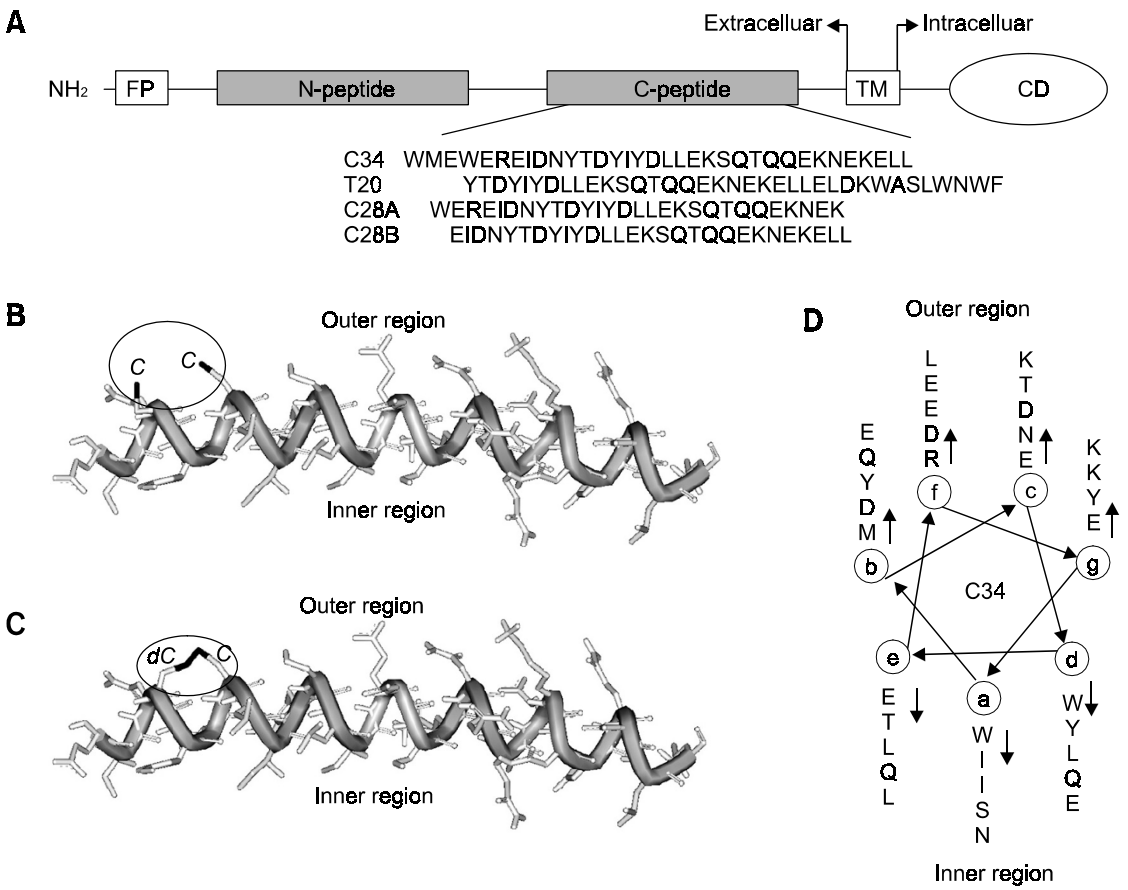


Figure 1. Schematic representation of HIV-1 gp41 and peptide sequences derived from C-peptide (A). Amino acids are represented by one-letter codes. FP, fusion peptide; TM, transmembrane domain; CD, cytoplasm domain; Ac, N-terminal acetyl group; NH_2, C-terminal amide group. Italicized amino acids represent changed residues of C28-89.6. The *C* and *C* (B) or *dC* and *C* (C) residues were introduced to the c and f positions in the α-helical structure of C28B, respectively. The distance between the sulfhydryl groups of *C* and *C* (B) are not enough to maintain a helical structure after forming an intramolecular disulfide bond, while the distance between the sulfhydryl groups of *dC* and *C* (C) are adequate. Heptad repeat-helical wheel diagram of C34 (D). The residues from the inner region (a, d, and e positions) interact with an N-peptide, whereas the residues from the outer region (b, c, f, and g positions) make contact with the surrounding solution. The *dC-C* disulfide bonds were introduced to the outer region. The arrows indicate the direction from the N-terminus to the C-terminus.

steps. Based on an α-helical structure (Figures 1B-D), the *i* and *i+3* positions are in close contact. A disulfide bond also provides a constrained force, but the distance between the two sulfhydryl groups of L-Cysteine (*C*) residues at positions *i* and *i+3* may not be enough to form an α-helical structure (Figure 1B). In a previous study (Pellegrini *et al.*, 1997), a 12mer peptide with a *C-C* disulfide bond at positions 5 and 8 did not display a helical structure, whereas a 12mer peptide with a D-cysteine (*dC*)-C disulfide bond at positions 5 and 8 did reveal a helical structure from position 2 to 9, but the structure was not an α-helix due to the *dC*. However, it has been suggested that the structure could serve as an α-helical nucleation core.

The present authors believe that a *dC-C* disulfide bond can provide an α-helix nucleation core, as proposed in Figure 1C, and enhance anti-HIV-1 activity. Based on its six-helix bundle structure (Figure 1D), a C-peptide can be divided into two regions. The inner region (a, d, and e positions) with conserved residues binds with an N-peptide, whereas the outer region (b, c, f, and g positions) with relatively variable residues contacts with the surrounding solution (Otaka *et al.*, 2002). Accordingly, since the inner region of a C-peptide is important for interacting with an N-peptide, disulfide bonds were introduced to the outer region of two 28mer peptides, C28A and C28B (Figure 1A), derived from HIV-$1_{89.6}$ gp41 to evaluate the effect on the antiviral activity. The secondary structures of the peptides were also investigated, along with their structure-activity relationships.

Materials and Methods

Peptide synthesis

The peptides were synthesized by solid phase methods (Merrifield, 1986) using 9-fluorenylmethoxycarbonyl (Fmoc) as the N_{α}-amino protecting group. The peptides were then purified by HPLC on a Varian reverse-phase-C18 column (10 × 250 mm) (Varian), and their purity confirmed by analytical HPLC on a Beckman reverse-phase-C18 column (4.6 × 250 mm) (Beckman). The identities and amounts of the synthesized peptides were confirmed by amino acid analyses and MALDI-TOF mass analyses. The peptides with *dC* and *C* were oxidized in 100 mM ammonium acetate, pH 8.5 with 5% DMSO overnight with mild shaking, then purified by reverse-phase HPLC. The sequences of the peptides used in this study are listed in Table 1.

Preparation of HIV-1/MuLV pseudotyped viruses

The anti-HIV-1 activity of the peptides was measured using a method reported previously (Lee *et al.*, 2002), as with the preparation of the HIV-1_{BaL}/MuLV and HIV-1_{LAI}/MuLV pseudotyped viruses (Lee *et al.*, 2002). Briefly, the plasmids (pcBaLenvD and pcIIIBenvD) were transfected into TELCeB6 cells (kindly provided by Dr. F. L. Cosset, Centre National de la Recherche Scientifique, Lyon, France) (Cosset *et al.*, 1995) based on calcium phosphate transfection (Kim *et al.*, 2004). The transfected cells were then cultured for 2 days, and the culture supernatant with the HIV-1/MuLV pseudotyped viruses collected by centrifugation and used without further purification. The HIV-1_{BaL}/MuLV pseudotypes were also prepared from a stable cell clone, TELCeB6/BaL20 (Lee *et al.*, 2002). The infectivity of the pseudotypes with the target cells was then tested using a method described previously (Lee *et al.*, 2002). Briefly, after incubating the HIV-1_{BaL}/MuLV and HIV-1_{LAI}/MuLV pseudotypes with the HOS-CD4-CCR5 and HOS-CD4-CXCR4 cells, respectively, for 36 to 40 h at 37°C, the infected target cells were monitored using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining, as described in the technical manual of Gibco-BRL. The blue-stained cells were counted under a microscope, and the titer of the pseudotyped virus represented by the infected units (blue-stained cells) per milliliter (IU/ml).

Measurement of anti-HIV-1 activities of peptides

The anti-HIV-1 activities of the peptides were measured based on the inhibition of the HIV-1/MuLV pseudotype infection in the target cells. The serially

Table 1. Amino acid sequences of the peptides used in this study.

Peptides	Amino acid sequence
C28A	WEREIDNYTDYIYDLLEKSQTQQEKNEK-NH_2
C28A1	WEREID*dC*YT*C*YIYDLLEKSQTQQEKNEK-NH_2
C28A2	WEREIDNYTDYIY*dC*LL*C*KSQTQQEKNEK-NH_2
C28A3	WEREIDNYTDYIYDLLEKSQ*dC*QQ*C*KNEK-NH_2
Ac-C28A	Ac-WEREIDNYTDYIYDLLEKSQTQQEKNEK-NH_2
Ac-C28A1	Ac-WEREID*dC*YT*C*YIYDLLEKSQTQQEKNEK-NH_2
Ac-C28An	Ac-*dC*WE*C*EIDNYTDYIYDLLEKSQTQQEKNEK-NH_2
Ac-C28Ac	Ac-WEREIDNYTDYIYDLLEKSQTQQE*dC*NE*C*-NH_2
C28B	EIDNYTDYIYDLLEKSQTQQEKNEKELL-NH_2
C28B1	EID*dC*YT*C*YIYDLLEKSQTQQEKNEKELL-NH_2
C28B2	EIDNYTDYIY*dC*LL*C*KSQTQQEKNEKELL-NH_2
C28B3	EIDNYTDYIYDLLEKSQ*dC*QQ*C*KNEKELL-NH_2
Ac-C28B	Ac-EIDNYTDYIYDLLEKSQTQQEKNEKELL-NH_2
Ac-C28B1	Ac-EID*dC*YT*C*YIYDLLEKSQTQQEKNEKELL-NH_2
Ac-C28Bn	Ac-*dC*ID*C*YTDYIYDLLEKSQTQQEKNEKELL-NH_2
Ac-C28Bc	Ac-EIDNYTDYIYDLLEKSQTQQEKNE*dC*EL*C*-NH_2

Ac, N-terminal acetyl group; NH_2, C-terminal amide group.
The underlined and italicized *dC* and *C* at positions *i* and *i+3*, respectively, indicate the residues introduced for formation of a *dC-C* disulfide bond.

diluted peptides were added to the HOS-CD4-CCR5 cells or HOS-CD4-CXCR4 cells in 96-well plates, then a 200-500 IU of the pseudotypes was added to each well. After incubating the cells for 36 to 40 h at 37°C, the infected cells were monitored using X-gal staining. Thereafter, 50% inhibition concentrations of the peptides were calculated from curve-fitting graphs analyzed using the Prizm 3.0 program.

CD spectra

The CD spectra of 0.1 mg/ml of the C28 peptides in 10 mM sodium phosphate, pH 7.0 containing 0%, 20% or 50% trifluoroethanol (TFE) were recorded on a Jasco J720 spectropolarimeter (Jasco, Japan). The spectra were measured at 25°C using a 1 mm path length cell, and the scan was repeated three times. The mean residue ellipticity (MRE, [θ]) given in deg · cm^2 · $dmol^{-1}$ was calculated as follows: [θ] = [θ]obs × (MRW/10*lc*), where [θ]obs is the observed ellipticity (millidegree), MRW is the mean residue molecular weight of the peptide, *l* is the pathlength of the cell (cm), and c is the peptide concentration (mg/ml).

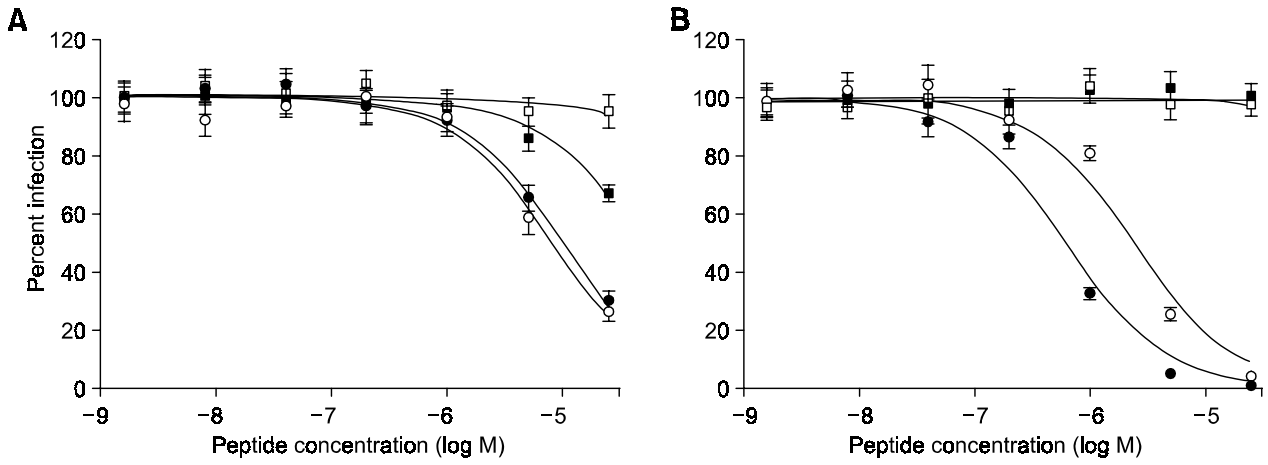
Results

Peptide syntheses and purification

We synthesized the 28 mer C-peptides and analog peptides derived from the HIV-$1_{89.6}$ strain. All the purified peptides showed more than 90% purity with HPLC, and the results for the amino acid compositions and molecular weights were as expected (data not shown). All the peptides with *dC* and *C* at positions *i* and *i+3*, respectively, were analyzed to form over 90% disulfide bond within a day in the incubation solution by HPLC, and elicited more than 90% purity after HPLC purification (data not shown). The disulfide bond formation was confirmed by MALDI-TOF mass analyses (data not shown). The peptides with disulfide bonds exhibited changed elution times when compared with the linear peptides, but these differences disappeared when treated with 10 mM dithiothreitol (data not shown).

Anti-HIV-1 activities of peptides

To analyze the anti-HIV-1 activity of the peptides, we initially measured inhibition of the HIV-1_{BaL}/MuLV



Peptides	C28A	C28A1	C28A2	C28A3
IC_{50} (μM)	8.299 ± 1.442	10.280 ± 1.467	NA	47.863 ± 8.259
Peptides	C28B	C28B1	C28B2	C28B3
IC_{50} (μM)	2.472 ± 0.452	0.615 ± 0.0973	NA	NA

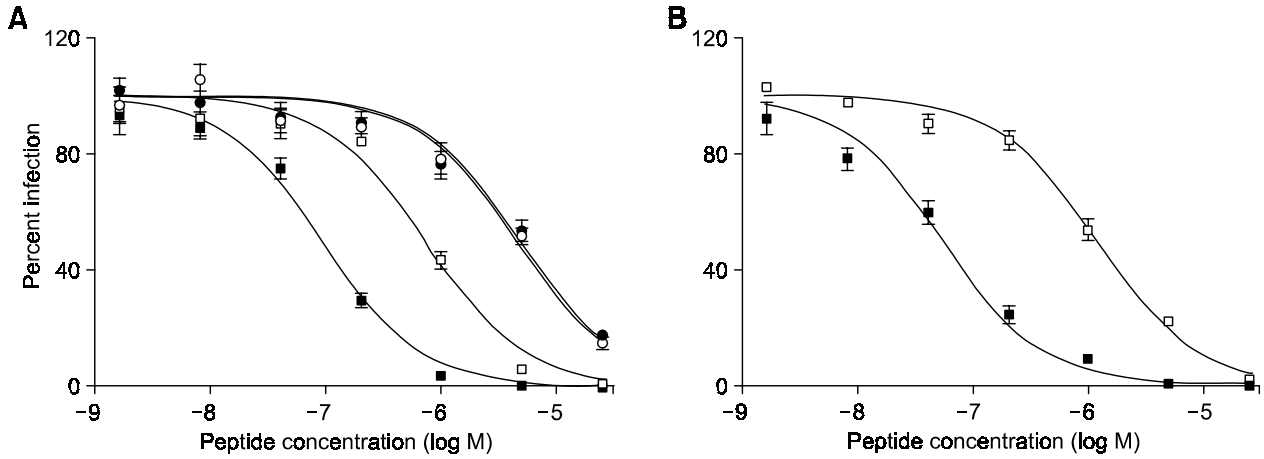
Figure 2. Inhibition by C28A and its analog peptides (A) and C28B and its analog peptides (B) of HIV-1_{BaL}/MuLV pseudotyped viral infection in HOS-CD4-CCR5 cells. The symbols in each panel represent the unmodified peptide (open circle); C28A (A) or C28B (B), and modified peptides (closed circle, open square, and closed square); C28A1 (A) or C28B1 (B), C28A2 (A) or C28B2 (B), and C28A3 (A) or C28B3 (B), respectively. One hundred percent infection indicates viral infection in the absence of the peptide, then the percentage of viral infection was calculated using the following equation: percentage of viral infection = (number of blue-stained cells in the presence of the peptide/number of blue-stained cells in the absence of the peptide) × 100. The IC_{50} values for the peptides are listed in the table below.

pseudotyped viral infection into the HOS-CD4-CCR5 cells. The viral infection decreased in a dose-dependent manner according to increase of the peptide concentration. The curve-fitting was performed using the Prism 3.0 program, and the 50% inhibitory concentration (IC_{50}) calculated for each peptide using the fitted curve.

The N-terminal unmodified C28A and C28B showed anti-HIV-1 activities (IC_{50} values, 8.299 μM and 2.472 μM, respectively) (Figures 2A and 2B), but their IC_{50} values were much lower than that of C34 derived from the HIV-$1_{89.6}$ strain (approximately 10 nM) (Seo *et al*., 2005).

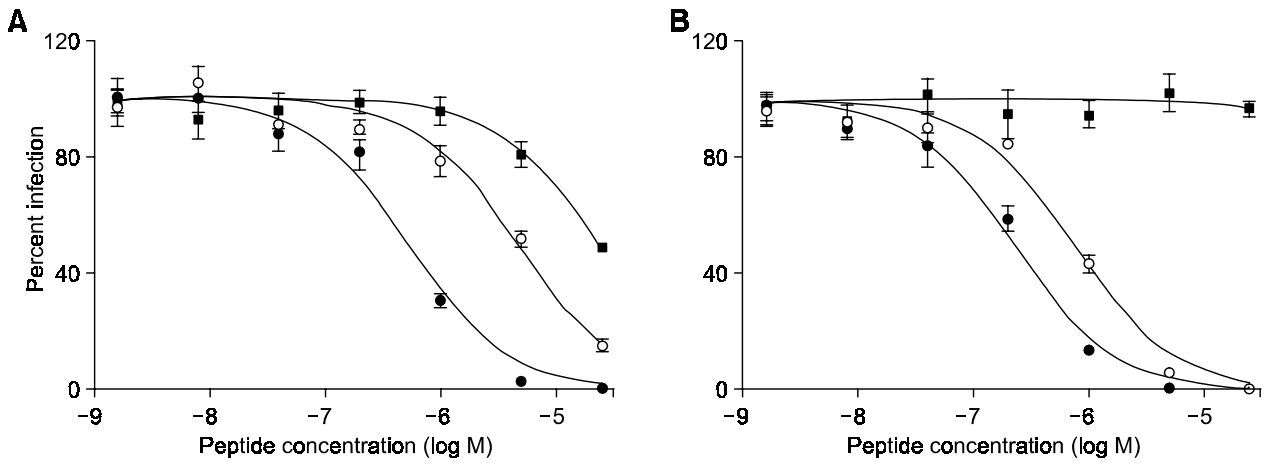
To identify the positional effects of the disulfide bonds, *dC-C* disulfide bonds were initially introduced to three different regions of C28A and C28B (Figure 1A). The residues, *dC* and *C*, were placed at positions c and f, respectively, since these positions are more separated from the inner region (Figure 1D). C28B1 displayed a 4 times stronger HIV-1 activity (IC_{50}, 0.615 μM) than its native form, whereas C28A1 with a bond at the same position exhibited a similar activity to its native form (Figure 2). However, all the other peptides, where the disulfide bonds were shifted to the C-terminus, i.e. C28A2, C28A3, C28B2, and C28B3, exhibited a much lower or almost no activity (Figure 2). A similar pattern was also found when using the N-terminal acetylated peptides. The anti-HIV-1 activities of the N-terminal acetylated peptides were stronger than those of the unmodified ones (Figures 2 and 3A). Ac-C28B1 showed an 8 times stronger anti-HIV-1 activity (IC_{50}, 0.0924 μM) than Ac-C28B, whereas Ac-C28A1 exhibited a similar activity to Ac-C28A and the N-terminal unmodified peptides (Figure 3A). Surprisingly, Ac-C28B1 showed a 20 times stronger inhibition of the HIV-1_{LAI} infection of the Hos-CD4-CXCR4 cells than Ac-C28B (Figure 3B).

To investigate the effect of a *dC-C* bond at the N- or C-terminal and whether it could provide an α-helical nucleation core, the analog peptides were synthesized with a terminal disulfide bond derived from Ac-C28A and Ac-28B (Figure 1A and Table 1). Since the first tryptophan (W) residue of Ac-C28A (position d, Figure 1D) has been reported to be critical for binding to an N-peptide [10], the *dC* was located before the residue. Interestingly, Ac-C28An and Ac-C28Bn showed a much stronger anti-HIV-1 activity than their native peptides, while Ac-C28Ac and Ac-C28Bc only showed 20% activity and no



Peptides	Ac−C28A (R5)	Ac−C28A1 (R5)		
IC_{50} (μM)	4.602 ± 0.760	4.909 ± 0.734		
Peptides	Ac−C28B (R5)	Ac−C28B1 (R5)	Ac−C28Bn (×4)	Ac−C28Bc (×4)
IC_{50} (μM)	0.741 ± 0.111	0.0924 ± 0.0109	1.169 ± 0.112	0.0541 ± 0.0072

Figure 3. Inhibition by Ac-C28A, Ac-C28A1, Ac-C28B, and Ac-C28B1 of HIV-1_{BaL}/MuLV pseudotyped viral infection in HOS-CD4-CCR5 cells (A) and inhibition by Ac-C28B and Ac-C28B1 of HIV-1_{LAI}/MuLV pseudotyped viral infection in HOS-CD4-CXCR4 cells (B). The symbols in panel A represent Ac-C28A (open circle), Ac-C28A1 (closed circle), Ac-C28B (open square), and Ac-C28B1 (closed square), while the symbols in panel B represent Ac-C28B (open square) and Ac-C28B1 (closed square). The percentage of viral infection was calculated using the same method as in Figure 2. The IC_{50} values for the peptides are listed in the table below.



Peptides	Ac−C28A	Ac−C28An	Ac−C28Ac
IC_{50} (μM)	4.602 ± 0.760	0.524 ± 0.088	22.751 ± 3.546
Peptides	Ac−C28B	Ac−C28Bn	Ac−C28Bc
IC_{50} (μM)	0.741 ± 0.111	0.226 ± 0.0307	NA

Figure 4. Inhibition by Ac-C28A and its terminal modified peptides (A) and Ac-C28B and its terminal modified peptides (B) of HIV-1_{BaL}/MuLV pseudotyped viral infection in HOS-CD4-CCR5 cells. The symbols in each panel represent the unmodified peptide (open circle), Ac-C28A (A), or Ac-C28B (B); the N-terminal modified peptide (closed circle), Ac-C28An (A), or Ac-C28Bn (B); and the C-terminal modified peptide (closed square), Ac-C28Ac (A), or Ac-C28Bc (B). The percentage of viral infection was calculated using the same method as in Figure 2. The IC_{50} values for the peptides are listed in the table below.

activity, respectively (Figure 4).

Structure-activity relationships of peptides

Since the anti-HIV-1 activity of a C-peptide is closely correlated with its α-helicity (Judice *et al.*, 1997), the α-helicity of the peptides was tested and the structure-activity relationships were analyzed. All the peptides tested showed a 20% or less α-helicity in an aqueous solution, while the α-helicity of the peptides increased in 20% and 50% TFE, with significant differences found in a 20% TFE solution. Interestingly, the peptides C28A2, C28B2, C28B3, and Ac-C28Bc with no anti-HIV-1 activity even at 25 μM (Figures 2 and 4B) also showed a much lower α-helicity than the unmodified ones (Table 2). Among the peptides that exhibited 3-fold or more anti-HIV-1 activities compared to their native counterparts, Ac-C28An, C28B1, and Ac-C28B1 showed a slight increase in α-heicity, but Ac-C28Bn displayed no significant increase. The other peptides showed no significant changes when compared with their native counterparts (Table 2).

Discussion

The structure of an anti-HIV-1 peptide can provide important information for improving its potency. From the six-helix bundle structure between the N- and C-peptides (Chan *et al.*, 1997), it has been proposed and proved that increasing the α-helicity of a C-peptide by a constrained force can enhance its anti-HIV-1 activity (Judice *et al.*, 1997). Cyclization via an amide linkage between the side chains at positions *i* and *i+3* or *i+7* is also a powerful method for enhancing the α-helicity (Judice *et al.*, 1997; Rivier *et al.*, 1998), but additional steps and expensive substrates are required for the peptide synthesis. In contrast, no additional step is required during the peptide synthesis to make a *dC-C* disulfide bond, only a disulfide linking step after the peptide synthesis. In the present study, the *dC* and *C* residues at positions *i* and *i+3* easily formed an intramolecular disulfide bond, regardless of the peptide sequences and disulfide bond locations, and no oligomeric peptide was formed by the intermolecular disulfide bond(s). Thus, the current results indicate that the sulfhydryl groups of the *dC* and *C* residues were able to contact each other easily in

Table 2. Percent α-helicity of the peptides used in this study.

Peptides	Percent α-helicity[a] in solutions		
	0% TFE	20% TFE	50% TFE
C28A	16.8	38.7	54.2
C28A1	11.5	48.9	53.9
C28A2	6.1	15.1	22.1
C28A3	13.2	43.0	44.1
Ac-C28A	13.3	36.6	52.3
Ac-C28A1	15.2	37.5	41.1
Ac-C28An	17.8	54.0	64.9
Ac-C28Ac	6.5	36.7	44.2
C28B	13.9	52.4	70.8
C28B1	16.4	62.5	75.3
C28B2	7.4	17.9	38.1
C28B3	8.4	42.3	42.1
Ac-C28B	10.6	53.4	70.1
Ac-C28B1	18.9	59.6	70.6
Ac-C28Bn	8.7	53.7	71.1
Ac-C28Bc	8.2	27.2	40.6

[a]Percent α-helicity of each peptide was calculated as following.
Percent α-helicity = 100 × ($[\theta]_{222}$ - $[\theta]^0_{222}$)/($[\theta]^{100}_{222}$ - $[\theta]^0_{222}$)
Where $[\theta]_{222}$ = the observed mean residue ellipticity per residue at 222 nm in deg • cm^2 • $dmol^{-1}$; $[\theta]^0_{222}$ = -3,000 deg • cm^2 • $dmol^{-1}$ (the estimated ellipticity corresponding to a ramdom coil structure); and $[\theta]^{100}_{222}$ = -33,000 deg • cm^2 • $dmol^{-1}$ (the estimated ellipticity corresponding to 100% α-helical peptide). $[\theta]_{222}$ was eastimated by the following equiation: $[\theta]_{222} = [\theta]_{222obs} \times (MRW/10lc)$ where $[\theta]_{222obs}$ is observed ellipticity at 222 nm; MRW is mean residue molecular weight of the peptide; *l* is pathlength of the cell; and c is the peptide concentration (mg/ml).

the solution, as proposed in Figure 1C.

In the present study, C28A and C28B derived from the HIV-$1_{89.6}$ strain were used as the template peptides, as the current authors previously found that the peptides derived from this strain had a stronger anti-HIV-1 activity than corresponding ones derived from the HIV-1_{LAI} strain (Seo *et al.*, 2005), which has been commonly used as a template strain in many previous studies (Chan *et al.*, 1997; Weissenhorn *et al.*, 1997; Jiang and Debnath, 2000). C34 can be separated into three domains according to the binding property with an N-peptide (Chan *et al.*, 1997; Tan *et al.*, 1997), where the hydrophobic interaction is critical for the N-terminal half [called the hydrophobic domain (HPD)], whereas the hydrogen interaction is more important for the C-terminal half [called the hydrogen domain (HGD)] (Tan *et al.*, 1997), while the middle domain is the interface of both interactions [called the interface domain (IFD)]. In this regard, C28A has a longer HPD than C28B, yet shorter HGD (Figure 1A and Table 1). The hydrophobic residues in the N-terminal pocket binding region of C34 are important for strengthening the anti-HIV-1 activity, and the fourth tryptophan residue (Trp4) plays the most important role over the other residues (Chan *et al.*, 1998). However, in the present study, C28A and Ac-C28A with Trp4 exhibited less activity and less α-helicity than C28B and Ac-C28B without Trp4 (Figures 2 and 3), respectively, indicating that the C-terminal three amino acid residues of C28B also play an important role in the anti-HIV-1 activity and stabilizing the α-helical structure.

The effects of the *dC-C* disulfide bonds varied according to their location, such as HPD, IFD, and HGD. The introduction of disulfide bonds to these three different locations in C28A or C28B elicited various results according to location as well as the peptide sequences. For example, a disulfide bond at HPD in C28B produced a positive effect on the anti-HIV-1 activity (see C28B1), yet there was no effect at the same location in C28A (see C28A1) (Figure 2). Plus, C28B1 exhibited a slightly higher α-helicity than C28B1, whereas C28A1 did not (Table 2). Therefore, these results suggest that the introduction of a disulfide bond to the shorter HPD in C28B was useful for improving the anti-HIV-1 activity, while a disulfide bond at the longer HPD in C28A was ineffective. However, the positional effect of the disulfide bond can not be totally excluded in C28A1, as the disulfide bond was shifted more to the central region than in C28B1. Although N-terminal acetylation increases hydrophobicity, it does not alter the patterns (Figure 3A), thereby supporting the effect of the disulfide bond at HPD. Plus, Ac-C28B1 with the highest activity among the peptides tested was also effective for inhibiting CXCR4 mediated infection (Figure 3B). As such, these results also prove that *dC-C* disulfide modification at HPD is useful for developing potent anti-HIV-1 drugs.

IFD and HGD in C28B are considered to play important roles in maintaining the structure and anti-HIV-1 activity. Previously, the current authors found that the C28B derived from the HIV-1_{LAI} strain (called C28B-LAI in this study) exhibited much less anti-HIV-1 activity than the C28B derived from the HIV-$1_{89.6}$ strain used in this study, indicating that the C-terminal half of C28B, including part of IFD and all of HGD, is related to higher activity (Seo *et al.*, 2005). In a recent paper (Otaka *et al.*, 2002), the salt bridge between the *i* and *i+4* positions in C34 was found to stabilize an α-helical conformation and enhance anti-HIV-1 activity. C28B has 3 possible regions for forming salt bridges, while C28B-LAI only has one (Seo *et al.*, 2005). Interestingly, the disulfide bonds introduced to IFD and HGD are located in

the regions for forming salt bridges (Table 1). In addition, if the hydrogen groups in a C-peptide play important roles for binding to an N-peptide, the modifications resulting from the introduction of a *dC-C* disulfide bond could destroy the intermolecular hydrogen interactions. From this perspective, the present data agree well with this assumption (Figure 2 and Table 2). The modifications at IFD in both C28A and C28B destroyed the α-helical conformation and anti-HIV-1 activity (Figure 2 and Table 2). C28B3 also showed a similar effect, while C28A3 exhibited a slightly lower activity and almost similar α-helicity when compared with C28A. As such, it is possible that the longer HPD in C28A3 may have compensated for the effect of the modifications.

Since the linear α-helical structure of a C-peptide is critical for binding with the coiled coil motif of an N-peptide (Chan *et al.*, 1997), it can be assumed that a kink structure in a C-peptide will inhibit the binding with an N-peptide. Thus, if the *dC-C* bridge induces a kink structure, the negative effect would be expected to appear in the middle region. The present data also agreed well with this assumption (Figure 2). However, a helix-nucleation core could be provided at the N- or C-terminus without the formation of a kink structure. To prove this effect, a bond was introduced at the N- and C-terminus in Ac-C28A and Ac-C28B, yet the results differed according to the location the bond was introduced (Figure 4). Positive effects were found when the bond was located at the N-terminus, whereas at the C-terminus, the bond had a negative effect on the anti-HIV-1 activity. Moreover, the C-terminal modification of Ac-C28B eliminated the anti-HIV-1 activity and greatly decreased the α-helicity. Interestingly, the results were similar to those shown in Figure 2, except for Ac-C28An with an additional residue for inducing a disulfide bond. Taken together, the results suggest that the effect of the *dC-C* bond may depend on the domain such as HPD and HGD rather than the localization in the domain.

In conclusion, the effects of a *dC-C* disulfide bond varied according to the location it was introduced. The modifications to HPD increased the anti-HIV-1 activity, whereas the modifications to IFD and HGD destroyed the activity. When the disulfide bond eliminated the anti-HIV-1 activity, the α-helicity of the modified peptide was also disrupted. The IC_{50} values for Ac-C28B1, which exhibited the strongest inhibitory activity among the peptides tested, were below 70 nM against both R5 and X4 tropic HIV-1 infections. Accordingly, the present results suggest that the introduction of a *dC-C* disulfide bond to HPD in a C-peptide may be useful for developing more potent anti-HIV-1 drugs.

Acknowledgement

The authors would like to thank Dr. F. Cosset for providing the TELCeB6 cells. This work was supported by grants NLM0010012 and NSW0270231 from the Korean Ministry of Science and Technology.

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