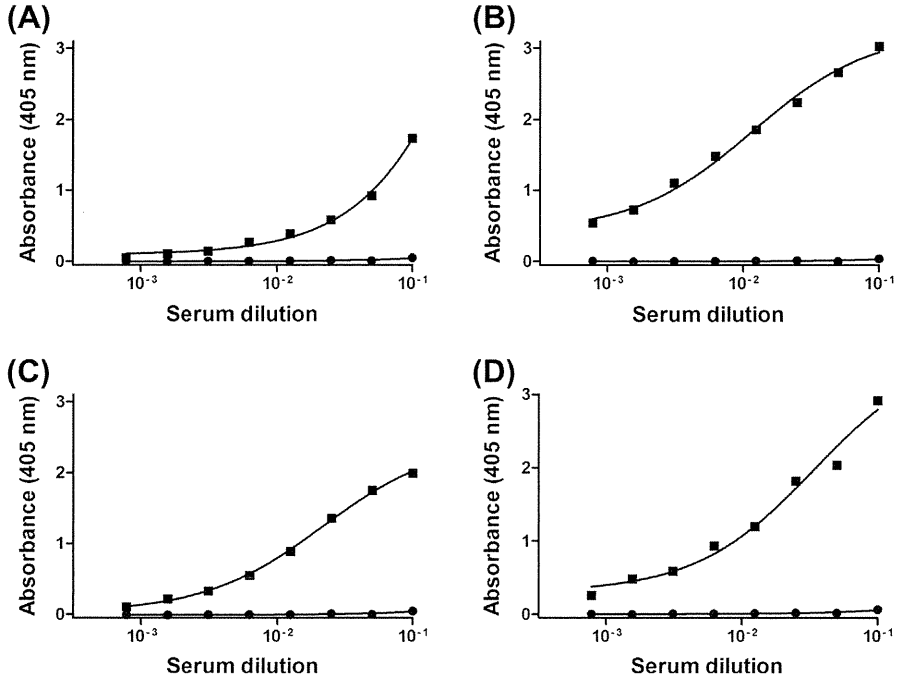

Figure 4. Results of serum titer ELISA of antisera collected from ECL-antigens-immunized mice at one week before immunization (●) and one week after 5th immunization (■) to determine the immunogenicity of designed antigens. The titers were evaluated based on binding to each corresponding monomer peptide; (A) antiserum against C1 tetramer; (B) antiserum against L1 tetramer, (C) antiserum against C2 tetramer, and (D) antiserum against L2 tetramer. The antibody response was elicited in a representative mouse among four mice used for experiments.

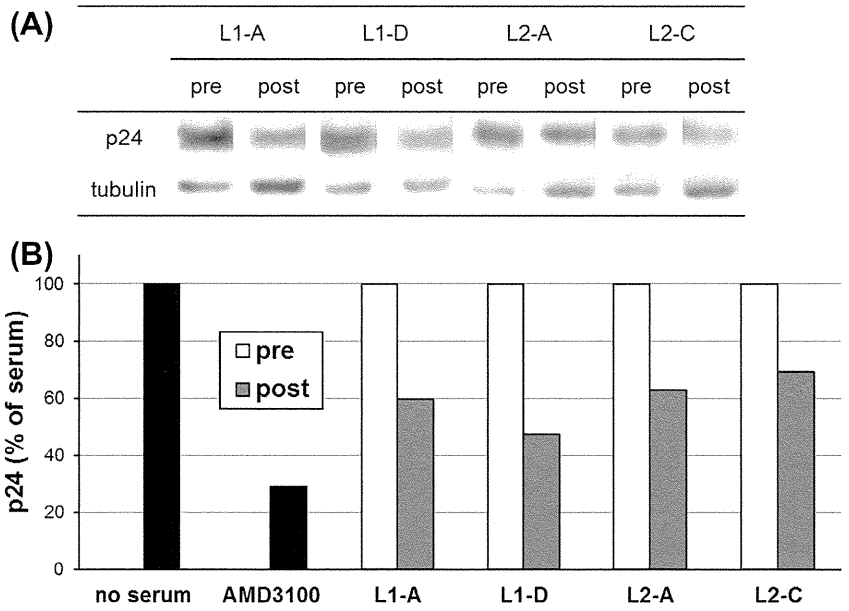

Figure 5. Results of a p24 assay using antisera collected from mice immunized with L1 and L2. (A) Two (L1-A and L1-D) of four (L1-A–D) suppressed p24 production, and two (L2-A and L2-C) of four (L2-A—L2-D) suppressed p24 production. (B) The average percentages of p24 production were calculated from the band intensities in panel A. Black bars are the data of experiments with no serum and with CXCR4 antagonist AMD3100 (1 μM). White Bars are the data of pre-immunization sera, and gray bars are the data of post-immunization sera, which were standardized based on the signals of tubulin. The statistical analyses are shown in Supplementary data.

assays utilizing the antisera bled from four mice for each antigen molecule. Two (L1-A, L1-D) of four (L1-A—L1-D) antisera produced in mice immunized with linear peptide L1 largely suppressed p24 production in comparison to pre-immunization sera (Fig. 5A). Two (L2-A, L2-C) of four (L2-A—L2-D) antisera produced in mice immunized with linear peptide L2 also largely suppressed p24 production compared to pre-immunization sera (Fig. 5A). The average percentages of p24 production were calculated from the intensities of the bands in panel A of Figure 5 (Fig. 5B). Anti-C1 and anti-C2 antibodies were produced but had no significant anti-HIV-1 activity. Possible reasons for this result are (i) the antibody titer levels of L1- and L2-induced sera are higher than that of C1- and C2-induced sera, and (ii) the region around the site of conjugation between C1/C2 and MAPs might behave as an effective epitope for inhibition of HIV-1 entry. In murine serum, several peptides and proteins, for example an antimicrobial peptide α-defensin,[41] have been

reported to have inhibitory activity against HIV-1 entry. The antisera collected at both pre-immunization and post-immunization phases may contain α-defensin, which may affect their HIV-1 inhibitory activity. In this assay, the difference between the antisera collected at both pre-immunization and post-immunization phases was evaluated.

The Env glycoprotein gp120 is known to be highly glycosylated, and its mutation allows the escape of HIV-1 from neutralization by antibodies.[42] However, several antibodies isolated from sera of an African donor such as PG9, PG16[43,44] and VRC01[45] show broad and potent neutralizing activity as a result of recognition of the steric structure of gp120. These antibodies can neutralize 70–80% of HIV-1 strains isolated across all clades with potency approximately one order of magnitude higher than that reported previously for broadly neutralizing monoclonal antibodies. These results suggest that there may be antibody production mechanisms which recognize epitope structures and the development of antigen molecules designed to induce structure-specific antibodies are required.[46,47] We previously synthesized antigen molecules N36 trimer mimic[48] and C34 trimer mimic, based on highly conserved regions of gp41.[49,50] Mice were immunized with each antigen, and anti-HIV-1 antibodies that recognized tertiary structures of each antigen were induced. Antibodies induced by the immunization of CXCR4-ED-derived antigen molecules may have less potent anti-HIV-1 activity than broadly neutralizing antibodies PG9, PG16 and VRC01. They bind to the quaternary neutralizing epitopes on gp120,[51] in contrast, anti-CXCR4-ED antibodies have no time and space barriers in the binding to CXCR4. The tip of the V3 loop is only 30 Å from the target cell membrane[52] and blocking the CXCR4-ED may be more efficient than blocking the V3 loop. Therefore, antigen molecules which can effectively induce anti-CCR5/CXCR4 antibodies might have promise as AIDS vaccines.

3. Conclusion

The HIV-1 co-receptor CXCR4 is an attractive drug target with which to overcome the mutability of HIV-1. In this study, the CXCR4-ED-derived peptides, excluding ECL3, were conjugated with MAPs to produce antigen molecules. Only N1-derived antigen (N1 tetramer) was produced using SPAAC between an N1 dimer bearing azide moiety and a diyne. With the exception of N2 and N3, the CXCR4-ED-derived antigen molecules significantly induced antibodies against each corresponding antigen molecule but only the antisera induced by immunization of the linear peptides based on ECL1 and ECL2 (L1 and L2) exhibited HIV-1 inhibitory activity. The design of antigen molecules mimicking the flexible structure of proteins such as CXCR4-ED is difficult owing to lack of structural information. The X-ray crystal structures of the complexes of CXCR4 with ligands have been reported,[53] but the ligand-free structure has not been published. The results of the present and a previous[30] study suggested that the rational design of the cyclic peptides based on ECL1/ECL2 is possible, but data supporting the rational design of ECL-derived cyclic peptides with MAPs is unavailable. It is clear that, to develop more effective ECL-derived antigen molecules, the length of the linkers on MAPs should be optimized to avoid steric hindrance. This study has provided promising examples for the design of AIDS vaccines targeting the human protein CXCR4 to overcome mutability of HIV-1.

4. Experimental

4.1. Synthesis of the MAPs

The 2-branched azide template and the $ClCH_2CO$-GABA MAP were synthesized on a Rink amide resin (0.74 mmol/g, or 0.6 mmol/g) and the $ClCH_2CO$-$(GABA)_2$ MAP and $BrCH_2CO$-$(GABA)_2$ MAP were synthesized on a NovaSyn® TGR resin (0.21 mmol/g) by standard Fmoc-SPPS with Fmoc-Lys(Fmoc)-OH in the branch position. The constructed peptide was treated with 40 equiv of monochloroacetic acid or monobromoacetic acid, 40 equiv of DIPCI and 40 equiv of HOBt·H_2O for 1 h. Cleavage of peptides from resins and side chain deprotection were carried out by stirring for 1.5 h with a mixture of TFA, thioanisole, H_2O, *m*-cresol and TIS (10/0.75/0.5/0.25/0.1, v/v, 10 mL).

The 2-branched azide template for the conjugation of N1 was synthesized on a Rink amide resin (0.74 mmol/g, 0.05 mmol scale). The product (24.3 mg, 48% yield) was purified by preparative HPLC and characterized by ESI-TOF-MS: *m/z* calcd for $C_{35}H_{60}Cl_2N_{14}O_{10}$ $[M+H]^+$ 907.41, found 907.40.

The $ClCH_2CO$-GABA MAP for the conjugation of N2/N3 was synthesized on a Rink amide resin (0.60 mmol/g, 0.10 mmol scale). The product (93.8 mg, 45%) was purified by preparative HPLC and characterized by ESI-TOF-MS: *m/z* calcd for $C_{70}H_{121}Cl_4N_{23}O_{20}$ $[M+H]^+$ 1743.79, found 1746.23.

The $ClCH_2CO$-$(GABA)_2$ MAP for the conjugation of C1/L1 was synthesized on a NovaSyn® TGR resin (0.21 mmol/g, 0.10 mmol scale). The product (90.5 mg, 37%) was purified by preparative HPLC and characterized by ESI-TOF-MS: *m/z* calcd for $C_{86}H_{150}Cl_4N_{27}O_{24}$ $[M+H]^+$ 2085.01, found 2086.56.

The $BrCH_2CO$-$(GABA)_2$ MAP for the conjugation of C2/L2 was synthesized on a NovaSyn® TGR resin (0.21 mmol/g, 0.10 mmol scale). The product (56.2 mg, 22%) was purified by preparative HPLC and characterized by ESI-TOF-MS: *m/z* calcd for $C_{86}H_{150}Br_4N_{27}O_{24}$ $[M+H]^+$ 2260.81, found 2262.87.

4.2. Synthesis of N1 tetramer

N1 (20.7 mg, 7.88 μmol), the 2-branched azide template (4.03 mg, 3.95 μmol), and KI (22.8 mg, 137 μmol) were dissolved in 0.1 M sodium phosphate buffer at pH 7.8 (2 mL) under N_2. After stirring overnight at room temperature, the N1 dimer product (8.89 mg, 36%) was purified by preparative HPLC and characterized by ESI-TOF-MS: *m/z* calcd for $C_{237}H_{370}N_{70}O_{92}S_6$ $[M+H]^+$ 5862.52, found 5862.57.

sym-Dibenzo-1,5-cyclooctadiene-3,7-diyne[37] (0.0620 mg, 0.310 μmol) in dry MeOH (55.1 μL) was added to the N1 dimer containing an azide moiety (4.80 mg, 0.760 μmol) in dry MeOH (800 μL). After stirring overnight at room temperature under N_2, the N1 tetramer ligation product (1.38 mg, 36%) was purified by semipreparative HPLC and characterized by ESI-TOF-MS: *m/z* calcd. for $C_{490}H_{748}N_{140}O_{184}S_{12}$ $[M+H]^+$ 11925.02, found 11928.58.

4.3. Synthesis of N2 and N3 tetramers

N2 (21.2 mg, 7.03 μmol) and $ClCH_2CO$-GABA MAP (1.55 mg, 0.745 μmol) were dissolved in 0.1 M sodium phosphate buffer pH 7.8 (2 mL). After stirring overnight at room temperature under N_2, the N2 tetramer ligation product (6.77 mg, 65%) was purified by preparative HPLC and characterized by ESI-TOF-MS: *m/z* calcd for $C_{506}H_{769}N_{147}O_{184}S_{12}$ $[M+H]^+$ 12236.31, found 12237.32.

N3 (19.5 mg, 6.31 μmol) and $ClCH_2CO$-GABA MAP (1.64 mg, 0.790 μmol)) were dissolved in 0.1 M sodium phosphate buffer pH 7.8 (2 mL). After stirring overnight at room temperature under N_2, the N3 tetramer ligation product (5.82 mg, 52%) was purified by preparative HPLC and characterized by ESI-TOF-MS: *m/z* calcd for $C_{518}H_{801}N_{155}O_{168}S_8$ $[M+H]^+$ 12140.79, found 12140.97.

4.4. Synthesis of C1 and L1 tetramers

C1 (25.8 mg, 7.44 μmol) and $ClCH_2CO$-$(GABA)_2$ MAP (4.5 mg, 1.86 μmol) were dissolved in a 3:2 mixture (3.0 mL) of 0.1 M

sodium phosphate buffer pH 7.8 (containing 5.0 mM ethylenediaminetetraacetic acid; EDTA) and CH_3CN. After stirring overnight at room temperature under N_2, the C1 tetramer ligation product (5.31 mg, 18%) was purified by preparative HPLC and characterized by ESI-TOF-MS: *m/z* calcd for $C_{590}H_{902}N_{187}O_{168}S_8$ $[M+H]^+$ 13550.58, found 13553.77.

L1 (20.0 mg, 5.93 μmol) and $ClCH_2CO$-$(GABA)_2$ MAP (2.0 mg, 0.83 μmol) were dissolved in a 3:2 mixture (3.0 mL) of 0.1 M sodium phosphate buffer at pH 7.8 (containing 5.0 mM EDTA) and CH_3CN. After stirring overnight at room temperature under N_2, the L1 tetramer ligation product (5.06 mg, 40%) was purified by preparative HPLC and characterized by ESI-TOF-MS: *m/z* calcd for $C_{578}H_{890}N_{183}O_{164}S_4$ $[M+H]^+$ 13146.58, found 13150.70.

4.5. Synthesis of C2 and L2 tetramers

C2 (10.5 mg, 2.5 μmol) and $BrCH_2CO$-$(GABA)_2$ MAP (1.0 mg, 0.41 μmol) were dissolved in a 3:2 mixture (1.0 mL) of 0.1 M sodium phosphate buffer at pH 7.2 (containing 5.0 mM EDTA) and DMF. After stirring overnight at room temperature under N_2, the mixed C2 trimer and tetramer ligation products (tetramer 0.44 mg; trimer 0.36 mg, total yield 8%) were purified by preparative HPLC and characterized by ESI-TOF-MS; tetramer: *m/z* calcdfor $C_{778}H_{1122}N_{219}O_{224}S_4$ $[M+H]^+$ 17256.30, found 17263.79; trimer: *m/z* calcd for $C_{605}H_{883}N_{171}O_{175}NaS_3$ $[M+Na]^+$ 13468.50, found 13469.10.

L2 (56 mg, 13.2 μmol) and $BrCH_2CO$-$(GABA)_2$ MAP (4.0 mg, 1.53 μmol) were dissolved and stirred in the same manner as in the synthesis of the C2 tetramer. The mixed L2 trimer; tetramer ligation products (tetramer 7.18 mg and trimer 1.79 mg, total yield 32%) were purified by preparative HPLC and characterized by ESI-TOF-MS; tetramer: *m/z* calcd for $C_{786}H_{1154}N_{223}O_{228}S_4$ $[M+H]^+$ 17492.46, found 17497.33; trimer: *m/z* calcd for $C_{611}H_{904}N_{174}O_{178}S_3$ $[M+H]^+$ 13622.63, found 13626.37.

4.6. Immunization

Five-week-old male Balb/C mice, purchased from CLEA Japan, Inc. (Tokyo, Japan), were maintained in an animal facility under specific pathogen-free conditions. The experimental protocol used was approved by the ethical review committee of Tokyo Medical and Dental University. Freund's incomplete adjuvant and PBS were purchased from Wako Pure Chemical Industries (Osaka, Japan), DMSO (endotoxin free) was purchased from Sigma–Aldrich (St. Louis, MO).

All mice were habituated for one week and all six-week-old mice were bled one week before immunization. Antigen molecule (100 μg) was dissolved in DMSO (1 μL) and PBS (49 μL). This solution was mixed with Freund's incomplete adjuvant (50 μL) and the mixture was injected subcutaneously under anesthesia on days 0, 7, 14, 21 and 28. Four mice for each immunization were bled on days 5, 12, 19, 26 and 33. Serum was separated by centrifugation (1500 rpm) at 4 °C for 10 min, and inactivated at 56 °C for 30 min. Sera were stored at −80 °C before use.

4.7. ELISA assay

Tween-20 (polyoxyethylene (20) sorbitan monolaurate) and H_2O_2 (30%) were purchased from Wako. ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt) was purchased from Sigma–Aldrich. Anti-mouse IgG (H+L)(goat)-HRP was purchased from EMD Chemicals (San Diego, CA). Ninety six-well microplates were coated with 25 μL of a synthetic peptide at 10 μg/mL in PBS at 4 °C overnight. The coated plates were washed 10 times with distilled water and blocked with 150 μL of blocking buffer (0.02% PBST, PBS with 0.02% Tween 20, containing 5% skim milk) at 37 °C for 1 h. The plates were washed 10 times with distilled water. Mice sera were diluted in binding buffer (0.02% PBST with 1% skim milk), and 50 μL of twofold serial dilutions of sera from 1/10 to 1/1280 were added to the wells and allowed to incubate at 37 °C for 2 h. The plates were washed 10 times with distilled water. Twenty-five microliters of HRP-conjugated anti-mouse IgG, diluted 1:2000 in 0.02% PBST, was added to each well. After 45 min incubation, the plates were washed 10 times with distilled water and 25 μL of HRP substrate, prepared by dissolving 10 mg ABTS in 200 μL of HRP staining buffer—a mixture of 0.5 M citrate buffer (pH 4.0, 1 mL), H_2O_2 (3 μL), and H_2O (8.8 mL)—was added. After 30 min incubation, the reaction was stopped by addition of 25 μL/well 0.5 M H_2SO_4, and optical densities were measured at 405 nm.

4.8. Anti-HIV-1 assay (p24 assay)

The experiments using HIV-1 were performed in the biosafety level 3 laboratory #5 at the National Institute of Infectious Diseases. For virus preparation, 293T cells in a 60 mm dish were transfected with 10 μg of the pNL4-3 construct by the calcium phosphate method. The supernatant was collected 48 h after transfection, passed through a 0.45 μm filter, and stored at −80 °C as the virus stock.

For the viral neutralizing assay, MT-4 cells (1×10^5 cells/100 μL) were incubated in medium (100 μL) containing 10 μL of sera from immunized or pre-immunized mice for 1 h at 37 °C. The pretreated MT-4 cells were infected with HIV-1 NL4-3 (MOI = 0.05). At 3 days after infection, the virus-infected cells were collected and lysed with lysing buffer. The processed samples were subjected to SDS–PAGE to perform Western blotting. The HIV-1 gag p24 was detected with HIV-1 neutralizing serum (NIH AIDS Research and Reference Reagent Program).[54] The intensity of p24 bands was obtained by measuring their chemiluminescence and quantified using LAS-3000 (Fuji Film). The level of p24 in the culture supernatant was determined by the p24 ELISA kit (ZeptoMetrix Corporation).

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Supplementary data

Supplementary data (HPLC charts of the synthetic compounds and HIV-1 inhibitory assay data (p24 Western)) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.09.037.

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Anti-HIV-1 Peptide Derivatives Based on the HIV-1 Co-receptor CXCR4

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The human immunodeficiency virus type 1 (HIV-1) uses CD4 and the co-receptor CCR5 or CXCR4 in the process of cell entry. The negatively charged extracellular domains of CXCR4 (CXCR4-ED) interact with positive charges on the V3 loop of gp120, facilitating binding via electrostatic interactions. The presence of highly conserved positively charged residues in the V3 loop suggests that CXCR4-ED-derived inhibitors might be broadly effective inhibitors. Synthetic peptide derivatives were evaluated for anti-HIV-1 activity. The 39-mer extracellular N-terminal region (NT) was divided into three fragments with 10-mer overlapping sites (**N1**–**N3**), and these linear peptides were synthesized. Peptide **N1** contains Met1–Asp20 and shows significant anti-HIV-1 activity. Extracellular loops 1 and 2 (ECL1 and 2) were mimicked by cyclic peptides **C1** and **C2**, which were synthesized by chemoselective cyclization. Cyclic peptides **C1** and **C2** show higher anti-HIV-1 activity than their linear peptide counterparts, **L1** and **L2**. The cytotoxicities of **C1** and **C2** are lower than those of **L1** and **L2**. These results indicate that Met1–Asp20 segments of the NT and cyclic peptides of ECL1 and ECL2 are potent anti-HIV-1 drug candidates.

Introduction

Almost 30 years have passed since the human immunodeficiency virus (HIV) was discovered to be responsible for acquired immunodeficiency syndrome (AIDS).[1] Infection by HIV-1 is initiated by adsorption with the primary host cell receptor CD4[2] and the co-receptors CCR5 or CXCR4.[3–8] Initially, the HIV-1 envelope glycoprotein gp120 interacts with CD4, triggering conformational changes within gp120 that result in exposure of the co-receptor binding site, which is composed of the V3 loop and the gp120 bridging sheet.[3] To date, various CCR5/CXCR4 antagonists have been developed as anti-HIV-1 agents.[9] Maraviroc (4,4-difluoro-*N*-[(1*S*)-3-[(3-*exo*)-3-[3-methyl-5-(1-methylethyl)-4*H*-1,2,4-triazol-4-yl]-8-azabicyclo-[3.2.1]oct-8-yl]-1-phenylpropyl]cyclohexanecarboxamide) was the first CCR5 antagonist approved by the FDA.[10] A potent CXCR4 antagonist T140, a 14-mer cyclic peptide derived from polyphemusin II, was developed by our research group,[11] and a 5-mer cyclic peptide FC131 was developed by further modification of T140.[12] Small-molecule CXCR4 antagonists including AMD3100,[13,14] Dpa–zinc complex,[15] azamacrocyclic–zinc complexes,[16] KRH-1636,[17] and other compounds[18–22] have been developed.

The HIV-1 co-receptors CCR5 and CXCR4 are classified as a member of the seven transmembrane G-protein-coupled receptor (7TMGPCR) family, and have an N-terminal extracellular region (NT) and three extracellular loops (ECL1–3) as the extracellular domains (ED) as shown in Figure 1a. CXCR4-ED has more negative charges than CCR5-ED, with net charges of 0 on the NT, −1 on ECL1, +5 on ECL2, and −1 on ECL3. CXCR4-ED has net charges of −6 on the NT, 0 on ECL1, −3 on ECL2, and

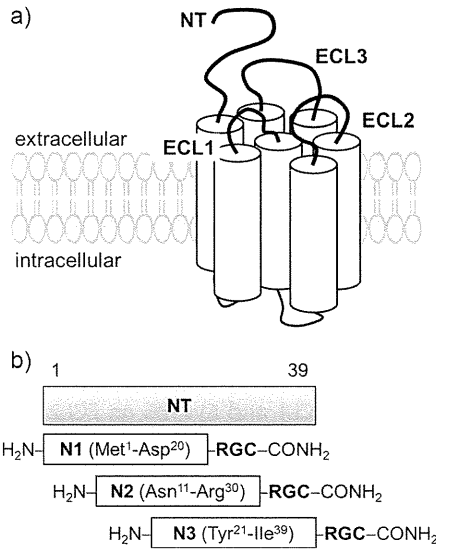

Figure 1. a) Schematic view of a 7TMGPCR, CXCR4. The extracellular N-terminal region (NT) and extracellular loops 1–3 (ECL1–ECL3) are indicated. b) The 39-mer peptide NT was divided into three fragment peptides, **N1**–**N3**.

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−1 on ECL3[23] and has electrostatic interactions with both its endogenous ligand CXCL12/SDF-1α[24] and the viral envelope protein gp120.[25–27] The negatively charged amino acid residues on CXCR4-ED are responsible for the binding of CXCL12/SDF-1α or gp120.

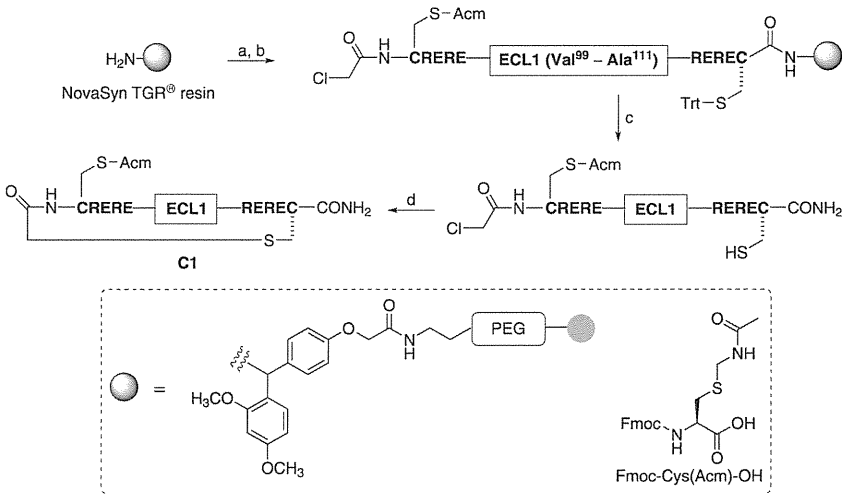
In the development of anti-HIV-1 drugs, viral mutation is the main obstacle, and it is therefore useful to target amino acid sequences that are conserved among various strains. The co-receptor binding site on the V3 loop is a variable region, but positively charged amino acid residues such as Arg 3, Arg/Lys 9–10, Arg 13, Arg 31, and His 34 that are responsible for binding to CCR5/CXCR4 are highly conserved amongst various strains,[28–30] and these positively charged amino acid residues can be an appropriate target for the development of HIV-1 entry inhibitors. Herein, we focus on peptidic inhibitors that bind to the conserved basic residues on the V3 loop of gp120, which might suppress the emergence of resistant viruses. Peptide derivatives based on CXCR4-ED were synthesized and their anti-HIV-1 activities have been evaluated in a search for drug candidates which can neutralize a broad variety of HIV-1 strains.

Results and Discussion

Design

Acidic amino acid residues on the NT are thought to be important for the interaction between the V3 loop and CXCR4.[26,31] The co-crystal structure (Figure 3 below) of CXCR4 and a T140[11]-derived CXCR4 antagonist CVX15 or a small molecule CXCR4 antagonist IT1t, shows that nine or seven amino acid residues on or around ECL1 and ECL2 of CXCR4-ED have polar interactions with these CXCR4 antagonists, while only one amino acid residue on ECL3 interacts with each antagonist.[32] Consequently, in this study, peptides derived from the NT, ECL1, and ECL2, not but ECL3, were synthesized in an attempt to develop entry inhibitors. As the NT is a linear peptide and ECLs form loop architectures (Figure 1 a), linear fragment peptides of NT and cyclic peptides derived from ECL1 and ECL2 were synthesized (Figure 1 b). The Cys residues involved in two disulfide bonds in CXCR4, between Cys 28 on the NT and Cys 274 on ECL3, and between Cys 109 on ECL1 and Cys 186 on ECL2 were replaced by Ser to avoid disulfide formation and reaction with a carrier protein or peptide. The 39-mer NT was segmented into NT-fragment peptides: **N1** (Met 1–Asp 20), **N2** (Asn 11–Arg 30), and **N3** (Tyr 21–Ile 39), retaining 10-mer overlapping sites. The tyrosine residues (Tyr 7, Tyr 12, and Tyr 21) in the NT are post-translationally sulfated, and are implicated in the reaction between CXCR4 and CXCL12/SDF-1α.[31,33,34] However, in the synthesis of sulfo-Tyr-containing peptides, the conditions for the cleavage of the peptides from the resin must be carefully selected because sulfonyl groups are acid labile.[35] Accordingly, NT-fragment peptides in which Tyr is substituted for sulfo-Tyr were synthesized. On the C terminus of each NT-fragment peptide, three additional amino acid residues were attached; Arg as a hydrophilic amino acid residue, Gly as a spacer, and Cys as a ligation site for a carrier such as BSA, KLH, or multi-antigen peptides (MAP) so that the antigenicity of these peptides could be evaluated in future studies (Figure 1 b).

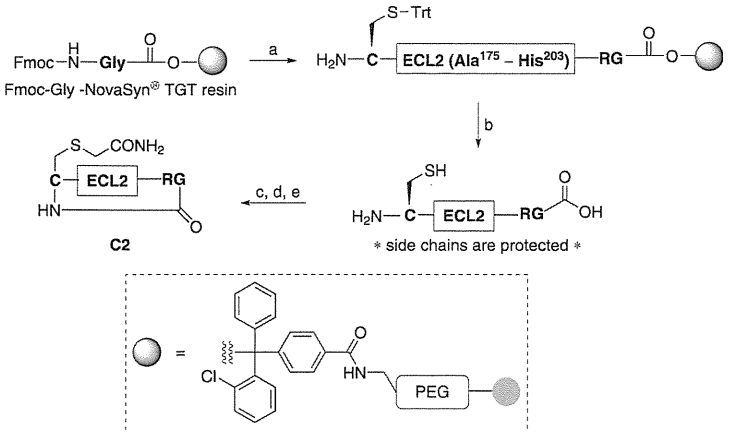
Two different types of cyclization strategies were used to synthesize cyclic peptides of ECL1 and ECL2 which mimic their loop architectures. A cyclic peptide based on ECL1 (**C1**) was designed with double repeats of Arg and Glu (RERE) at both the N and C termini to increase the aqueous solubility and to be cyclized by a reaction between the N-terminal chloroacetyl group and the C-terminal Cys thiol group in a dilute solution, pH 7.8 (Scheme 1). As in the NT-fragment peptides, another Cys residue was fused to the N terminus to create the ligation site with a carrier. Accordingly, an Fmoc-Cys(Acm)-OH was condensed at the N terminus, in which the Cys side chain was protected with an acetamidemethyl group (Acm) that can be cleaved with silver trifluoromethanesulfonate (AgOTf) in TFA, and an Fmoc-Cys(Trt)-OH was condensed at the C terminus to avoid reactions between the thiol group on the N-terminal Cys and the chloroacetyl group.



Scheme 1. Synthesis of a cyclic peptide of ECL1, **C1**. *Reagents and conditions*: a) Fmoc SPPS; b) 2-chloroacetic acid, HOBt·H_2O, DIPCI, DMF; c) TFA, thioanisole, *m*-cresol, EDT, H_2O, TIS; d) H_2O, CH_3CN, pH 7.8 adjusted with $NH_{3(aq)}$.

A cyclic peptide based on ECL2 (**C2**) could not be synthesized in this way (data not shown), and a different **C2** variant was designed to cyclize in a head-to-tail cyclization using HOBt/HBTU/DIEA as shown in Scheme 2. The N-terminal Cys was fused as a ligation site with a carrier, and the C-terminal Arg was fused as a hydrophilic residue and the C-terminal Gly as a spacer.

Additionally, all peptides, with the exception of **C1**, were treated with iodoacetamide in 0.1 M sodium phosphate buffer



Scheme 2. Synthesis of a cyclic peptide of ECL2, **C2**. *Reagents and conditions*: a) Fmoc SPPS; b) AcOH, TFE, CH_2Cl_2; c) HOBt·H_2O, HBTU, DIPEA, DMF; d) TFA, thioanisole, *m*-cresol, EDT, H_2O, TIS; e) iodoacetamide, 0.1 M sodium phosphate buffer, pH 7.8.

at pH 7.8 to cap thiol groups of Cys residues fused as ligation sites with a carrier (Figure 2). The thiol group of the N-terminal Cys on **C1** is protected with Acm.

Peptide synthesis

All peptides were synthesized by standard Fmoc solid-phase peptide synthesis (SPPS) and characterized by ToF-ESIMS. The three NT-fragment peptides (**N1**–**N3**) containing 10-mer overlapping regions to facilitate investigation of active sites were synthesized on NovaSyn TGR resin. After HPLC purification, free thiol groups on the C terminus were capped with iodoacetamide in 0.1 M sodium phosphate buffer at pH 7.8, producing **N1**–**N3** as colorless powders (Supporting Information figure 1).

C1, a cyclic peptide based on ECL1, was synthesized on a NovaSyn TGR resin, and then cleaved from the resin with a mixture of TFA/thioanisole/*m*-cresol/EDT/TIS/H_2O. After deprotection and cleavage from the resin, the **C1** linear peptide was dissolved in a dilute solution of CH_3CN/H_2O, and the solution was adjusted to pH 7.8 with an aqueous ammonia solution. The crude peptide was purified to obtain a cyclic peptide **C1** as a colorless powder.

C2, a cyclic peptide based on ECL2, was synthesized on an Fmoc-Gly NovaSyn TGT resin. The synthetic peptide on the resin was treated with AcOH/TFE/CH_2Cl_2 to obtain the protected crude peptide, and this was followed by cyclization involving condensation of the N-terminal amino group with the C-terminal carboxyl group using HOBt/HBTU/DIEA. The side chains of the cyclic peptides were deprotected with TFA/thioanisole/*m*-cresol/EDT/TIS/H_2O and the crude peptides were purified by HPLC. Subsequently, the free thiol group on the N terminus was capped with iodoacetamide in 0.1 M sodium phosphate buffer at pH 7.8 to produce a cyclic peptide **C2** as a colorless powder. Linear peptides of ECL1 (**L1**) and ECL2 (**L2**) were synthesized as controls for cyclic peptides **C1** and **C2** (Supporting Information figures 3 and 5). The amino acid sequences of thiol-capped CXCR4-ED-derived peptides are shown in Figure 2.

Anti-HIV-1 activity

The anti-HIV-1 activity and cytotoxicity of the synthetic peptides were assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and the results are presented in Table 1. Of the synthetic peptides, **N1** showed the highest anti-HIV-1 activity (EC_{50} = 0.88 μM), and did not show significant cytotoxicity at concentrations below 10 μM. **N2** and **N3** failed to show either significant anti-HIV-1 activity or cytotoxicity, indicating that **N2** and **N3** cannot bind to the V3 loop of gp120. The negatively charged amino acid residues of the extracellular domains of CXCR4 interact with the positively charged amino acid residues on the V3 loop of gp120. Of the peptides **N1**–**N3**, **N1** has the greatest number of negative charges and it was hypothesized that it might bind strongly to the V3 loop and thus inhibit HIV-1 entry. The amino acid sequence of the fragment peptide **N1** (Met1–Asp20) contains two sulfo-Tyr, Tyr7 and Tyr12 and sulfotyrosines on the NT of

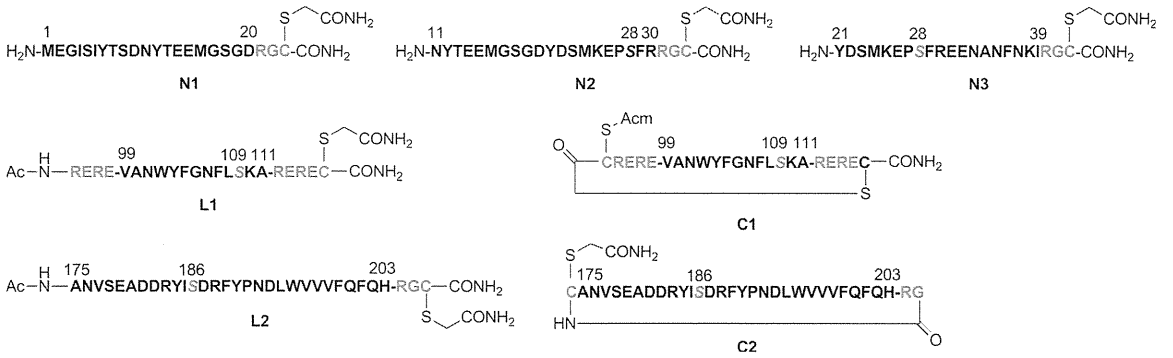


Figure 2. Structures of synthetic CXCR4-ED-derived peptides. **L1** is a thiol-capped linear peptide of ECL1, **C1** is a thiol-capped cyclic peptide of ECL1, **L2** is a thiol-capped linear peptide of ECL2, and **C2** is a thiol-capped cyclic peptide of ECL2. R and E (in grey) are Arg and Glu residues, respectively, added to increase solubility. C (in grey) is used as a ligation site with multi-antigen peptides (MAP). Cys109 on ECL1 and Cys186 on ECL2 were mutated to Ser (S, grey italics) to avoid reaction with MAP at these sites.

Table 1. Anti-HIV-1 activity and cytotoxicity of CXCR4-ED-derived peptides.[a]

Compd	EC_{50} [μM]	CC_{50} [μM]	Compd	EC_{50} [μM]	CC_{50} [μM]
N1	0.88	>10	**L1**	50[b]	61
N2	>10	>10	**L2**	>25	35
N3	>10	>10	**C1**	35	>200
AMD3100	0.06	>20	**C2**	32	133
			AMD3100	0.096	18

[a] The number of viable cells was determined using the MTT method. A CXCR4 antagonist (AMD3100) was used as positive control. EC_{50} values are the concentrations resulting in 50% protection from HIV-1-induced cytopathogenicity in MT-4 cells. CC_{50} values are the concentrations required for a 50% decrease in MT-4 cell viability. All data are the mean values from three independent experiments. The data on the left and right columns are from two independent assays under identical conditions. [b] 48% inhibition.

CCR5 have been reported to be critical for specific binding to the V3 loop.[36] In contrast, the fragment peptide **N1** (Met1–Asp20), which has no sulfo-Tyr, has significant anti-HIV-1 activity. This result indicates that the NT of CCR5, with less negatively charged amino acid residues than that of CXCR4, might require sulfo-Tyr for the interaction between the NT and the V3 loop, because the sulfate groups bear a negative charge. Sulfo-Tyr residues on CXCR4-NT might be related to the interaction between CXCR4 and gp120, or an endogeneous ligand CXCL12/SDF-1α.[37,38] Sulfo-Tyr-containing peptides however are difficult to handle due to the sensitivity of sulfonyl groups to acidic conditions.[35] This makes sulfo-Tyr-containing peptides difficult to develop as drugs and **N1** might be a potent anti-HIV-1 drug candidate because it contains no sulfo-Tyr. The cytotoxicity of the NT-fragment peptides is minimal.

The cyclic peptides **C1** and **C2** are less cytotoxic and more potent than their corresponding linear peptides **L1** and **L2**. If ECL-derived peptides are to be developed as entry inhibitors, they might inhibit the interaction between CXCR4 and gp120 by their binding to the V3 loop of gp120. Cyclic peptides **C1** and **C2** were thought to bind to the V3 loop with higher binding affinity than linear peptides **L1** and **L2** indicating that **C1** and **C2** might be able to mimic the structures of native ECL1 and ECL2 effectively. Cyclization also affected the cytotoxicities of ECL-derived peptides. The cytotoxicity of **C1** and **C2** were lower than **L1** and **L2**. The V3 loop is thought to be composed of three regions: 1) the base of the loop containing a disulfide bond; 2) the stem, a conformationally flexible region; and 3) the tip, a conserved region (Figure 3a).[36,39] According to models of CXCR4 with gp120 by Wu et al., the NT of CXCR4-ED initially binds to the V3 base, and then the ligand-binding cavity, the V3 stem and tip bind to ECL2 and ECL3 (Figure 3a).[32] The NT can bind to the V3 base alone, however, amino acid residues related to the V3 stem and tip binding dot on sequentially discontinuous regions. This might explain why **N1** has potent anti-HIV-1 activity whereas **C1** and **C2** have only moderate anti-HIV-1 activity.

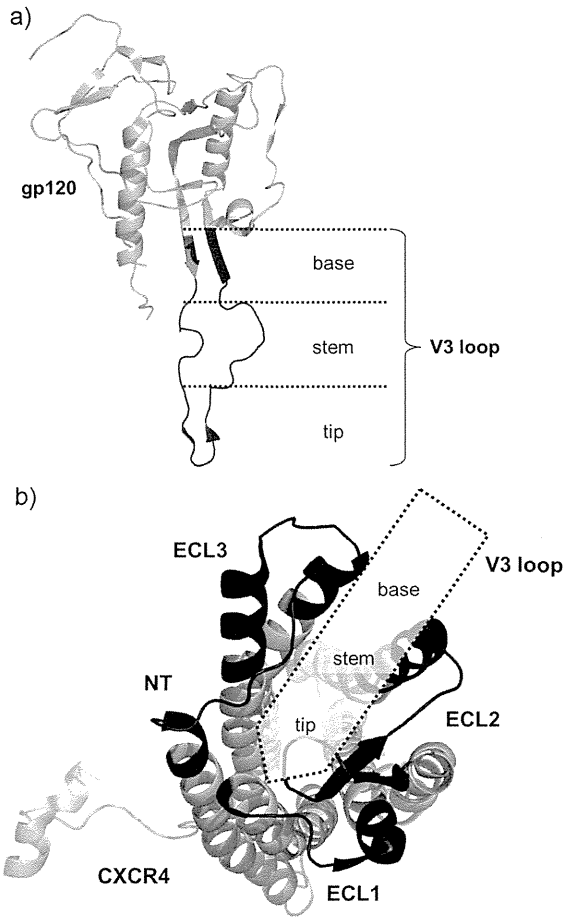


Figure 3. a) X-ray crystal structure of the gp120 V3 loop (PDB ID: 2B4C):[40] gp120 is depicted in light grey, and the section Lys117–Gln203 is hidden to show the V3 loop clearly. b) Predicted binding of CXCR4 (PDB ID: 3ODU)[32] and the V3 loop: the NT is indicated, and Met1–Glu26 is truncated because these residues with undefined density are presumed to be disordered. ECL1–3 are also indicated; Wu et al.[32] predicted that the V3 tip is inserted into the ligand binding pocket on CXCR4, the V3 base interacts with NT, and the V3 stem/tip interact with ECL1–3. These images were generated with PyMOL.[41]

Conclusions

Peptidic inhibitors focusing on electrostatic interactions between CXCR4 and the gp120 V3 loop of the HIV-1 envelope protein have been synthesized. Anti-HIV-1 agents targeting the V3 loop, whose basic amino acid residues are highly conserved among various strains, might have a significant advantage in suppressing the emergence of drug resistant viruses. The NT, ECL1, and ECL2 of CXCR4-ED are thought to interact with the V3 loop, and accordingly peptides derived from these regions were synthesized and their anti-HIV-1 activity was evaluated to identify regions associated with anti-HIV-1 activity. An NT-fragment peptide, **N1** showed significant anti-HIV-1 activity. Cyclic peptides of ECL1 and ECL2, which mimic the structures of the native ECL1 and ECL2, showed higher anti-HIV-1 activity than the corresponding linear peptides. The **N1** peptide appears to bind to the V3 base and behave as an inhibitor of HIV-1 entry. Other candidates might be found based on ECL1 and ECL2 by

optimizing the appropriate cyclization. CXCR4-ED-derived peptidic inhibitors could be potent HIV-1 entry inhibitors, suppressing the emergence of drug resistant strains.

Experimental Section

Peptide synthesis: Peptides were synthesized by using the standard Fmoc solid-phase protocol, purified by RP-HPLC, and characterized by ToF-ESIMS. Detailed procedures and data are provided in the Supporting Information.

Biological assays: Anti-HIV-1 activity was assessed as protection against HIV-1 (NL4-3 strain)-induced cytopathogenicity in MT-4 cells. Mixtures of various concentrations of test peptide solutions and HIV-1 were added to MT-4 cells at a multiplicity of infection (MOI) of 0.001 and placed in a 96-well microplate. After a five-day incubation period at 37 °C in a CO_2 incubator, the number of viable cells was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and EC_{50} values were calculated. Cytotoxicity was determined based on the viability of mock-infected cells using the MTT method (CC_{50}). Each experiment was performed three times independently.

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Keywords: antiviral agents · CXCR4 · HIV-1 entry inhibitors · extracellular domains · peptides

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Multimerized CHR-derived peptides as HIV-1 fusion inhibitors ☆

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ABSTRACT

To date, several HIV-1 fusion inhibitors based on the carboxy-terminal leucine/isoleucine heptad repeat (CHR) region of an HIV-1 envelope protein gp41 have been discovered. We have shown that a synthetic peptide mimetic of a trimer form of the CHR-derived peptide C34 has potent inhibitory activity against the HIV-1 fusion mechanism, compared to a monomer C34 peptide. The present study revealed that a dimeric form of C34 is evidently structurally critical for fusion inhibitors, and that the activity of multimerized CHR-derived peptides in fusion inhibition is affected by the properties of the unit peptides C34, SC34EK, and T20. The fluorescence-based study suggested that the N36-interactive sites of the C34 trimer, including hydrophobic residues, are exposed outside the trimer and that trimerization of C34 caused a remarkable increase in fusion inhibitory activity. The present results could be useful in the design of fusion inhibitors against viral infections which proceed via membrane fusion with host cells.

1. Introduction

Newly developed anti-HIV-1 drugs such as protease inhibitors and integrase inhibitors have contributed to the highly active anti-retroviral therapy (HAART) for AIDS.[1] Entry of human immunodeficiency virus type 1 (HIV-1) into target cells is mediated by its envelope glycoproteins (Env), which are type I transmembrane proteins consisting of a surface subunit gp120 and a non-covalently associated transmembrane subunit gp41.[2] Sequential binding of HIV-1 gp120 to its cell receptor CD4 and a co-receptor (CCR5 or CXCR4) can trigger a series of conformational rearrangements in gp41 to mediate fusion between viral and cellular membranes.[3–5] The protein gp41 is hidden beneath gp120 and its ectodomain contains the helical amino-terminal and carboxy-terminal leucine/isoleucine heptad repeat domains NHR and CHR. Regions of NHR and CHR are involved in membrane fusion, and 36-mer and 34-mer peptides, derived from NHR and CHR, have been designated as the N-terminal helix (N36) and C-terminal helix (C34), respectively. In the membrane fusion of HIV-1, these helices assemble to form a six-helical bundle (6-HB) consisting of a central parallel trimer of N36 surrounded by three strands of C34 in an antiparallel hairpin fashion. Synthetic peptides derived from these helices have potent antiviral activity both against laboratory-adapted strains and against primary isolates of HIV-1.[6–9] They inhibit the membrane fusion stage of HIV-1 infection in a dominant-negative manner by binding to the counterpart regions of gp41 (NHR or CHR) thereby blocking formation of the viral gp41 core. Several potent anti-HIV-1 peptides based on the CHR region have been discovered[7,8] and T20 has subsequently been developed by Roche/Trimeris as the clinical anti-HIV-1 drug, enfuvirtide.[8,10–13] T20 is a 36-mer peptide derived from the gp41 CHR sequence and can bind to the NHR region to inhibit the formation of the 6-HB structure in a dominant-negative fashion.[10] T20 therapy has brought safety, potent antiretroviral activity, and immunological benefit to patients, but its clinical application is limited by resistance development. The C-terminal helix C34 is also a CHR-derived peptide, and contains the amino acid residues required for the peptide to dock into the hydrophobic pocket, termed the 'deep pocket' of the trimer of the NHR region. This peptide potently inhibits HIV-1 fusion in vitro.[14] To date, several gp41 mimetics, especially for N36 regions, which assemble these helical peptides with branched peptide-linkers have been synthesized as antigens.[15–19] Our three-helix bundle mimetic, which corresponds to the trimeric form of N36, with a novel template containing C3-symmetric linkers of equal lengths, showed significant potency as a peptide antigen that can produce antibodies with structural-preference.[20] In terms of inhibitory activity the N36 trimer peptide, compared to the N36 monomer, showed only a stoichiometric increase in inhibition of HIV-1 fusion, a phenomenon consistent with the results of other studies.[21–23] The utilization

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of our C3-symmetric linkers was expanded to C34 sequences in the design of the trimer mimics of gp41. It is noteworthy that the trimer form of C34 showed a 100-fold increase in inhibition of fusion compared to the monomer form.[24] Multimerization of functional units, such as synthetic ligands against receptors, generally shows synergistic binding and high binding affinity.[25] To explore the mechanism of action of fusion inhibition by the C34 trimer, C34-derived peptides in the form of monomer, dimer, or trimer were prepared in this study and as CHR-derived peptides, SC34EK[26] and T20 peptides were also studied (Fig. 1). It has been reported that the sequence differences of these peptides show different interaction modes with N36 trimers. Thus, the effects of increased activity in HIV-1 fusion inhibition were investigated by comparison of the activities of the multimerized CHR-derived peptides.

2. Results and discussion

2.1. Chemistry

The C-terminal region of gp41 is known to be an assembly site involving a trimeric coiled-coil conformation. In our synthetic CHR-derived peptides, CHR-thioester and CHR-REG (Fig. 2A), the triplet repeat of arginine and glutamic acid (RERERE) was added to the C-terminal end of the C34 sequence (residues 628–661) to increase the solubility in buffer solution, and in thioesterified CHR-derived peptides, glycine thioester was fused to the C-terminus. The peptides were synthesized by standard Fmoc solid-phase peptide synthesis. The C3-symmetric template was utilized to form a triple helix corresponding precisely to the gp41 pre-fusion form. The template linker has three branches of equal length and possesses a hydrophilic structure and a ligation site for coupling with thioesterified CHR-derived peptides. The template was synthesized as reported previously[24] and for chemoselective coupling between C34 peptides and the template, native chemical ligation was performed between an unprotected CHR-REG-thioester and a three-armed cysteine scaffold (Fig. 2B, C).[27,28] The trimer forms of other CHR-derived peptides, SC34EK and T20, were similarly prepared. For the synthesis of the dimer forms of these peptides, the ratio of the peptides and the template was controlled in a stoichiometric manner (Fig. 2B). After the native chemical ligation, the free thiol groups were blocked by carboxymethylation with iodoacetamide in the SH-capped peptides. The products were purified by preparative HPLC and characterized by ESI-TOF-MS.

2.2. Anti-HIV assays

The fusion inhibitory activity was evaluated. In our previous study, the C34 trimer, named triC34e, contained free thiol groups at the ligation sites, and a concern with intermolecular or intramolecular formation of disulfide bonds remained. Thus, in this study, dimer and trimer peptides with and without carboxymethylation at the thiol groups, SH-capped and SH-free peptides, respectively, were prepared. In these dimer and trimer peptides, the inhibitory activities of the SH-capped peptides were comparable with or slightly higher than those of the corresponding SH-free peptides. The presence of the carboxymethyl groups did not affect the fusion inhibitory activity and consequently, the IC_{50} values of the SH-capped peptides are felt to be more reliable. Of the C34 unit peptides, the dimer form peptide, the C34 dimer (SH-capped), showed a significant increase in inhibitory activity compared to the C34 monomer (Table 1). Its IC_{50} value was nearly equal to that of the C34 trimer (SH-capped). These results suggest that the C34 units in the dimer form can bind to the gp41 N36 region in a cooperative manner. Compared to the C34 unit peptides, SC34EK and T20 units showed different activity phenomena in multimerized forms. For SC34EK, the monomer unit showed very high activity in fusion inhibition. It is known that the SC34EK obtains higher helicity by the introduction of salt bridges between lysine and glutamic acid in positions at *i* and *i+4*. The positions of substitution of these residues are selected as those having no interaction with N36 trimers. Thus, SC34EK is suspected to form an amphipathic helix. In the dimer and trimer forms of SC34EK, the hydrophilic face should be exposed to buffer solvents and the hydrophobic face, which can interact with the N36 pocket, might be packed inside. The decrease in inhibitory activity, especially in the trimer form (5.1-fold, SH-capped), stemmed from this formation of SC34EK multimers, compared to that of the SC34EK monomer. For T20 peptides, the C-terminal region has been shown to have interactions with membranes. The dimer and trimer of T20 peptides showed 4.3- and 8.6-fold (SH-capped) increases in fusion inhibitory activity, respectively, compared to the T20 monomer. Although some increase in activity was observed, the effect of multimerization did not reveal any cooperative interaction. For all of the peptides, significant cytotoxicity was not observed at a concentration below 5 μM.

2.3. Circular dichroism (CD) spectroscopy

To investigate different effects of multimerization of CHR-derived peptides, folded structures of multimerized peptides were estimated by analysis of CD spectroscopy (Fig. 3). The peptides were dissolved in 50 mM sodium phosphate buffer, pH 7.2 with 150 mM NaCl. In our previous study, it was observed that C34 peptides tended to form random structures both in the monomer and in the trimer.[24] The spectra of the C34 monomer, dimer, and trimer (both, SH-capped) displayed minima around 200 nm, indicating that these peptides form random structures (Fig. 3A). We previously reported that the N36 monomer N36RE and the N36 trimer triN36e form a highly structured α-helix and that the helical content of triN36e was higher than that of N36RE.[20,29] In C34 peptides, multimerization of the peptides did not induce an increase in the α-helicity of the peptides. On the contrary, SC34EK and T20 peptides showed an increase of minima around 208 and 222

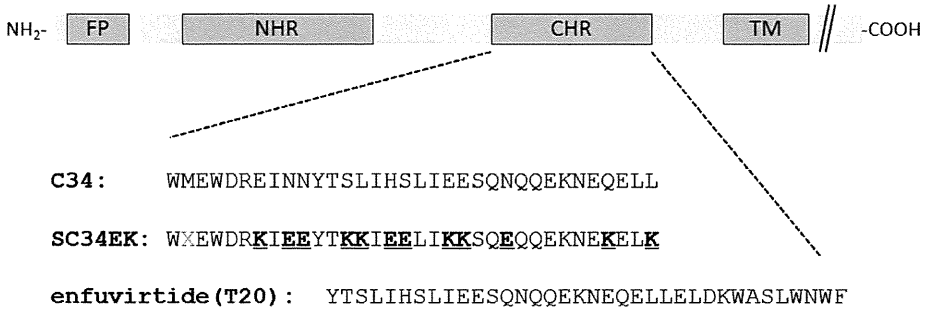

Figure 1. Schematic illustration of HIV-1 gp41 and amino acid sequences of C34, SC34, and enfuvirtide (T20). FP and TM represent a hydrophobic fusion peptide and a transmembrane domain, respectively. In the sequence of SC34EK, X represents norleucine.

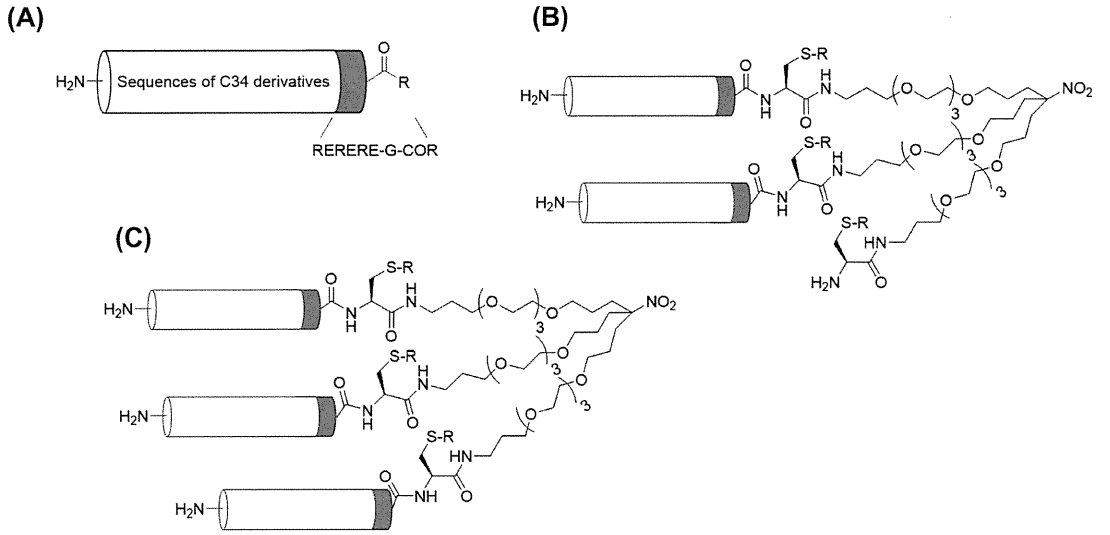

Figure 2. (A) Schematic illustration of CHR-derived peptides. The monomer peptides obtain carboxylate at the C-terminus (R = OH). The monomer units for native chemical ligation with the templates obtain thioester at the C-terminus (R = $SC_2H_4COOC_2H_5$). (B) Structures of dimers of CHR-derived peptides. The R represent the state of thiol groups, SH-free or SH-capped by iodoacetamide (R = H or CH_2CONH_2, respectively). (C) Structures of trimers of CHR-derived peptides. The R represent the state of thiol groups, SH-free or SH-capped by iodoacetamide (R = H or CH_2CONH_2, respectively).

Table 1
Fusion inhibitory activity and cytotoxicity of monomer or multimerized CHR-derived peptides

	IC_{50}[a] (nM)	CC_{50}[b] (μM)
C34 monomer	152	>5
C34 dimer (SH-free)	1.62	>5
C34 dimer (SH-capped)	1.27	>5
C34 trimer (SH-free)	2.25	>5
C34 trimer (SH-capped)	1.28	>5
SC34EK monomer	1.82	>5
SC34EK dimer (SH-capped)	2.14	>5
SC34EK trimer (SH-free)	8.20	>5
SC34EK trimer (SH-capped)	9.34	>5
T20 monomer	465	>5
T20 dimer (SH-capped)	107	>5
T20 trimer (SH-free)	66.8	>5
T20 trimer (SH-capped)	54.1	>5
T20 (NIH)[c]	45.2	>5

[a] IC_{50} values are based on luciferase signals in TZM-bl cells infected with HIV-1 (NL4-3 strain)

[b] CC_{50} values are based on the reduction of the viability of TZM-bl cells. All data are the mean values from at least three experiments.

[c] The sample was obtained through the NIH AIDS Research and Reference Reagent Program, which did not contain RERERE or glycine thioester.

(nm) indicating an increase of α-helicity as the level of unit peptides increases (Fig. 3B, C). SC34EK is known to form more stable α-helix by the effects of salt bridges[26] and the SC34EK monomer peptide failed to show such effects. The SC34EK monomer has three repeats of Arg-Glu at the C-terminus and a corresponding increase in solubility. Thus, the hydrophilic residues might inhibit efficient formation of α-helix in the monomer form or in the assembled forms. In the dimer and trimer forms of SC34EK, the peptides showed a significant increase of α-helicity (Fig. 3B). These multimers are assembled by covalent bonds via the template and the spectra indicated that the peptides could interact with one another to form stable α-helices. The same effect was observed for multimerization of the T20 peptides (Fig. 3C). Multimerization of SC34EK and T20 affected fusion inhibitory activity differently (Table 1). Thus, this difference might result from a different interaction with N36 peptides. To investigate the difference, the CD spectra of the complex with N36 peptides were investigated.

When complexed with N36RE which is an N36 derivative with the triplet repeat of Arg and Glu (RERERE) at the N-terminus,[20] all of the CHR-derived peptides showed similar spectra independent of the difference of the numbers of unit peptides, that is, monomer, dimer and trimer. The spectra indicated the complexes of the C34 and N36RE peptides form a stable α-helix (Fig. 4A). The spectrum of N36RE displayed weak Cotton effects at 208 and 222 (nm). Compared to the previous study, the solvent was changed from 40% methanol to PBS only with possible weakening of the formation of α-helices. The spectra of the C34 peptides suggest that the equi-

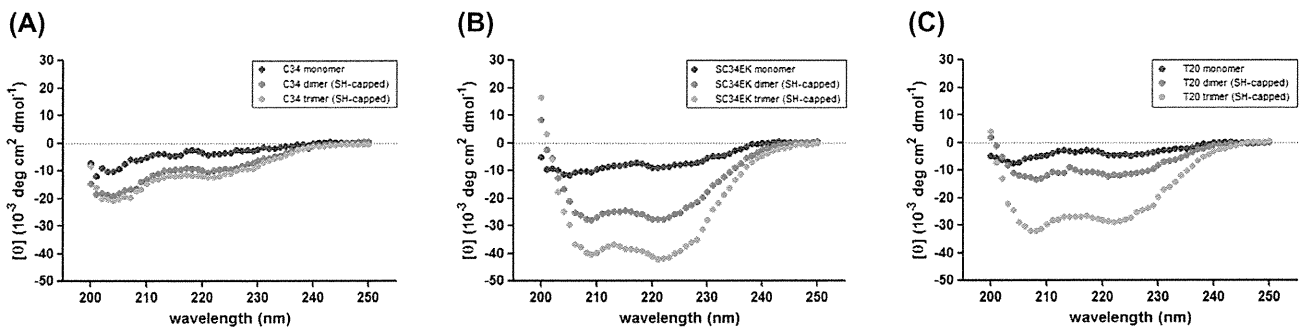

Figure 3. Circular dichroism (CD) spectra of monomer (blue dot), dimer (red dot), and trimer (green dot) of CHR-derived peptides. Panels (A–C) depict the spectra for C34 (A), SC34EK (B), and T20 (C). The concentrations of peptides are 6, 3, and 2 (μM) for monomer, dimer, and trimer (SH-capped), respectively.

librium states of complexes with N36RE are similar in the three types of peptides. For SC34EK, in comparison between monomer and dimer, the dimer complex showed higher α-helicity (Fig. 4B). It is difficult to conclude that the monomer and dimer complexes form different complex states and the difference in α-helicity might be caused by the SC34EK peptides that are not included in the complex with the N36RE peptide. The spectra of the T20 peptides showed that the three types of peptides form similar complexes with the N36RE peptide (Fig. 4C) and individually, the T20 peptides showed a significant increase of α-helicity paralleling the increase of unit numbers (Fig. 3C). In the complexes with the N36RE peptide, it was suggested that the equilibrium states are similar in each of the three types of peptides. The α-helicities of the dimer and the trimer of T20 in the complexes with the N36RE peptide are lower than those of the corresponding dimer and trimer of T20 alone, respectively (Figs. 3C and 4C). This indicates that the increased α-helicity of the T20 peptides by multimerization was disrupted by the interaction with the N36RE peptide, and that the T20 peptide shows a interaction mode with N36RE that is different from the formation of stable α-helical complexes as observed in the C34 peptides.

2.4. Fluorescence-based assays

To explore the reason for the remarkable decrease in fusion inhibitory activity by trimerization of SC34EK, the interaction among unit peptide strands was investigated. The SC34EK peptides are thought to form stable helical bundles through packing of hydrophobic residues exposing the hydrophilic faces formed by the introduction of salt bridges between Glu and Lys. The formation of the stable packing structure of the SC34EK peptides could weaken interactions with the N36 peptide and to evaluate the packing effect of the multimerized SC34EK peptides, analysis with a fluorescent dye, utilized for estimation of the folding stability of proteins, was applied. In the due course of denaturing by increasing temperatures, hydrophobic residues are exposed.[30] A hydrophobic dye, SYPRO orange,[31] can interact with hydrophobic residues thereby increasing the fluorescence intensity. By taking advantage of this phenomena, the states of the multimerized C34 and SC34EK peptides can be estimated. If the hydrophobic residues are packed in the assembled form of SC34EK, the interaction of the SC34EK trimer with SYPRO orange could be decreased compared to that of the C34 timer. As shown in Figure 5A, the monomers of C34 and SC34EK showed similar spectra with low levels of fluorescent intensity. The trimers of C34 and SC34EK showed spectra with remarkably higher levels of fluorescent intensity than the corresponding monomers. In addition, the wavelength showing maximum intensity in the spectra of these trimers was shifted from 625 to 602 nm. These data suggest that the surfaces of these trimers have higher hydrophobicity than those of the corresponding monomers. The C34 trimer showed approximately 1.5-fold stronger fluorescent intensity compared to the SC34EK trimer at the maxima in the fluorescence spectra (602 nm). High fluorescent intensity was not observed in an octa-arginine peptide. Thus, this 1.5-fold difference of fluorescent intensity is due to the difference of hydrophobicity in the surfaces caused by formation of multimerization of C34 and SC34EK peptides. In the SC34EK trimer, since the hydrophobic residues might be efficiently packed inside helical bundles by the trimerization, the increase of hydrophobicity of the peptide surfaces was totally suppressed. Thus, in comparison with the helix sites of the C34 trimer, the N36-interactive sites of the SC34EK strands tend to be buried inside the trimer, and the solvent-accessible sites having E-K salt bridges tend to be exposed outside the trimer. As a result, the SC34EK trimer has more difficulty than the SC34EK monomer accessing N36, and thereby the inhibitory activity of the SC34EK trimer is lower than that of the SC34EK monomer.

2.5. Biological discussion

Based on our previous study indicating the increased activity in HIV-1 fusion inhibition by a cooperative action of C34 in the trimer form, the different sets of CHR-derived peptides in forms of monomer, dimer, and trimer forms were synthesized and evaluated. In the fusion inhibition assays, the following noteworthy effects were revealed; (1) dimerization of C34 shows the same cooperative effect in increases of fusion inhibition as are shown by the C34 trimerization, and (2) the activity of multimerized CHR-derived peptides in fusion inhibition is affected by the properties of unit peptides C34, SC34EK, and T20.

In the previous study, a dimeric C37 (residues 625–661) variant did not show any significant difference in IC_{50} values of anti-HIV-1 activity compared to a wild type C37 monomer although the dimer peptide showed tighter binding than the C37 monomer to the gp41 NHR coiled coil.[32] In this study, the C37 dimer was formed *via* a disulfide bond at the C-terminus after six histidine residues. In our dimer peptides, the unit peptides are linked by the template structure and, because of the stretched distance between the unit peptides in the complex formation with the N36 peptide, the unit peptides have greater flexibility than the C37 dimeric structure. Such an effect might lead to activity of the dimeric form of the C34 peptides that is different from that of C37 peptides. It has been shown that T-1249, an analogous peptide of enfuvirtide (T20), and its hydrophobic C-terminal peptide inhibit HIV-1 fusion by interacting with lipid bilayers.[33] The tryptophan-rich domain of T-1249 was shown to play important roles in HIV-1 fusion.[34–36] Since enfuvirtide shows a weak interaction with the gp41 core structure and the C34 sequence lacks the C-terminal lipid binding

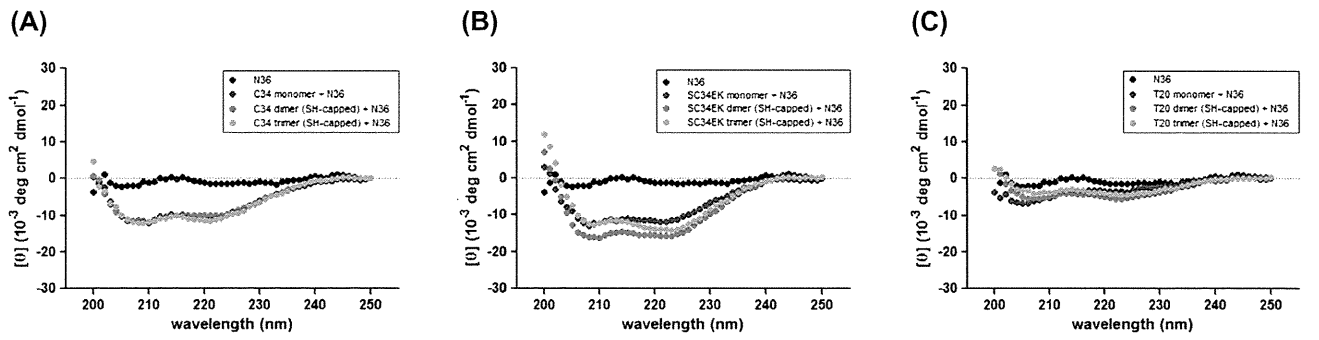

Figure 4. Circular dichroism (CD) spectra of N36RE (black dot) and the complexes of N36RE[21] with monomer (blue dot), dimer (red dot), and trimer (green dot) of CHR-derived peptides. Panels (A)-(C) depict the spectra for C34 (A), SC34EK (B), and T20 (C). The concentrations of CHR-derived peptides are 6, 3, and 2 (μM) for monomer, dimer, and trimer (SH-capped), respectively. The concentration of the N36RE peptide is 6 μM.

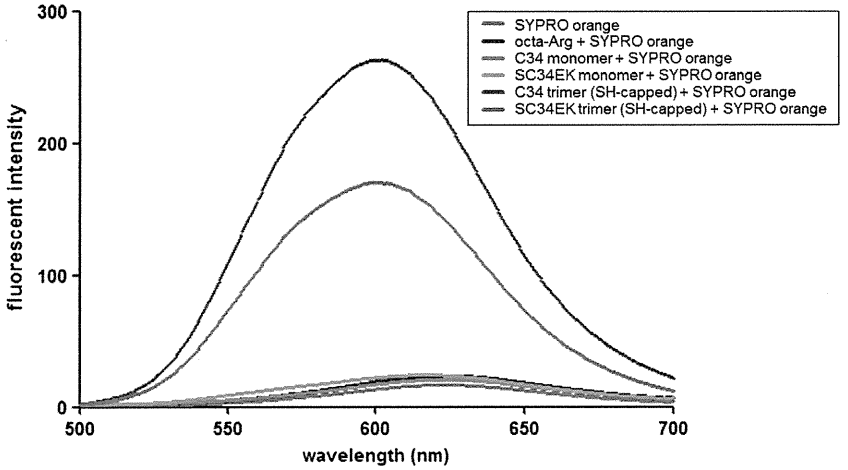

Figure 5. Fluorescent analysis utilizing SYPRO orange. The spectra are shown as follows; green line, SYPRO orange; blue line, octa-Arg + SYPRO orange; red line, C34 monomer + SYPRO orange; orange line, SC34EK monomer + SYPRO orange; purple line, C34 trimer (SH-capped) + SYPRO orange; pink line, SC34EK trimer (SH-capped) + SYPRO orange. The peptide concentration is 6 (μM) in all experiments.

domain, it has been suggested that C34 has a mechanism of action distinct from that of enfuvirtide.[37] Our study on the complex structures of the multimerized CHR-derived peptides and the N36 peptides was performed in aqueous buffer condition. The state of N36 is totally different from the state of gp41 in the membrane fusion of HIV-1 and host cells. The CHR-derived peptides could access the N36 peptide more easily than the whole of gp41 because the N36 sequence only contains interactive residues. The complex form of the CHR-derived peptides could be different from the complex form in the membrane fusion of HIV-1. If analytical methods are available with which to observe the state of the complex of the gp41 trimer and the CHR-derived peptides, more detailed information concerning the effects of cooperative interactions in multimerized forms of C34 will be obtained. Fluorescence-based analyses could be useful to analyze detailed mechanisms of actions for multimerized C34 peptides in fusion inhibition and recently, a fluorescence-based study observing the interaction of gp41 in the surface of cells was reported.[38] The present fluorescence-based study suggested that the N36-interactive sites of the C34 trimer including hydrophobic residues are exposed outside the trimer, and that the surface state of the C34 trimer is different from that of the SC34EK trimer. Thus, trimerization of C34 caused a remarkable increase in fusion inhibitory activity whereas trimerization of SC34EK caused a remarkable decrease in fusion inhibitory activity. It was shown that C34 and T20 have similar structures in the equilibrium states of the complexes with N36. For C34, the large increases in fusion inhibition in dimer and trimer forms might be due to the cooperative binding of monomer units. Since the complex formations with N36 are thought to be similar in the monomer, dimer and trimer forms, the difference of fusion inhibitory activity might be due to the difference of kinetics of the interaction with the N36 peptide. In the interaction with gp41 at the fusion step, it might be difficult for CHR-derived peptides to form the stable complexes with the trimeric form of gp41 at the NHR region. The C34 units could interact cooperatively with the trimer form of gp41 from the specific side. In the dimer or trimer forms of C34, this cooperative interaction is plausible, judging by the results of inhibition assays and CD measurement for the complex with N36. T20 peptides showed similar structures in the complex with the N36 peptide. However, the dimer and trimer forms of T20 did not show cooperative effects in inhibitory activity. T20 peptides contains hydrophobic residues at their C-terminus, and it has been suggested that these residues interact with lipid membranes. It has been indicated that the interaction with lipid bilayers is important as well as the interaction with the N36 region in the mechanism of action for the T20 monomer peptide in fusion inhibition.

3. Conclusion

By utilizing the different sets of CHR-derived peptides with multimerized forms as fusion inhibitors and a new action mechanism, different effects in multimerization which depend on the properties of unit peptides have been revealed. Our template has three branched linkers of equal lengths that precisely mimic the native assembly of C-peptides. The native chemical ligation effectively proceeds by chemoselective coupling in an aqueous medium of a three-cysteine-armed scaffold with unprotected CHR-derived peptides containing a C-terminal-thioester. A dimeric form of C34 is evidently critical as a necessary active structure of fusion inhibitors. This effect should provide useful information for the facile design of highly potent fusion inhibitors. As shown in studies of the soluble C34 derivative, the different form of interaction of SC34EK peptides could lead to highly inhibitory effects against enfuvirtide-resistant viruses.[26,39] As indicated, the stable helical form of unit peptides could lead to the decreased effect of multimerization of SC34EK units. However, since the activity of multimers is highly dependent on the sequences of unit peptides, the further optimization of sequences of unit peptides might be possible. Furthermore, the trimeric C34 was utilized for production of antibodies against HIV-1 infection although the antisera did not show distinct neutralizing activity as performed with the N36 trimer.[20,40] It would be of interest to utilize those synthetic multimers of C34 derivatives as peptide antigens. In combination with investigation of the effects against enfuvirtide-resistant strains, the designs of inhibitors targeting the dynamic supramolecular mechanism of HIV-1 fusion will be optimized in future studies.

4. Experiments

4.1. Synthesis of trimers of CHR-derived peptides (SH-free peptides)

TCEP·HCl (3.43 mg, 12.0 μmol), 4-mercaptophenylacetic acid (MPAA) (5.04 mg, 30.0 μmol), 8 M NaOH aq (7 μL) and CH_3CN (68 μL) were dissolved in 0.133 M sodium phosphate buffer (450 μL, pH 8.9) containing 8 M urea and 2.67 mM EDTA. CHR-derived peptide thioester (C34 thioester: 9.0 mg, 1.48 μmol, SC34EK

thioester: 10.2 mg, 1.48 μmol or T20 thioester: 9.3 mg, 1.48 μmol), CH_3CN (45 μL) and the template (30 μL, 0.450 μmol) dissolved in 50% aqueous CH_3CN (0.1% TFA) were then added and the mixture was stirred for 2 h at 30 °C under a nitrogen atmosphere and monitored by HPLC. The obtained ligation products were purified by reverse phase HPLC. The purified products were lyophilized and identified by ESI-TOF-MS; C34 trimer (SH-free): *m/z* calcd for $C_{703}H_{1108}N_{205}O_{245}S_6$ $[M+H]^+$ 16533.9, found: 16542.9 (3.0 mg, 0.16 μmol, 35% yield). SC34EK trimer (SH-free): *m/z* calcd for $C_{739}H_{1212}N_{208}O_{239}S_3$ $[M+H]^+$ 16921.9, found: 16930.5 (1.9 mg, 0.09 μmol, 20% yield). T20 trimer (SH-free): *m/z* calcd for $C_{757}H_{1161}N_{205}O_{245}S_3$ $[M+H]^+$ 17140.4, found: 17148.8 (2.9 mg, 0.15 μmol, 33% yield).

4.2. Synthesis of dimers of CHR-derived peptides (SH-free peptides)

In the synthesis of C34 dimer (SH-free), TCEP·HCl (2.06 mg, 7.2 μmol), 4-mercaptophenylacetic acid (MPAA) (3.03 mg, 18.0 μmol), 8 M NaOH aq (4 μL) and CH_3CN (41 μL) were dissolved in 0.133 M sodium phosphate buffer (270 μL, pH 8.9) containing 8 M urea and 2.67 mM EDTA. The thioesterified C34 peptide (2.5 mg, 0.41 μmol), CH_3CN (27 μL) and the template (18 μL, 0.27 μmol) dissolved in 50% CH_3CN aq (0.1% TFA) were then added to the mixture. The reaction mixture was stirred for 1.5 h at 30 °C under a nitrogen atmosphere and monitored by HPLC. The obtained product was purified by reverse phase HPLC. The purified product was lyophilized and identified by ESI-TOF-MS; C34 dimer (SH-free): *m/z* calcd for $C_{484}H_{769}N_{139}O_{169}S_5$ $[M+H]^+$ 11392.4, found 11399.1 (0.5 mg, 0.04 μmol, 14% yield).

4.3. Synthesis of dimers and trimers with capped thiol groups at ligation sites (SH-capped peptides)

TCEP·HCl (3.43 mg, 12.0 μmol), MPAA (5.04 mg, 30.0 μmol), 8 M NaOH aq (7 μL) and CH_3CN (68 μL) were dissolved in 0.133 M sodium phosphate buffer (450 μL, pH 8.9) containing 8 M urea and 2.67 mM EDTA. CHR-derived peptide thioester (C34 thioester: 4.1 mg, 0.67 μmol, SC34EK thioester: 4.6 mg, 0.67 μmol or T20 thioester: 4.2 mg, 0.67 μmol), CH_3CN (45 μL) and the template (30 μL, 0.45 μmol) dissolved in 50% aqueous CH_3CN (0.1% TFA) were then added to the mixture. After stirring for 1 h at 30 °C under a nitrogen atmosphere, iodoacetamide (12.5 mg, 67.4 μmol) was added to the mixture, which was stirred for 30 min at 30 °C and monitored by HPLC. The obtained products were purified by reverse phase HPLC. The purified products were lyophilized and identified by ESI-TOF-MS; C34 dimer (SH-capped): *m/z* calcd for $C_{490}H_{778}N_{142}O_{172}S_5$ $[M+H]^+$ 11563.5, found: 11571.5 (1.1 mg, 0.08 μmol, 18% yield). C34 trimer (SH-capped): *m/z* calcd for $C_{709}H_{1116}N_{208}O_{248}S_6$ $[M+H]^+$ 16703.9, found: 16715.3 (0.5 mg, 0.03 μmol, 6% yield). SC34EK dimer (SH-capped): *m/z* calcd for $C_{478}H_{848}N_{144}O_{168}S_3$ $[M+H]^+$ 11823.2, found: 11829.5 (1.2 mg, 0.08 μmol, 18% yield). SC34EK trimer (SH-capped): *m/z* calcd for $C_{691}H_{1221}N_{211}O_{242}S_3$ $[M+H]^+$ 17091.9, found: 17101.4 (0.5 mg, 0.02 μmol, 5% yield). T20 dimer (SH-capped): *m/z* calcd for $C_{526}H_{814}N_{142}O_{172}S_3$ $[M+H]^+$ 11967.9, found: 11976.1 (1.0 mg, 0.07 μmol, 16% yield). T20 trimer (SH-capped): *m/z* calcd for $C_{763}H_{1170}N_{208}O_{248}S_3$ $[M+H]^+$ 17310.5, found 17321.6 (1.5 mg, 0.08 μmol, 17% yield).

4.4. CD spectra

CD measurements were performed with a J-720 circular dichroism spectropolarimeter equipped with a thermo-regulator (JASCO). The wavelength dependence of molar ellipticity $[\theta]$ was monitored at 25 °C from 195 to 250 nm. The peptides were dissolved in PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.2).

4.5. Virus preparation

For virus preparation, 293T/17 cells in a T-75 flask were transfected with 10 μg of the pNL4-3 construct by the calcium phosphate method. The supernatant was collected 48 h after transfection, passed through a 0.45 μm filter, and stored at −80 °C as the virus stock.

4.6. Anti-HIV-1 assay

For the viral fusion inhibitory assay, TZM-bl cells (1×10^4 cells/100 μL) were cultivated with the NL4-3 virus (10 ng of p24) and serially diluted peptides. After 48 h cultivation, cells were lysed and the luciferase activity was determined with the Steady-Glo luciferase assay system (Promega, WI)[41]

4.7. Cytotoxicity assay

The cytotoxic effects of peptides were determined by CellTiter 96 Non-Radioactive Cell Proliferation assay system (Promega) under the same conditions as the anti-HIV assay but in the absence of viral infection.

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Supplementary data

Supplementary data (peptide synthesis, mass data and HPLC charts of compounds) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.05.060.

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