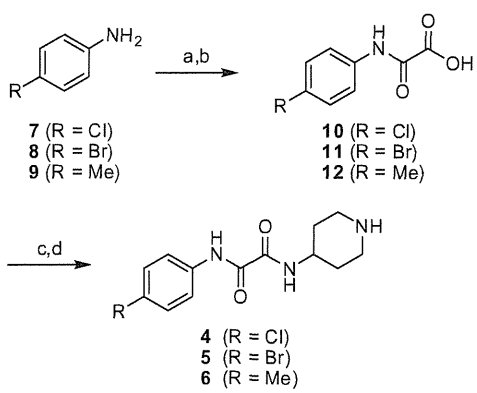
hybrid molecules that combined CD4 mimic analogs with a selective CXCR4 antagonist were also synthesized and bioevaluated.

For the design of novel CD4 mimic analogs, we initially tried to directly derivatize the nitrogen atom of piperidine group. However, direct alkylation and acylation of **1** failed probably as a result of steric hindrance from the methyl groups on the piperidine ring so we synthesized several derivatives lacking the methyl groups and evaluated their anti-HIV activity, cytotoxicity, and ability to mimic CD4. According to the previous SAR study,[4e] the *p*-Cl (**4**), *p*-Br (**5**) and *p*-methyl derivatives (**6**) lacking the methyl groups on the piperidine ring were prepared. Compounds **4–6** were synthesized by published methods as shown in Scheme 1. Briefly, coupling of aniline derivatives with ethyl chloroglyoxalate in the presence of Et_3N and subsequent saponification gave the corresponding acids (**10–12**). Condensation of these acids with 4-amino-*N*-benzylpiperidine in the presence of EDC–HOBt system, followed by debenzylation under von Braun conditions with 1-chloroethyl choroformate[6] produced the desired compounds **4–6**.[7]

The anti-HIV activity of each of the synthetic compounds was evaluated against MNA (R5) strain, with the results shown in Table 1. IC_{50} values were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method[8] as the concentrations of the compounds which conferred 50% protection against HIV-1-induced cytopathogenicity in PM1/CCR5 cells. Cytotoxicity of the compounds based on the viability of mock-infected PM1/CCR5 cells was also evaluated using the MTT method. CC_{50} values, the concentrations achieving 50% reduction of the viability of mock-infected cells, were also determined. Compounds **1** and **3** showed potent anti-HIV activity. The anti-HIV IC_{50} of compound **2** was previously reported to be comparable to that of compound **1**, and thus was not determined in this study. Novel derivatives **4** and **6** without the methyl groups on the piperidine ring, showed significant anti-HIV activity comparable to that of the parent compounds **1** and **3**, respectively. The *p*-methyl derivative **6** has slightly lower activity than the *p*-Cl derivative **4** and the *p*-Br derivative **5**. These results are consistent with our previous SAR studies on the parent compounds **1–3**. Compound **5** was found to exhibit relatively strong cytotoxicity (CC_{50} = 50 μM) and compounds **4** and **6** have cytotoxicities comparable to that of compounds **1** and **3**, respectively. This observation indicates that the methyl groups on the piperidine ring do not contribute significantly to the anti-HIV activity or the cytotoxicity.



Scheme 1. Synthesis of compounds **4–6**. Reagents and conditions: (a) ethyl chloroglyoxylate, Et_3N, THF; (b) 1 M aq NaOH, THF, 67%–quant.; (c) 1-benzyl-4-aminopiperidine, EDC·HCl, HOBt·H_2O, Et_3N, THF; (d) (i) 1-chloroethyl chloroformate, CH_2Cl_2; (ii) MeOH, 8–47%.

Table 1
Effects of the methyl groups on anti-HIV activity and cytotoxicity of CD4 mimic analogs[a]

Compd	R	IC_{50} (μM) MNA (R5)	CC_{50} (μM)
1	Cl	12	110
2	Br	ND	93
3	Me	15	210
4	Cl	8	100
5	Br	6	50
6	Me	20	190

[a] All data with standard deviation are the mean values for at least three independent experiments (ND = not determined).

Compound **1** and the newly synthesized derivatives **4–6** were also evaluated for their effects on conformational changes of gp120 by a fluorescence activated cell sorting (FACS) analysis. The profile of binding of an anti-envelope CD4-induced monoclonal antibody (4C11) to the Env-expressing cell surface (an R5-HIV-1 strain, JR-FL, -infected PM1 cells) pretreated with the above derivatives was examined. Comparison of the binding of 4C11 to the cell surface was measured in terms of the mean fluorescence intensity (MFI), as shown in Figure 2. Pretreatment of the Env-expressing cell surface with compound **1** (MFI = 53.66) produced a significant increase in binding affinity for 4C11, consistent with that reported previously.[4e] This indicates that compound **1** enhances the binding affinity of gp120 with the 17b monoclonal antibody which recognizes CD4-induced epitopes on gp120. The Env-expressing cells without CD4 mimic-pretreatment failed to show significant binding affinity to 4C11. On the other hand, the profiles of the binding of 4C11 to the Env-expressing cell surface pretreated with compound **4** (Cl derivative) and **5** (Br derivative) (MFI = 49.88 and 52.34) were similar to that of compound **1**. Pretreatment of the cell surface with compound **6** (Me derivative) (MFI = 45.99) produced slightly lower enhancement but significant levels of binding affinity for 4C11, compared to that of compound **1** as pretreatments. These results suggested that the removal of the methyl groups on the piperidine moiety might not affect the CD4 mimicry effects on conformational changes of gp120 and it was conjectured that the phenyl ring of CD4 mimic might be a key moiety for the interaction with gp120 to induce the conformational changes of gp120. This is consistent with the results in the previous paper where it was reported that CD4 mimics having suitable substituent(s) on the phenyl ring cause a conformational change, resulting in external exposure of the co-receptor-binding site of gp120.[4e]

Based on these results, a series of N-alkylated and N-acylated piperidine derivatives **13–18** with no methyl groups were prepared. Several compounds with 6-membered rings were also prepared to determine whether or not the piperidine ring is mandatory. The synthesis of these derivatives is shown in Scheme 2. Since the *p*-Cl derivative **4** showed potent anti-HIV activity and relatively low cytotoxicity, compared to the *p*-Br derivative **5**, chlorine was selected as the substituent at the *p*-position of the phenyl ring. The N-methyl derivative **13** was synthesized by coupling of **10** with 4-amine-1-methylpiperidine. Alkylation of **4** with *tert*-butyl bromoacetate, followed by deprotection of *tert*-butyl ester provided compound **14**. The N-isopropyl derivative **15** was prepared by reductive amination of **4** with isopropyl aldehyde. The N-acyl derivatives **16–18** were prepared by simple acylation or condensation with the corresponding substrate. The synthesis of other derivatives **19–23** with different 6-membered rings is depicted in Scheme 3. The 6-membered ring derivatives with the exception of **21** were prepared by coupling of acid **10** with the corresponding amines. Compound **21** was prepared by reaction of **10** with thionyl chloride to give the corresponding acid chloride, which was subsequently coupled with 4-aminopyridine.

Compounds **1**, **3**, and **13–18** were evaluated for their CD4 mimicry effects on conformational changes of gp120 by the FACS anal-

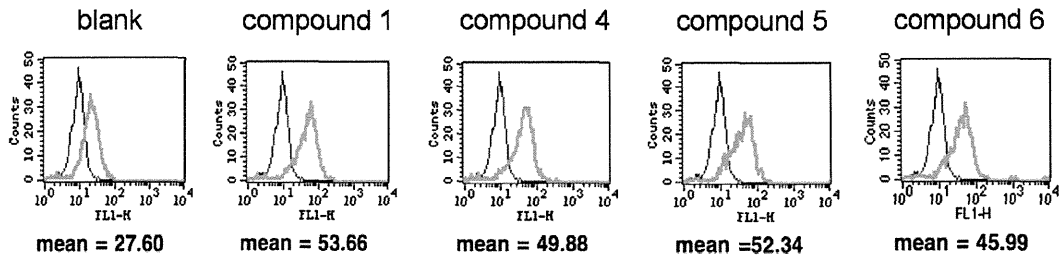
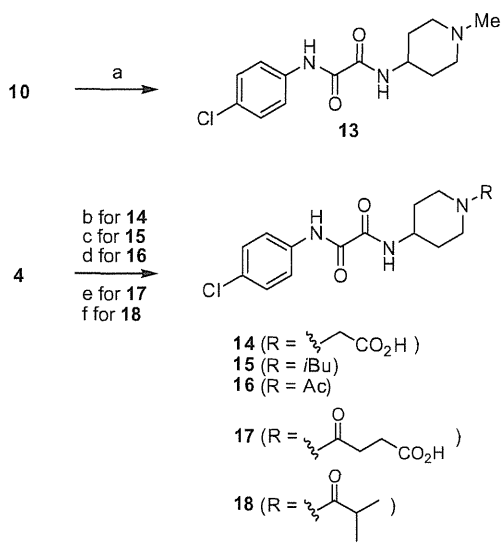
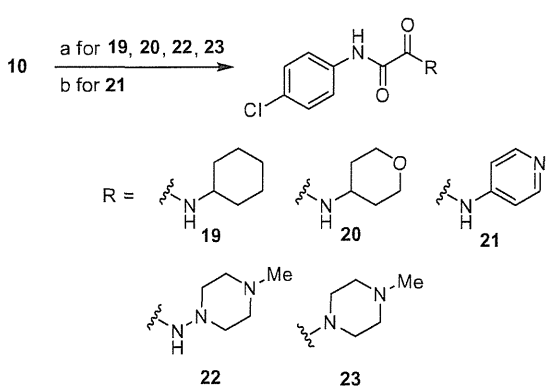


Figure 2. FACS analysis of compounds **1** and **4–6**. JR-FL (R5, Sub B) chronically infected PM1 cells were preincubated with 100 μM of a CD4 mimic for 15 min, and then incubated with an anti-HIV-1 mAb, 4C11, at 4 °C for 15 min. The cells were washed with PBS, and fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG antibody was used for antibody-staining. Flow cytometry data for the binding of 4C11 (green lines) to the Env-expressing cell surface in the presence of a CD4 mimic are shown among gated PM1 cells along with a control antibody (anti-human CD19:black lines). Data are representative of the results from a minimum of two independent experiments. The number at the bottom of each graph shows the mean fluorescence intensity (MFI) of the antibody 4C11.



Scheme 2. Synthesis of N-alkylated and N-acylated piperidine derivatives **13–18**. Reagents and conditions: (a) 4-amine-1-methylpiperidine, EDC·HCl, HOBt·H_2O, Et_3N, THF, 16%; (b) (i) *tert*-butyl bromoacetate, NaH, DMF; (ii) TFA, 6%; (c) isobutylaldehyde, $NaBH(OAc)_3$, AcOH, DCE, quant.; (d) acetyl chloride, Et_3N, DMF, quant.; (e) succinic anhydride, Et_3N, THF, 37%; (f) isobutyric acid, EDC·HCl, HOBt·H_2O, Et_3N, THF, 95%.



Scheme 3. Synthesis of 6-membered ring derivatives **19–23**. Reagents and conditions: (a) the corresponding amine, EDC·HCl, HOBt·H_2O, Et_3N, THF, 22%–quant.; (b) 4-aminopyridine, $SOCl_2$, MeOH, 38%.

ysis, and the results are shown in Figure 3. Pretreatment of the Env-expressing cells with the N-substituted compounds **13**, **15**, **16**, and **18** produced a notable increase in binding affinity to 4C11, similar to that observed in the pretreatment with compound **1**. The profile of the binding of 4C11 to the cell surface pretreated with compounds **14** and **17** was similar to that of controls, suggesting that these derivatives offer no significant enhancement of binding affinity for 4C11 and that the carboxylic moiety in the terminal of piperidine ring is not suited to CD4 mimicry. It is hypothesized that the carboxylic moieties of compounds **14** and **17** might prevent the interaction of CD4 mimic with gp120 by their multiple contacts with side chain(s) of amino acid(s) around the Phe43 cavity, such as Asp^{368} and Glu^{370}. Replacement of the piperidine moiety with the different 6-membered rings resulted in a significant loss of binding affinity for 4C11 in the FACS analysis of compound **19–23** (MFI(**19**) = 11.44, MFI(**20**) = 12.84, MFI(**21**) = 12.47, in MFI (blank) = 11.34; MFI(**22**) = 26.67, MFI(**23**) = 20.21, in MFI(blank) = 26.79, data not shown), indicating a significant contribution from the piperidine ring which interacts with gp120 inducing conformational changes.

In view of their ability to induce conformational changes of gp120, the anti-HIV activity and cytotoxicity of the piperidine derivatives **13–18** were further evaluated, with the results shown in Table 2. The anti-HIV activity of the synthetic compounds was evaluated against various viral strains including both laboratory and primary isolates and IC_{50} and CC_{50} values were determined as those of compounds **4–6**. The *N*-methylpiperidine compound **13**, was not found to possess significant anti-HIV activity against a primary isolate, but was found to possess moderate anti-HIV activity against a laboratory isolate, a IIIB strain (IC_{50} = 67 μM). Anti-HIV activity was not observed however, even at concentrations of 100 μM of **13** against an 89.6 strain. The potency was approximately eight-fold lower than that of the parent compound **1** (IC_{50} = 8 μM), indicating a partial contribution of the hydrogen atom of the amino group of the piperidine ring to the bioactivity of CD4 mimic. Although compound **15**, with an *N*-isobutylpiperidine moiety, failed to show significant anti-HIV activity against laboratory isolates, relativity potent activity was observed against a primary isolate, an MTA strain (IC_{50} = 28 μM). Compounds **16** and **18**, which are *N*-acylpiperidines, were tested against laboratory isolates and significant anti-HIV activity was not observed even at 100 μM. Compounds **14** and **17**, with the carboxylic moieties, failed to show significant anti-HIV activity against laboratory isolates even at 100 μM, which are compatible with the FACS analysis. These results suggest that the N-substituent on the piperidine ring of CD4 mimic analogs may contribute to a critical interaction required for binding to gp120. Compounds **19–23** showed no significant anti-HIV activity against a IIIB strain even at 100 μM, which are compatible with the FACS analysis (data not shown).

All but one of the compounds **13–18** have no significant cytotoxicity to PM1/CCR5 cells ($CC_{50} \geqslant 260$ μM); the exception is compound **18** (CC_{50} = 45 μM). Compounds **13** and **15** show relatively potent anti-HIV activity without significant cytotoxicity.

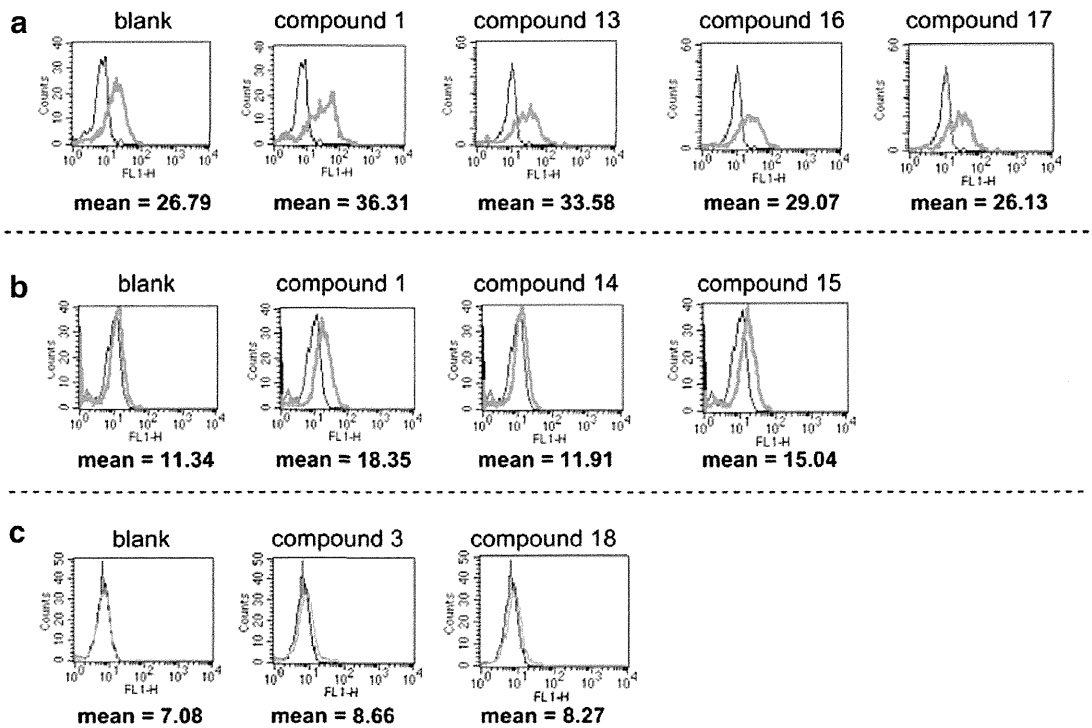


Figure 3. FACS analysis of compounds **1**, **3**, and **13–18**. The experimental procedures are described in Figure 2. The lanes of (a), (b) and (c) show independent experiments.

Table 2
Anti-HIV activity and cytotoxicity of compounds **13–18**[a]

Compd	R	IC_{50} (μM)			CC_{50} (μM)
		Laboratory isolates		Primary isolates	
		IIIB (X4)	89.6 (dual)	MTA (R5)	
1		8	10	ND	150
4	H	ND	ND	ND	100
13	Me	67	>100	ND	>300
14	CH_2CO_2H	>100	ND	ND	260
15	*i*Bu	>100	ND	28	>300
16	Ac	>100	>100	ND	>300
17	$C(O)CH_2CH_2CO_2H$	>100	>100	ND	>300
18	C(O)*i*Pr	>100	ND	ND	45

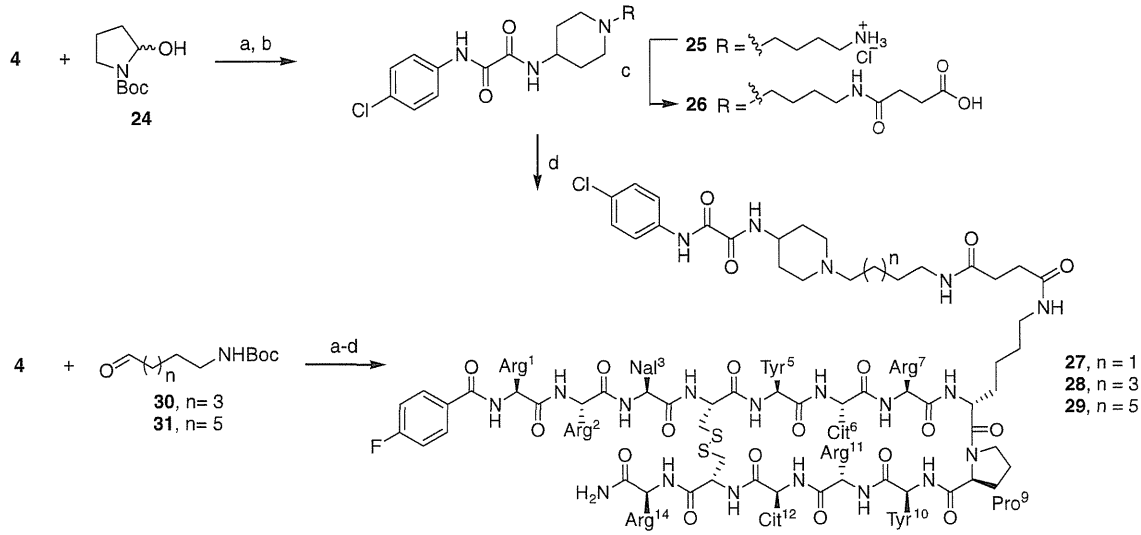
[a] All data with standard deviation are the mean values for at least three independent experiments.

The results for **15** showed it to have 3–6 times less cytotoxicity than **4** and **18**. This observation indicates that the alkylation of the piperidine nitrogen may be favorable because it lowers the cytotoxicity of CD4 mimic analogs.

In the course of the SAR studies on CD4 mimic analogs, we have already found that a CD4 mimic or sCD4 exhibited a remarkable synergistic effect[4e] with a 14-mer peptide CXCR4 antagonist T140.[9] This result indicates that the interaction of CD4 mimic with gp120 could facilitate the approach of CXCR4 to gp120 by exposing the co-receptor binding site of gp120. It was thought that the CD4 mimic analogs conjugated with a selective CXCR4 antagonist might as a consequence show a higher synergistic effect for the improvement of anti-HIV activity. In this context, efforts were made to synthesize and bioevaluate hybrid molecules that combined a CD4 mimic analog with 4F-benzoyl-TZ14011, which is a derivative of T140 optimized for CXCR4 binding and stability in vivo.[10]

The synthesis of hybrid molecules **27–29** is outlined in Scheme 4. To examine the influence of the linker length on anti-HIV activity and cytotoxicity, three hybrid molecules with linkers of different lengths were designed. Based on the fact that alkylation of the piperidine nitrogen, having no deleterious effects on bioactivity, is an acceptable modification of CD4 mimic analogs, the alkylamine moiety was incorporated into the nitrogen atom of the piperidine moiety to conjugate CD4 mimic analogs with 4F-benzoyl-TZ14011. Reductive alkylation of **4** with N^{α}-Boc-pyrrolidin-2-ol **24**, which exists in equilibrium with the corresponding aldehyde, and successive treatment with TFA and HCl/dioxane provided the amine hydrochloride **25**. Treatment of **25** with succinic anhydride under basic condition gave the corresponding acid **26**, which was subjected to condensation with the side chain of D-Lys[8] of 4F-benzoyl-TZ14011 in an EDC–HOBt system to give the desired hybrid molecule **27** with a tetramethylene linker.[11] Other hybrid molecules **28** and **29** bearing hexa- and octamethylene linkers, respectively, were prepared using the corresponding aldehydes **30** and **31**.

The assay results for these hybrid molecules **27–29** are shown in Table 3. To investigate the effect of conjugation of two molecules on binding activity against CXCR4, the inhibitory potency against



Scheme 4. Synthesis of hybrid molecules **27–29**. Reagents and conditions: (a) $NaBH(OAc)_3$, AcOH, DCE; (b) TFA, then 4 M HCl/1,4-dioxane; (c) succinic anhydride, pyridine, DMF, then 4 M HCl/1,4-dioxane; (d) 4F-benzoyl-TZ14011, EDC·HCl, HOBt·H_2O, Et_3N, DMF. Nal = L-3-(2-naphthyl)alanine, Cit = L-citrulline.

Table 3
CXCR4-binding activity, anti-HIV activity and cytotoxicity of hybrid molecules **27–29**[a]

Compd	EC_{50}[b] (μM)	IC_{50}[c] (μM)	CC_{50}[d] (μM)	SI (CC_{50}/IC_{50})
4F-benzoyl-TZ14011	0.0059	0.0131	ND	ND
1 (NBD-556)	ND	0 2.10	ND	19.2[e]
27 (C4)	0.0044	0.0509	8.60	169
28 (C6)	0.0187	0.0365	8.00	219
29 (C8)	0.0071	0.0353	8.60	244
AZT	ND	0.0493	ND	ND

[a] All data with standard deviation are the mean values for at least three independent experiments.
[b] EC_{50} values are based on the inhibition of [^{125}I]-SDF-1α binding to CXCR4 transfectants of CHO cells.
[c] IC_{50} values are based on the inhibition of HIV-1-induced cytopathogenicity in MT-2 cells.
[d] CC_{50} values are based on the reduction of the viability of mock-infected MT-2 cells.
[e] This value is based on the CC_{50} and IC_{50} values from Table 1.

binding of [^{125}I]-SDF-1α to CXCR4 was measured. All the hybrid molecules **27–29** significantly inhibited the SDF-1α binding to CXCR4. The corresponding EC_{50} values are: EC_{50}(**27**) = 0.0044 μM; EC_{50}(**28**) = 0.0187 μM; EC_{50}(**29**) = 0.0071 μM. These potencies are comparable to that of 4F-benzoyl-TZ14011 (EC_{50} = 0.0059 μM), indicating that introduction of the CD4 mimic analog into the D-Lys^8 residue of 4F-benzoyl-TZ14011 does not affect binding activity against CXCR4. Comparison of the binding activities of **27–29** showed that all hybrid molecules were essentially equipotent in inhibition of the binding of SDF-1α to CXCR4. This observation indicates that the linker length between two molecules has no effect on the binding inhibition.

Anti-HIV activity based on the inhibition of HIV-1 entry into the target cells was examined by the MTT assay using a IIIB(X4) strain. In this assay, the IC_{50} value of 4F-benzoyl-TZ14011 was 0.0131 μM. All hybrid molecules **27–29** showed significant anti-HIV activity [IC_{50}(**27**) = 0.0509 μM; IC_{50}(**28**) = 0.0365 μM; IC_{50}(**29**) = 0.0353 μM]; however, the potency was 2- to 4-fold lower than that of the parent compound 4F-benzoyl-TZ14011, indicating that the conjugation of CD4 mimic with a CXCR4 antagonist did not provide a significant synergistic effect. In view of the fact that the combinational uses of CD4 mimic with T140 produced a highly remarkable synergistic effect, the lower potency of hybrid molecules may be attributed to the inadequacy in the structure and/or the characters of the linkers. All the hybrid molecules **27–29** have relatively strong cytotoxicity [CC_{50}(**27**) = 8.6 μM; CC_{50}(**28**) = 8.0 μM; CC_{50}(**29**) = 8.6 μM]. However, selectivity indexes (SI = CC_{50}/IC_{50}) were 169 for **27**, 219 for **28**, and 244 for **29**, all 9–13 times higher than that of **1** (SI = 9.2). This result indicates that conjugation of a CD4 mimic analog with a selective CXCR4 antagonist can improve the SI of CD4 mimic.

The SAR study of a series of CD4 mimic analogs was conducted to investigate the contribution of the piperidine moiety of **1** to anti-HIV activity, cytotoxicity, and CD4 mimicry on conformational changes of gp120. The results indicate that (i) the methyl groups on the piperidine ring of **1** have no great influence on the activities of CD4 mimic; (ii) the presence of piperidine moiety is important for the CD4 mimicry; and (iii) N-substituents of the piperidine moiety contribute significantly to anti-HIV activity and cytotoxicity, as observed with N-alkyl groups such as methyl and isobutyl groups which show moderate anti-HIV activity and lower cytotoxicity.

Several hybrid molecules based on conjugation of a CD4 mimic with a selective CXCR4 antagonist were also synthesized and bioevaluated. All the hybrid molecules showed significant binding activity against CXCR4 comparable to the parent antagonist and exhibited potent anti-HIV activity. Although no significant synergistic effect was observed, conjugation of a CD4 mimic with a selective CXCR4 antagonist might lead to the development of novel type of CD4 mimic-based HIV-1 entry inhibitors, which possess higher selective indexes than a simple CD4 mimic. These results will be useful for the rational design and synthesis of a new type of HIV-1 entry inhibitors. Further structural modification studies of CD4 mimic are the subject of an ongoing project.

Acknowledgements

This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, Japan Human Science Foundation, and Health and Labour Sciences Research Grants from Japanese Ministry of Health, Labor, and Welfare. T.T. and N.O. are grateful for the JSPS Reseach Fellowships for Young Scientist.

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7. The synthesis of compound **4**: To the solution of compound **10** (104 mg, 0.52 mmol) in dry THF (4.0 mL), Et_3N (159 μL, 1.15 mmol), HOBt·H_2O (87 mg, 0.57 mmol), EDCI·HCl (109 mg, 0.57 mmol) and 4-amino-1-benzylpiperidine (109 μL, 0.57 mmol) were added with stirring at 0 °C, and continuously stirred for 6 h with warming to room temperature under N_2 atmosphere. After concentration under reduced pressure, the residue was extracted with EtOAc. The extract was washed with aq saturated $NaHCO_3$ and brine, and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with $CHCl_3$–MeOH (20:1) including 1% Et_3N gave the crude benzyl amine as a white powder. To the solution of the above crude benzyl amine (95 mg, 0.26 mmol) in dry CH_2Cl_2 (10 mL), 1-chloroethyl chloroformate (110 μL, 0.68 mmol) was added dropwise with stirring at 0 °C. The mixture was then refluxed for 3 h under N_2 atmosphere. After concentration under reduced pressure, the residue was resolved in MeOH (10 mL) and then refluxed for 1 h. Concentration under reduced pressure gave a crude product. Reprecipitation with MeOH–Et_2O afforded a white powder of the title compound **4** (33 mg, 46% yield). δ_H(400 MHz; CD_3OD) 1.83–1.92 (2H, m, CH_2), 2.10–2.17 (2H, m, CH_2), 3.13 (2H, t, *J* 12.5, CH_2), 3.34 (1H, m, NH), 3.42–3.49 (1H, m, CH_2), 4.04 (1H, m, CH), 7.34 (2H, m, ArH), 7.51 (1H, m, NH), 7.73 (2H, m, ArH), 8.84 (1H, m, NH); LRMS (ESI), *m*/*z* calcd for $C_{13}H_{17}ClN_3O_2$ $(MH)^+$ 282.10, found 282.14.
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11. The synthesis of a hybrid molecule **27**: To the solution of compound **26** (2.6 mg, 4.6 μmol) in DMF (1.0 mL), Et_3N (26 μL, 92 μmol), HOBt·H_2O (3.5 mg, 23 μmol) and EDCI·HCl (4.5 mg, 23 μmol) were added with stirring at 0 °C, and stirred for 1 h at room temperature. To the mixture 4F-benzoyl-TZ14011 (15 mg, 4.1 μmol) was then added and the mixture was stirred for 24 h at room temperature under N_2 atmosphere. After concentration under reduced pressure, the residue was purified by reversed phase HPLC (t_R = 23 min, elution: a linear gradient of 27–31% acetonitrile containing 0.1% TFA over 30 min) to afford a fluffy white powder of the desired compound **27** (1.3 mg, 9.8%). LRMS (ESI), *m*/*z* 2621.20 $[M+H]^+$, calcd 2620.25.

Synthesis and biological evaluation of selective CXCR4 antagonists containing alkene dipeptide isosteres†

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Received 21st August 2009, Accepted 31st October 2009
First published as an Advance Article on the web 4th December 2009
DOI: 10.1039/b917236j

A set of cyclic peptide analogues of a selective CXCR4 antagonist FC131 [*cyclo*(-D-Tyr-Arg-Arg-Nal-Gly-)] were synthesized and bioevaluated. Using (*E*)-alkene and (*Z*)-fluoroalkene dipeptide isosteres for Arg-Arg and Arg-Nal substructures, indispensable or the partial contribution of the two peptide bonds to the CXCR4 antagonism and anti-HIV activity was demonstrated. FC131 and the analogues were shown to selectively inhibit SDF-1 binding to CXCR4, whereas no inhibition of binding of SDF-1 to CXCR7 was observed.

Introduction

Chemokine receptor CXCR4 belongs to the G-protein coupled receptor family[1] and plays important roles in physiological functions including angiogenesis,[2] chemotaxis,[3] and neurogenesis.[4] CXCR4 is associated with various pathological conditions including cancer metastasis,[5] HIV-1 infection[6] and rheumatoid arthritis.[7] The broad spectrum of biological activities has led to extensive research towards the development of specific inhibitors directed against CXCR4.[8,9]

We have previously identified a highly potent CXCR4 antagonist, T140 **1**, which is a β-sheet-like 14-mer peptide with a single disulfide bridge (Fig. 1).[10] The indispensable residues for bioactivity are four amino acids positioned across the disulfide bridge: Arg2, L-3-(2-naphthyl)alanine3 (Nal3), Tyr5 and Arg14. These residues were used for further molecular-size reductions. Using these critical residues for a characteristic combination of cyclic pentapeptide libraries, a potent CXCR4 antagonist FC131 **2** was identified, which exerts comparable anti-HIV activity to T140.[11]

Structure–activity relationship (SAR) studies of FC131 by various modifications such as amino acid substitution,[12] tuning of the ring structure,[13] and backbone modifications,[14,15] demonstrated that the potent bioactivity of FC131 is attributed to the ideal spatial dispositions of the side-chain functional groups. For example, *N*-methylation of the peptide bonds of FC131 and the epimeric congeners significantly altered the bioactivity.[14] The appropriate combination of sequence, chirality and auxiliary groups on the cyclic pentapeptide backbone can accommodate the bioactive conformations.

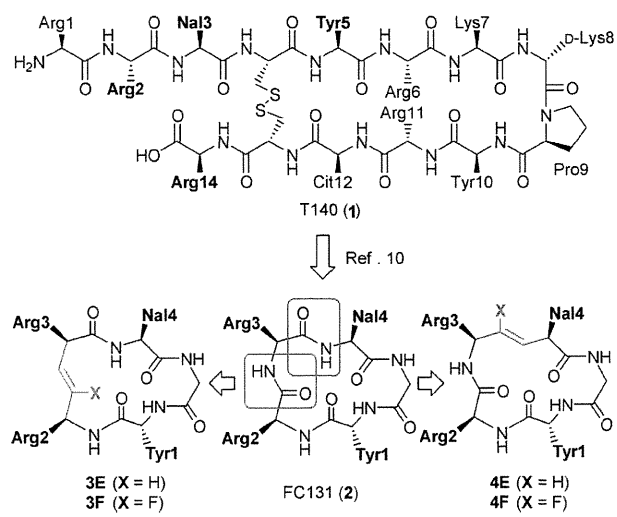

Fig. 1 Structures of T140 (**1**), FC131 (**2**), and the (*E*)-alkene and (*Z*)-fluoroalkene FC131 analogues. Bold residues of **1** are indispensable for the potent CXCR4-antagonistic activity. Nal = L-3-(2-naphthyl)alanine.

Replacement of the planar amide bond with a surrogate alkene substructure, including unsubstituted,[15,16] fluorinated,[17] multi-substituted,[18] and trifluoromethylated[19] alkenes, represents a promising approach to probe structural and electrostatic requirements in bioactive peptides. In particular, fluorinated or substituted alkene isosteres are considered to be more appropriate peptide bond mimetics when compared with unsubstituted alkene isosteres because of the favorable electrostatic and steric properties.[20] In this study, the contributions of the Arg2-Arg3 and Arg3-Nal4 peptide bonds to the bioactivity of FC131 were investigated through the synthesis and bioevaluation of alkene analogues of FC131, *cyclo*[(-D-Tyr-Arg-ψ[*trans*-CX=CH]-Arg-Nal-Gly-)] **3E/3F** and *cyclo*[(-D-Tyr-Arg-Arg-ψ[(*trans*-CX=CH]-Nal-Gly-)] **4E/4F** (X = H or F). The comparative study using unsubstituted and fluorinated isosteres aimed to reveal the electrostatic contributions of the amide carbonyl groups of these peptide bonds to the bioactivity of FC131.

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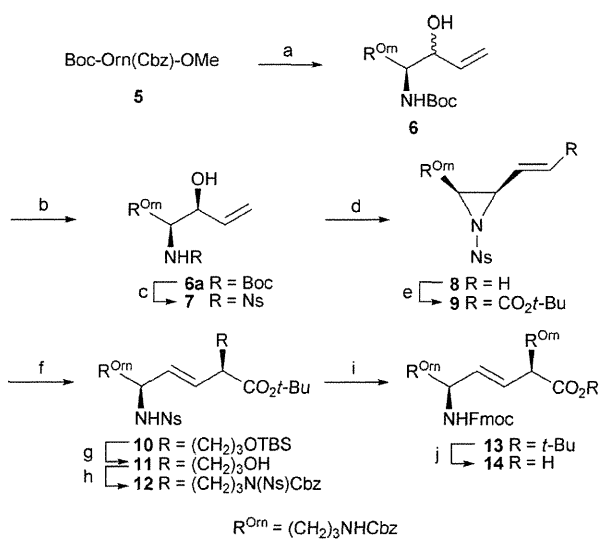
† Electronic supplementary information (ESI) available: Additional experimental procedures, NMR spectra and HPLC charts. See DOI: 10.1039/b917236j

Results and discussion

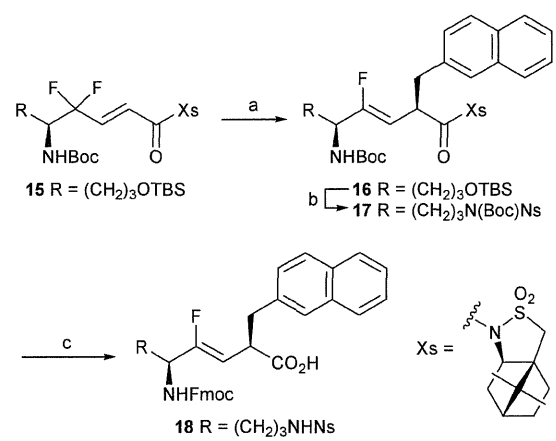
Synthesis of alkene dipeptide isosteres and the application to FC131 analogues

In our previous synthesis of the Arg-Nal type (*E*)-alkene dipeptide isostere (EADI),[15] a protected arginine was employed as the starting material. However, the derivatives were not experimentally tractable in the same synthetic process due to the presence of the protected guanidino group. Consequently, the synthesis of FC131 analogue **3E** bearing Arg-Arg type EADI began with Boc-Orn(Cbz)-OMe **5** (Orn = L-ornithine, Scheme 1). Ornithine includes a 3-aminoprop-1-yl group that can be used as a precursor of the arginine side-chain. Successive treatment of the ester **5** with diisobutylaluminium hydride (DIBAL-H) and vinylzinc chloride, gave a *syn* and *anti*-mixture of allylic alcohols **6** (*syn*:*anti* = 87:13). The *syn*-isomer **6a** was obtained by recrystallization. Boc cleavage of **6a** with TFA followed by *N*-2-nitrobenzenesulfonyl (Ns) protection produced a Ns-amide **7**. The intramolecular Mitsunobu reaction of **7** proceeded to provide 2,3-*cis*-aziridine **8** in high yield. Ozonolysis of **8** and the subsequent Horner–Wadsworth–Emmons reaction predominantly afforded the (*E*)-isomer of β-aziridinyl-α,β-enoate **9** in 57% yield. Organocopper-mediated *anti*-S_N2' type alkylation of **9** gave the α-alkylated product **10** with a TBS-protected 3-hydroxyprop-1-yl group, that can be modified to provide another Arg side-chain. Transformation to the Orn side-chain was performed by TBAF-mediated deprotection of **10** and the subsequent Mitsunobu reaction using CbzNHNs to give a bis(sulfonamide) **12**. The expected Fmoc-Orn(Cbz)-ψ[(*E*)-CH=CH]-Orn(Cbz)-OH **14** was obtained by sequential manipulation of the protecting groups including cleavage of two Ns groups in **12** and *N*-Fmoc protection and deprotection of the *t*-Bu ester.



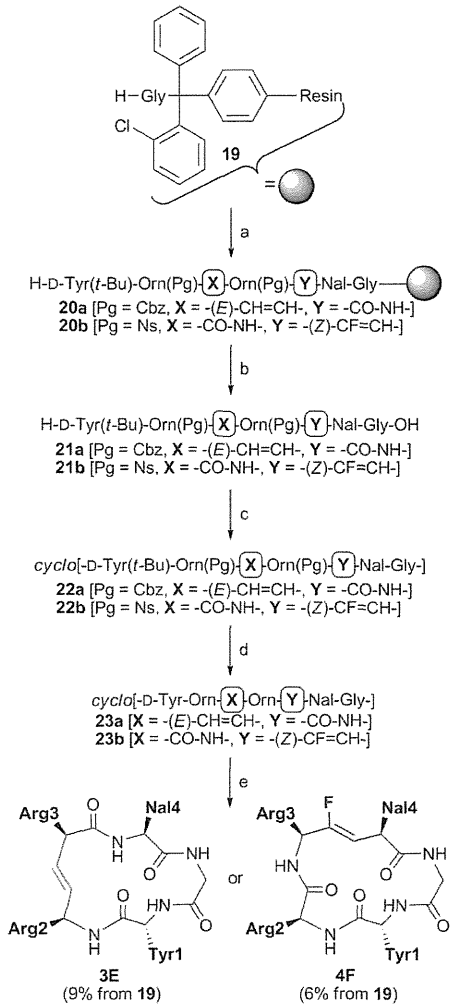
Scheme 1 Synthesis of the Orn-Orn-type (*E*)-alkene dipeptide isostere. Reagents and conditions: (a) (i) Diisobutylaluminium hydride (DIBAL-H), CH_2Cl_2–toluene, –78 °C, 1 h; (ii) H_2C=CHMgCl, $ZnCl_2$, LiCl, –78 °C, 3 h (42%, *syn*:*anti* = 87:13); (b) recrystallization; (c) (i) TFA, CH_2Cl_2, 0 °C, 1 h; (ii) 2-nitrobenzenesulfonyl chloride (NsCl), Et_3N, CH_2Cl_2, rt, 1 h (74%); (d) diethyl azodicarboxylate (DEAD), PPh_3, THF, rt, 9 h (93%); (e) (i) O_3, EtOAc, –78 °C, then Me_2S; (ii) $(EtO)_2P(O)CH_2CO_2t$-Bu, LiCl, (*i*-Pr)$_2$NEt, MeCN, 0 °C, 4 h (57%); (f) $TBSO(CH_2)_3Li$, CuCN, LiCl, THF–Et_2O–*n*-pentane, –78 °C, 2 h (66%); (g) tetrabutylammonium fluoride (TBAF), THF, 0 °C, 14 h (85%); (h) CbzNHNs, DEAD, PPh_3, THF, 0 °C, 24 h (93%); (i) (i) PhSH, K_2CO_3, MeCN–DMSO, 50 °C, 2 h; (ii) *N*-(9-fluorenylmethoxycarbonyloxy)succinimide (Fmoc-OSu), Et_3N, THF–H_2O, 0 °C, 4 h (quant); (j) 4 N HCl–dioxane, rt, 8 h (65%).

Diastereoselective synthesis of (*Z*)-fluoroalkene dipeptide isosteres (FADI) has recently been accomplished.[17e] The key step in this synthesis is the one-pot reaction involving organocopper-mediated reduction/asymmetric alkylation *via* transmetalation to establish the α-alkylated isostere with appropriate configuration. According to the previous synthetic study of peptide **3F** bearing the Arg-Arg type FADI,[17i] the preparation of the Orn-Nal type FADI was carried out (Scheme 2). The one-pot reaction of γ,γ-difluoro-α,β-enoyl sultam **15**[17i] with 2-(bromomethyl)naphthalene yielded the corresponding α-alkylated sultam **16**. Cleavage of the TBS group with aqueous H_2SiF_6 followed by the Mitsunobu reaction with BocNHNs afforded the sulfonamide **17**. The sulfonamide **17** was converted to the Fmoc-protected FADI **18** by a standard deprotection/protection manipulation.



Scheme 2 Synthesis of the Orn-Nal-type (*Z*)-fluoroalkene dipeptide isostere. Reagents and conditions: (a) (i) $Me_2CuLi{\cdot}LiI{\cdot}2LiBr$, THF–$Et_2O$, –78 °C, 0.5 h; (ii) Hexamethylphosphoric triamide (HMPA), –78 °C, 0.5 h; (iii) Ph_3SnCl, THF, –40 °C, 10 min; (iv) 2-(bromomethyl)naphthalene, –40 °C, 20 h (79%); (b) (i) H_2SiF_6 aq. MeCN–MeOH, 0 °C, 1 h; (ii) BocNHNs, DEAD, PPh_3, THF, rt, 12 h (98%); (c) (i) 1 N LiOH, H_2O_2, THF–H_2O, rt, 2 h; (ii) TFA, CH_2Cl_2, rt, 0.5 h; (iii) Fmoc-OSu, Et_3N, DMF–H_2O–MeCN, rt, 12 h (85%).

The resulting isosteres **14** and **18** were incorporated into the peptide-chain by standard Fmoc-based solid-phase peptide synthesis (Scheme 3). Briefly, the protected peptides **21a,b** were cleaved off the resins **20a,b** with 1,1,1,3,3,3-hexafluoroisopropanol (HFIP). After diphenylphosphoryl azide (DPPA)-mediated cyclization, the Cbz- or Ns-groups on the ornithine δ-amino group(s) of **22a,b** were deprotected by treatment with 1 M TMSBr/thioanisole in TFA or with 95% aqueous TFA followed by 2-mercaptoethanol/1,8-diazabicyclo[5,4,0]-7-undecene (DBU), respectively. Subsequently, the amino group(s) of **23a,b** were modified using 1*H*-pyrazole-1-carboxamidine to provide the expected peptidomimetics **3E** and **4F** with the Arg guanidino group(s).



Scheme 3 Synthesis of the alkene analogues of FC131. Reagents and conditions: (a) Fmoc-based SPPS; (b) 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), CH_2Cl_2; (c) diphenylphosphoryl azide (DPPA), $NaHCO_3$, DMF, −40 °C to rt; (d) **23a**: 1 M TMSBr/thioanisole in TFA, *m*-cresol, 1,2-ethandithiol, 6 h; **23b**: (i) TFA-H_2O, 3 h; (ii) 2-mercaptoethanol, 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU), DMF, 50 °C, 2.5 h; (e) **3E**: 1*H*-pyrazole-1-carboxamidine·HCl, $(i\text{-Pr})_2NEt$, DMF; **4F**: 1*H*-pyrazole-1-carboxamidine·HCl, Et_3N, DMF.

Biological evaluation of FC131 analogues with EADI and FADI

The biological activities of cyclic pseudopeptides **3E**/**3F**[17i] and **4E**[15]/**4F** were comparatively evaluated, in which the Arg2-Arg3 and Arg3-Nal4 dipeptide sites were substituted with EADI or FADI. The inhibitory potency against [^{125}I]-SDF-1-binding to CXCR4 or CXCR7 was measured (Table 1). Both EADI and FADI analogues (**3E** and **3F**) with substitution at the Arg2-Arg3 dipeptide moderately inhibited the SDF-1 binding to CXCR4 [IC_{50}(**3E**) = 1.46 μM; IC_{50}(**3F**) = 1.78 μM]. The potency was approximately 20-fold lower than the original FC131 **2** [IC_{50}(**2**) = 0.068 μM], indicating the partial contribution of the amide bond within the Arg2-Arg3 dipeptide to the bioactivity of FC131. This is consistent with the bioactivity of the FC131 analogue containing the Arg2-MeArg3 dipeptide substructure,[14] suggesting that the less potent activity may be attributed to the loss of the H-bonding amide hydrogen of Arg3 and/or the conformational change by the backbone modification. Comparison of the biological activities of the two analogues **3E** and **3F** showed that the unsubstituted alkene analogue **3E** was essentially equipotent in inhibiting the binding of SDF-1 to CXCR4 to the fluoroalkene analogue **3F**. This observation indicates that the presence of the fluorine atom did not aid the appropriate mimicry of the steric and electrostatic effects of the Arg2 carbonyl group.

Table 1 Inhibitory activity of FC131 and the derivatives against [^{125}I]-SDF-1 binding to CXCR4 and CXCR7

Peptide	IC_{50}/μM[c] CXCR4	CXCR7
FC131 **2**	0.068	> 10
cyclo[(-D-Tyr-Arg-Ψ^E-Arg-Nal-Gly-)] **3E**[a]	1.46	> 10
cyclo[(-D-Tyr-Arg-Ψ^F-Arg-Nal-Gly-)] **3F**[b]	1.78	> 10
cyclo[(-D-Tyr-Arg-Arg-Ψ^E-Nal-Gly-)] **4E**[a]	> 10	> 10
cyclo[(-D-Tyr-Arg-Arg-Ψ^F-Nal-Gly-)] **4F**[b]	> 10	> 10

[a] The Ψ^E indicates the isosteric ψ[(*E*)-CH=CH] substructure. [b] The Ψ^F indicates the isosteric ψ[(*Z*)-CF=CH] substructure. [c] IC_{50} values are the concentrations for 50% inhibition of the [^{125}I]-SDF-1α binding to CXCR4 or CXCR7 transfectants of CHO-K1 cells.

Our previous studies on *N*-methylamino acid-scanning[14] and EADI replacement[15] (**4E**) revealed that the modification of Arg3-Nal4 peptide bond resulted in a significant loss of CXCR4-binding inhibition activity. This is possibly due to the absence of the amide hydrogen and/or the dissolution of the pseudo-1,3-allylic strain between the Arg3 carbonyl group and the Nal4 side chain. Although the possible mimicking ability of the fluorine atom was expected,[20] the introduction of the FADI into the Arg3-Nal4 dipeptide (**4F**) also led to the loss of CXCR4-binding activity again [IC_{50}(**4F**) > 10 μM]. This result indicates that the amide hydrogen within the Arg3-Nal4 dipeptide of FC131 may contribute to a critical interaction required for binding to CXCR4.

Furthermore, inhibitory activity of the peptides for CXCR7, which is also a target receptor of SDF-1, was also examined; however, no inhibition was observed even at 10 μM. This observation showed that FC131 and the related analogues are selective CXCR4 antagonists and show similar target specificity as the T140 derivatives.[21]

Anti-HIV activity based on the inhibition of HIV-1 entry into the target cells was examined by the MAGI assay using three strains including NL4-3, IIIB and Ba-L (Table 2). As in the case of CXCR4-binding inhibition, moderate anti-HIV activity against NL4-3 and IIIB strains was observed for peptides **3E**/**3F** containing EADI and FADI for the Arg2-Arg3 dipeptide

Table 2 Anti-HIV activities of FC131 and the derivatives

Peptide	EC_{50}/μM[a] NL4-3	IIIB	Ba-L
2	0.014 ± 0.002	0.019 ± 0.003	> 10
3E	0.234 ± 0.004	0.295 ± 0.069	> 10
3F	0.332 ± 0.073	0.403 ± 0.051	> 10
4E	> 10	> 10	> 10
4F	> 10	> 10	> 10

[a] EC_{50} is the concentration that blocks HIV-1 infection by 50%.

[IC_{50}(**3E**) = 0.234 μM (NL4-3) and 0.295 μM (IIIB); IC_{50}(**3F**) = 0.332 μM (NL4-3) and 0.403 μM (IIIB)]. The potency was significantly less compared with the original FC131 **2** [IC_{50}(**2**) = 0.014 μM (NL4-3) and 0.019 μM (IIIB)]. Substitutions of Arg3-Nal4 dipeptides with EADI and FADI resulted in the loss of the anti-HIV activity [IC_{50}(**4E**/**4F**) > 10 μM (NL4-3 and IIIB)], which also correlates with the observation of no CXCR4 antagonistic activity of these peptides. For the Ba-L strain, that utilizes CCR5 for entry, all peptides showed no inhibitory activity at 10 μM.

Conclusions

In conclusion, Orn-Orn type EADI **14** and Orn-Nal type FADI **18** were synthesized and incorporated into FC131 analogues. Comparative bioevaluation of a set of peptides containing EADI or FADI at Arg2-Arg3 and Arg3-Nal4 positions revealed the significant contribution of these peptide bonds to FC131 bioactivity. Although substitutions with alkene isosteres resulted in a decrease in bioactivity, the structural and functional requirements of the corresponding amide bonds to biological activity was shown. The results will be useful for the development of cyclic pentapeptide-based CXCR4 antagonists. Additionally, it was demonstrated that FC131 and the analogous were selective CXCR4 antagonists, which did not inhibit SDF-1 binding to CXCR7. Further studies on the synthesis and biological evaluation of CXCR4 antagonists with peptide bond mimetics are the subject of an ongoing investigation.

Experimental

Synthesis

***tert*-Butyl (2*R*,5*S*,3*E*)-8-[*N*-(benzyloxycarbonyl)amino]-2-[3-(*tert*-butyldimethylsiloxy)prop-1-yl]-5-[*N*-(*o*-nitrobenzenesulfonyl)amino]oct-3-enoate (10).** 1.57 M *t*-BuLi in *n*-pentane solution (28.7 cm^3, 45 mmol) was added dropwise to a stirred solution of $I(CH_2)_3OTBS$ (6.78 g, 22.5 mmol) in dry Et_2O (10.6 cm^3) under argon at −78 °C. Following stirring at −78 °C for 30 min, the mixture was stirred at room temperature for 10 min. To a stirred solution of CuCN (1.26 g, 14.1 mmol) and LiCl (1.19 g, 28.1 mmol) in dry THF (20 cm^3) under argon at −78 °C, the above 0.5 M $TBSO(CH_2)_3Li$ in THF–Et_2O–*n*-pentane solution (28.2 cm^3) was added dropwise, and the mixture was further stirred at 0 °C for 10 min. To the above mixture, a solution of the enoate **9** (1.92 g, 3.51 mmol) in dry THF (20 cm^3) was added dropwise at −78 °C, and the mixture was further stirred for 2 h at −78 °C. The reaction was quenched by the addition of a saturated NH_4Cl/28% NH_4OH solution (1/1, 30 cm^3), with additional stirring at room temperature for 1 h. After the mixture was concentrated under reduced pressure, the residue was extracted with Et_2O. The extract was washed with water and brine, and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc–*n*-hexane (1/5) gave the title compound **10** (1.68 g, 66%) as a colorless oil: $[\alpha]_D^{24}$ −89.8 (*c* 1.00, $CHCl_3$); δ_H (500 MHz, $CDCl_3$, Me_4Si) 0.00 (6 H, s), 0.85 (9 H, s), 1.22–1.26 (2 H, m), 1.34 (9 H, s), 1.46–1.51 (6 H, m), 2.59–2.64 (1 H, m), 3.12–3.14 (2 H, m), 3.45–3.48 (2 H, m), 3.89–3.93 (1 H, m), 4.79–4.87 (1 H, m), 5.04 (2 H, s), 5.22 (1 H, dd, *J* 15.5 and 7.4), 5.34 (1H, dd, *J* 15.5 and 8.6), 5.42 (1 H, d, *J* 8.0), 7.23–7.31 (5 H, m), 7.61–7.65 (2 H, m), 7.74–7.80 (1 H, m) and 7.99–8.06 (1 H, m); δ_C (125 MHz, $CDCl_3$, Me_4Si) −5.4 (2 C), 18.2, 25.9 (3 C), 26.0, 27.9 (3 C), 28.7, 29.9, 33.0, 40.3, 49.0, 56.5, 62.5, 66.5, 80.6, 125.2, 128.0 (3 C), 128.4 (2 C), 130.9, 131.2, 132.8, 133.2, 133.3, 134.8, 136.5, 147.7, 156.4 and 172.6; HRMS (FAB), *m/z* calcd for $C_{35}H_{52}N_3O_9SSi$ ([M − H]$^-$) 718.3199, found 718.3190.

(2*R*,5*S*,3*E*)-8-[*N*-(Benzyloxycarbonyl)amino]-2-{3-[*N*-(benzyloxycarbonyl) amino]prop-1-yl}-5-[*N*-(fluorenylmethoxycarbonyl)amino]oct-3-enoic acid (14). Compound **13** (610 mg, 0.790 mmol) was dissolved in 4 N HCl–dioxane (8 cm^3) and the mixture was stirred at room temperature for 8 h. After the mixture was concentrated under reduced pressure, the residue was extracted with EtOAc. The extract was washed with 1 N HCl and brine, and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc–*n*-hexane–AcOH (1/1/0.02) gave the title compound **14** (367 mg, 65%) as a white solid: mp 162–163 °C; $[\alpha]_D^{24}$ −16.6 (*c* 1.02, DMSO); δ_H (500 MHz, DMSO, Me_4Si) 1.38–1.40 (7 H, m), 1.55–1.66 (1 H, m), 2.87 (1 H, m), 2.97 (4 H, m), 3.93 (1 H, m), 4.17–4.24 (1 H, m), 4.24–4.31 (1 H, m), 4.96–5.03 (5 H, m), 5.47 (2 H, m), 7.28–7.41 (17 H, m), 7.65–7.69 (2 H, m), 7.86–7.88 (2 H, m) and 12.20 (1 H, s); δ_C (125 MHz, DMSO, Me_4Si) 26.1, 27.0, 29.2, 30.9, 40.0 (2 C), 46.7, 47.8, 51.9, 65.1, 65.2 (2 C), 120.0 (2 C), 125.2 (2 C), 127.0 (2 C), 127.5 (2 C), 127.6 (3 C), 127.7 (3 C), 128.3 (4 C), 133.2, 137.2 (2 C), 140.7, 143.8 (2 C), 143.9 (2 C), 156.1 (3 C) and 174.8; HRMS (FAB), *m/z* calcd for $C_{42}H_{44}N_3O_8$ ([M − H]$^-$) 718.3134, found 718.3125.

(2*R*,5*S*,3*Z*)-5-[(*tert*-Butoxycarbonyl)amino]-8-(*tert*-butyldimethylsiloxy)-4-fluoro-2-(naphthalen-2-ylmethyl)oct-3-enoyl (*S*)-sultam (16). To a suspension of CuI (2.22 g, 11.6 mmol) in THF (250 cm^3) at −78 °C under argon was added dropwise a solution of MeLi·LiBr complex in Et_2O (1.5 M, 15.5 cm^3, 23.2 mmol), and the mixture was stirred for 10 min at 0 °C. To the solution of the above organocopper reagent at −78 °C was added dropwise a solution of the *N*-enoyl sultam **15** (1.80 g, 2.90 mmol) in THF (70 cm^3). The mixture was stirred for 30 min at −78 °C and HMPA (8.31 cm^3, 46.4 mmol) was added dropwise to the mixture. After stirring for 30 min at −78 °C, a solution of triphenyltin chloride (2.24 g, 5.80 mmol) in THF (20 cm^3) was added dropwise, and the mixture was subsequently stirred for 10 min at −40 °C. 2-(Bromomethyl)naphthalene (5.13 g, 23.2 mmol) in THF (30 cm^3) was added dropwise and the mixture was stirred for 20 h at −40 °C. The reaction was quenched at −40 °C by the addition of a saturated NH_4Cl/28% NH_4OH solution (1/1, 50 cm^3) and the mixture was stirred at room temperature for an additional 30 min. The mixture was extracted with Et_2O and the extract was washed with brine and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc–*n*-hexane (1/3) gave the title compound **16** (1.71 g, 79%) as a colorless oil: $[\alpha]_D^{24}$ −74.3 (*c* 1.00, $CHCl_3$); δ_H (500 MHz, $CDCl_3$, Me_4Si) 0.02 (6 H, s), 0.30 (3 H, s), 0.76 (3 H, s), 0.88 (9 H, s), 1.18–1.30 (2 H, m), 1.38–1.48 (11 H, m), 1.52–1.66 (4 H, m), 1.70–1.82 (2 H, m), 1.91 (1 H, dd, *J* 13.7 and 8.0), 2.97 (1 H, dd, *J* 13.7 and 6.9), 3.24–3.36 (3 H, m), 3.46–3.56 (2 H, m), 3.64–3.79 (1 H, m), 4.08–4.21 (1 H, m), 4.48–4.60 (1 H, m), 4.67 (1 H, d, *J* 8.6), 5.06 (1 H, dd, *J* 36.1 and 9.2), 7.38–7.44 (3 H, m), 7.64 (1 H, s) and 7.71–7.78 (3 H, m); δ_C (125 MHz, $CDCl_3$, Me_4Si) −5.3

(2 C), 18.3, 19.6, 19.8, 26.0 (3 C), 26.3, 28.3 (3 C), 28.6, 28.7, 32.8, 38.2, 40.6 (d, *J* 2.4), 43.0, 44.5, 47.3, 48.0, 51.7, 52.9, 62.5, 64.9, 79.6, 103.7 (d, *J* 13.1), 125.3, 125.7, 127.5, 127.6, 127.8, 127.9, 127.9, 132.4, 133.4, 135.1, 154.9, 158.8 (d, *J* 261.1) and 172.2; δ_F (125 MHz, $CDCl_3$, $CFCl_3$) −119.5; HRMS (FAB), *m/z* calcd for $C_{40}H_{58}FN_2O_6SSi$ ([M − H]$^-$) 741.3774, found: 741.3768.

(2*R*,5*S*,3*Z*)-5-[*N*-(Fluorenylmethoxycarbonyl)amino]-4-fluoro-2-(naphthalen-2-ylmethyl)-8-[*N*-(*o*-nitrobenzenesulfonyl)amino]oct-3-enoic acid (18). To a solution of the sultam **17** (986 mg, 1.08 mmol) and aqueous 50% H_2O_2 (0.383 cm^3, 5.62 mmol) in THF–H_2O (5/1, 15 cm^3) at 0 °C was added aqueous 1 N LiOH (2.16 cm^3, 2.16 mmol). The mixture was stirred at room temperature for 2 h. Following dilution with EtOAc (50 cm^3), the mixture was washed with 0.1 N HCl and dried over $MgSO_4$. Concentration under reduced pressure gave the corresponding acid, which was used in the next step without purification. TFA (5 cm^3) was added to a solution of the acid in CH_2Cl_2 (5 cm^3) at 0 °C, and the mixture was stirred at room temperature for 30 min. Concentration under reduced pressure gave an oily residue, which was dissolved in MeCN–DMF–H_2O (10/9/1, 40 cm^3). Fmoc-OSu (584 mg, 1.73 mmol) and Et_3N (0.332 cm^3, 2.38 mmol) were added to the mixture at 0 °C and the mixture was stirred at room temperature for 12 h. After being diluted with EtOAc (280 cm^3), the reaction mixture was washed with 1 N HCl and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc–*n*-hexane–AcOH (1/1/0.02) gave the title compound **18** (673 mg, 85%) as a colorless semisolid: $[\alpha]_D^{25}$ −27.4 (*c* 1.00, $CHCl_3$); δ_H (500 MHz, $CDCl_3$, Me_4Si) 1.31–1.40 (2 H, m), 1.41–1.55 (2 H, m), 2.93–2.99 (3 H, m), 3.28 (1 H, dd, *J* 13.7 and 6.3), 3.78–3.87 (1 H, m), 4.08–4.16 (2 H, m), 4.28 (1 H, dd, *J* 10.3 and 6.9), 4.40 (1 H, dd, *J* 10.3 and 6.9 Hz), 4.81 (1 H, d, *J* 9.2), 4.93 (1 H, dd, *J* 36.1 and 9.7), 5.38 (1 H, t, *J* 5.7), 7.26–7.79 (18 H, m) and 8.02–8.07 (1 H, m); δ_C (125 MHz, $CDCl_3$, Me_4Si) 25.6, 28.9, 38.4, 42.9, 47.1, 51.6 (d, *J* 27.6), 62.3, 66.7, 104.7 (d, *J* 14.4), 120.0 (2 C), 124.4, 124.9, 125.0 (2 C), 125.2 (2 C), 125.5 (2 C), 125.9, 127.1, 127.5 (2 C), 127.7, 127.8, 130.9, 132.2, 132.7, 133.3, 133.5, 133.5, 135.6, 141.3 (2 C), 143.7, 143.8, 147.9, 158.0 (d, *J* 262.0), 163.0 and 177.0; δ_F (125 MHz, $CDCl_3$, $CFCl_3$) −120.8; HRMS (FAB), *m/z* calcd for $C_{40}H_{35}FN_3O_8S$ ([M − H]$^-$) 736.2134, found: 736.2137.

Peptide synthesis

The protected linear peptides **20a,b** were constructed on H–Gly–(2-Cl)Trt resin (0.8 mmol g^{-1}, 38 mg, 0.03 mmol). *t*-Bu was employed for Tyr side-chain protection. Fmoc-protected amino acids (0.3 mmol) were coupled by using DIC (0.046 cm^3, 0.3 mmol) and HOBt·H_2O (46 mg, 0.3 mmol) in DMF. Coupling of EADI **14** (33mg, 0.045 mmol) was carried out with HOAt (6.3 mg, 0.045 mmol), HATU (17 mg, 0.045 mmol) and (*i*-Pr)$_2$NEt (0.009 cm^3, 0.045 mmol). Completion of each coupling reaction was ascertained using the Kaiser ninhydrin test. The Fmoc-protecting group was removed by treating the resin with a DMF/piperidine solution (80/20, v/v).

***cyclo*(–D–Tyr–Arg–Ψ[(*E*)-CH=CH]–Arg–Nal–Gly–)·2TFA (3E).** The obtained resin **20a** was treated with HFIP/CH_2Cl_2 (2/8, 15 cm^3) at room temperature for 2 h. After removal of the resin by filtration, the filtrate solution was concentrated under reduced pressure to give a crude protected peptide **21a**. To a mixture of **21a** and $NaHCO_3$ (21 mg, 0.25 mmol) in DMF (20 cm^3) was added DPPA (0.0270 cm^3, 0.13 mmol) at −40 °C. The mixture was stirred for 66 h with warming to room temperature and then filtered. The filtrate was concentrated under reduced pressure to give the protected cyclic peptide **22a**. The peptide **22a** was treated with 1 M TMSBr/thioanisole in TFA (10 cm^3) in the presence of *m*-cresol and 1,2-ethandithiol (0.117 cm^3) for 6 h at 0 °C. The mixture was poured into ice-cold dry Et_2O. The resulting powder was collected and washed three times with ice-cold dry Et_2O. To a stirred solution of the precipitant **23a** in DMF (1 cm^3) were added (*i*-Pr)$_2$NEt (0.014 cm^3, 0.08 mmol) and 1*H*-pyrazole-1-carboxamidine·HCl (12 mg, 0.04 mmol), and the mixture was stirred at room temperature for 60 h. After concentration under reduced pressure, purification by preparative HPLC gave the bis-trifluoroacetate salt of the title peptide **3E** (1.9 mg, 9% yield based on H–Gly–(2-Cl)Trt resin, >98% purity by HPLC analysis) as a colorless freeze-dried powder: HRMS (FAB), *m/z* calcd for $C_{37}H_{49}N_{10}O_5$ ([M+H]$^+$) 713.3882, found 713.3886.

***cyclo*(–D–Tyr–Arg–Arg–Ψ[(*Z*)-CF=CH]–Nal–Gly–)·2TFA (4F).** Cyclic peptide **4F** was synthesized by a procedure identical with that described for the synthesis of **3E**. The protected peptide **22b** (32.0 mg, 0.0270 mmol) was treated with aqueous TFA/H_2O (95/5, 10 cm^3) for 3 h. Concentration under reduced pressure gave an oily residue. To a solution of the residue in DMF (8 cm^3) were added 2-mercaptoethanol (0.0191 cm^3, 0.270 mmol) and DBU (0.0809 cm^3, 0.540 mmol), and the mixture was stirred at 50 °C for 2.5 h. After concentration under reduced pressure, the residue **23b** was treated with Et_3N (0.112 cm^3, 0.810 mmol) and 1*H*-pyrazole-1-carboxamidine·HCl (39.6 mg, 0.270 mmol) in DMF (2 cm^3). After concentration under reduced pressure, purification by preparative HPLC gave the bis-trifluoroacetate salt of the title peptide **4F** (3.6 mg, 6% yield based on H–Gly–(2-Cl)Trt resin, 89% purity by HPLC analysis): HRMS (FAB), *m/z* calcd for $C_{37}H_{48}FN_{10}O_5$ ([M+H]$^+$) 731.3788, found 731.3796.

[^{125}I]-SDF-1 binding and displacement

Membrane extracts were prepared from CHO-K1 cell lines expressing either CXCR4 or CXCR7. For ligand binding, 0.050 cm^3 of the inhibitor, 0.025 cm^3 of [^{125}I]-SDF-1α (0.3 nM, Perkin-Elmer Life Sciences) and 0.025 cm^3 of the membrane/beads mixture [CXCR4: 7.5 μg well^{-1} of membrane, 0.5 mg well^{-1} of PVT WGA beads (Amersham); CXCR7: 3 μg well^{-1} of membrane, 0.25 mg well^{-1} of PVT-PEI type A beads (Amersham)] in assay buffer (25 mM HEPES pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 140 mM NaCl, 250 mM sucrose, 0.5% BSA) were incubated in the wells of an Optiplate places (Perkin-Elmer Life Sciences) at room temperature for 1 h. The bound radioactivity was counted for 1 min well^{-1} in a TopCount (Packard). Inhibitory activity of the test compounds was determined based on the inhibition of [^{125}I]-SDF-1 binding to the receptors (IC_{50}).

Determination of anti-HIV activity

The peptide sensitivity of three HIV-1 strains was determined by the MAGI assay with some modifications.[22] Briefly, the target cells (HeLa-CD4/CCR5-LTR-β-gal; 10^4 cells well^{-1}) were plated in 96-well flat microtiter culture plates. On the following day,

the cells were inoculated with the HIV-1 (60 MAGI U/well, giving 60 blue cells after 48 h of incubation) and cultured in the presence of various concentrations of the drugs in fresh medium. Forty-eight hours after viral exposure, all the blue cells stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were counted in each well. The activity of test compounds was determined as the concentration that blocked HIV-1 replication by 50% (50% effective concentration [EC_{50}]).

Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research from MEXT, Japan, and Health and Labour Science Research Grants (Research on HIV/AIDS). T.N. and K.T. are grateful for the JSPS Research Fellowships for Young Scientists. We are grateful to Prof. Hirokazu Tamamura and Mr. Tomohiro Tanaka for helpful discussions.

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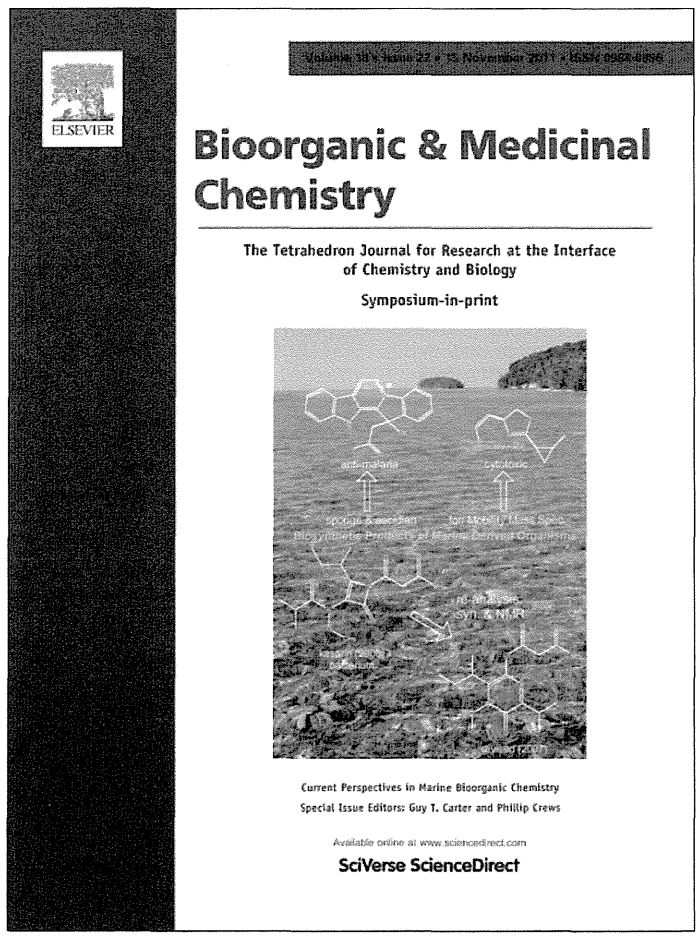

Bioorganic & Medicinal Chemistry 19 (2011) 6735–6742

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

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Small molecular CD4 mimics as HIV entry inhibitors

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ARTICLE INFO

Article history:
Received 5 August 2011
Revised 23 September 2011
Accepted 24 September 2011
Available online 29 September 2011

Keywords:
CD4 mimic
HIV entry
gp120-CD4 interaction
Phe43 cavity

ABSTRACT

Derivatives of CD4 mimics were designed and synthesized to interact with the conserved residues of the Phe43 cavity in gp120 to investigate their anti-HIV activity, cytotoxicity, and CD4 mimicry effects on conformational changes of gp120. Significant potency gains were made by installation of bulky hydrophobic groups into the piperidine moiety, resulting in discovery of a potent compound with a higher selective index and CD4 mimicry. The current study identified a novel lead compound **11** with significant anti-HIV activity and lower cytotoxicity than those of known CD4 mimics.

1. Introduction

The dynamic supramolecular mechanism of HIV cellular invasion has emerged as a key target for blocking HIV entry into host cells.[1] HIV entry begins with the interaction of a viral envelope glycoprotein gp120 and a cell surface protein CD4.[2] This triggers extensive conformational changes in gp120 exposing co-receptor binding domains and allowing the subsequent binding of gp120 to a co-receptor, CCR5[3]/CXCR4.[4] Following the viral attachment and co-receptor binding, gp41, another viral envelope glycoprotein mediates the fusion of the viral and cell membranes, thus completing the infection. Molecules interacting with each of these steps are potential candidates for anti-HIV-1 drugs. In particular, discovery and development of novel drugs that inhibit the viral attachment are required for blocking the HIV infection at an early stage.[5]

In 2005, small molecular CD4 mimics targeting the viral attachment were identified by an HIV syncytium formation assay and shown to bind within the Phe43 cavity, a highly conserved pocket on gp120,[6] which is a hydrophobic cavity occupied by the aromatic ring of Phe43 of CD4.[7] These molecules are comprised of three essential moieties: an aromatic ring, an oxalamide linker, and a piperidine ring (Fig. 1) and show micromolar order potency against diverse HIV-1 strains including laboratory and primary isolates. Furthermore, they possess the unique ability to induce the conformational changes in gp120 required for binding with soluble CD4.[8] Such CD4 mimicry can be an advantage for rendering the envelope more sensitive to neutralizing antibodies.[9] While such properties are promising for the development of HIV entry inhibitors and the use combinatorially with neutralizing antibodies, cytotoxicity is one of the drawbacks of CD4 mimics.

To date, we and others have performed structure–activity relationship (SAR) studies of CD4 mimics based on modifications of the aromatic ring, the oxalamide linker, and the piperidine moiety of CD4 mimics. In an initial survey of SAR studies of NBD-556 and NBD-557, Madani et al. revealed that potency (i.e., CD4 binding and mimicry) was highly sensitive to modifications of the aromatic ring, which is thought to bind in the Phe43 cavity of gp120 (Fig. 1). The CD4 mimic analogs (JRC-II-191) with a *para*-chloro-*meta*-fluorophenyl ring had significantly increased affinity for gp120.[10] Our SAR studies also revealed that a certain size and electron-withdrawing ability of the *para*-substituents are indispensable for potent anti-HIV activity.[11] Furthermore, the replacement of the chlorine group at the *para* position with a methyl group which is almost as bulky as a bromine atom leads to improvement of solubility of the compounds in buffer to provide the reproducibility in the biological studies with comparable biological activities.

Further SAR studies were focused on the piperidine moiety of CD4 mimics to investigate its contribution to biological activities, and we found that the piperidine ring is critical for the CD4 mimicry on the conformational changes in gp120 and that substituents on the nitrogen of the piperidine moiety can contribute significantly to both anti-HIV activity and cytotoxicity.[12] Based on these SARs and our modeling study, we speculate that interactions of the piperidine moiety with several amino acids in the vicinity of the Phe43 cavity in gp120, specifically an electrostatic interaction with

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doi:10.1016/j.bmc.2011.09.045

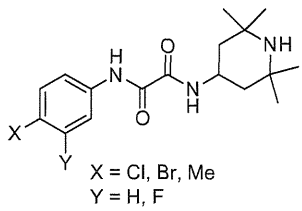


Figure 1. CD4 mimics.

Asp368 and a hydrophobic interaction with Val430, are critical for biological activity. LaLonde et al. focused on modifications of the piperidine moiety using computational approaches, adducing evidence for the importance of these interactions to the binding affinity against gp120.[13] Based on these results, we envisioned that an enhancement of the interaction of CD4 mimics with residues associated with the Phe43 cavity in gp120 would lead to the increase of their potency and CD4 mimicry inducing the conformational changes of gp120, and the decrease of their cytotoxicity. Thus, in this study a series of CD4 mimics, which were designed to interact with the conserved residues in the Phe43 cavity, were synthesized to increase binding affinity for gp120, and the appropriate SAR studies were performed.

2. Results and discussion

Two types of CD4 mimic analogs were designed: (1) CD4 mimics with the ability to interact electrostatically with Asp368, and (2) CD4 mimics with the ability to interact hydrophobically with Val430 (Fig. 2). The X-ray structure of gp120 bound to soluble CD4 (PDB: 1RZJ) revealed that the guanidino group of Arg59 of CD4 is involved in a hydrogen bond with Asp368 of gp120. In order to mimic this interaction, a guanidino and related groups such as thiourea and urea were introduced to the piperidine moiety of the CD4 mimic derivative COC-021, which was developed in order to modify the nitrogen of the piperidine moiety and which showed biological activity, including anti-HIV activity and CD4 mimicry, similar to that of the parent compound NBD-556.[12] Furthermore, to interact with Val430 by hydrophobic interaction, the methyl groups on the piperidine ring were replaced with cyclohexyl groups to prepare a novel CD4 mimic analog with enhanced hydrophobicity.

2.1. Chemistry

The syntheses of CD4 mimics are outlined in Scheme 1. CD4 mimics with guanidine, thiourea, and urea groups on the piperidine moiety were prepared using our previously reported method.[12] Coupling of *p*-chloroaniline with ethyl chloroglyoxylate followed by aminolysis of the ethyl ester with 4-amino-*N*-benzylpiperidine under microwave conditions (150 °C, 3 h) gave the corresponding amide. Removal of the benzyl group with 1-chloroethyl chloroformate[14] gave the free piperidine moiety, which was modified to produce the desired compounds **4–8** (Scheme 1).

For synthesis of a CD4 mimic derivative with two cyclohexyl groups, treatment of 2,2,6,6-tetramethylpiperidin-4-one **9** with cyclohexanone in the presence of ammonium chloride furnished a 2,6-substituted piperidin-4-one derivative,[15] and reductive amination with benzylamine and subsequent removal of benzyl group provided a primary amine **10**. Microwave-assisted aminolysis of ester **2** with amine **10** yielded the desired dicyclohexyl-substituted analog **11** (Scheme 2). The synthesis of the other compounds is described in Supplementary data.

2.2. Biological studies

The anti-HIV activity of synthetic CD4 mimics was evaluated in a single-round viral infective assay. Inhibition of HIV-1 infection was measured as reduction in β-galactosidase gene expression after a single-round of virus infection of TZM-bl cells as described previously.[9] IC_{50} was defined as the concentration that caused a 50% reduction in the β-galactosidase activity (relative light units [RLU]) compared to virus control wells. Cytotoxicity

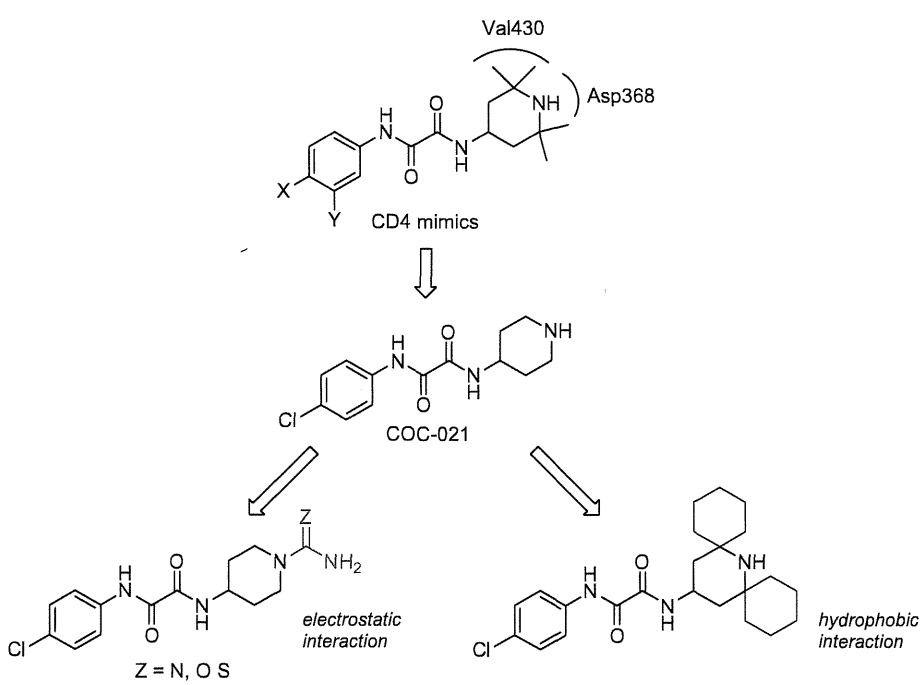
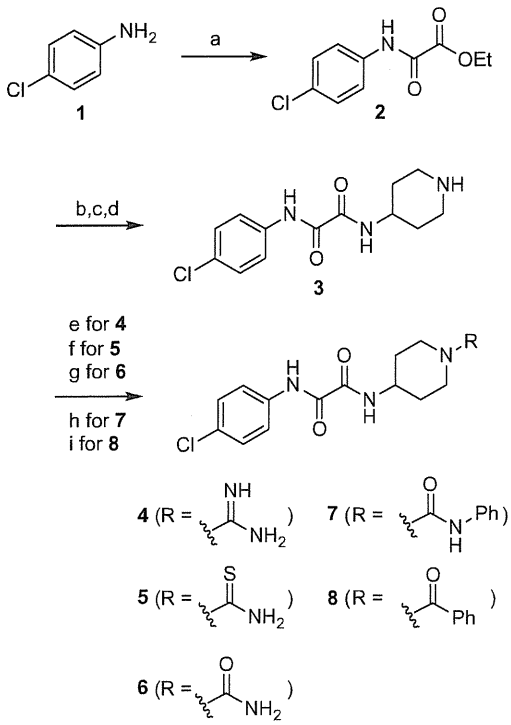
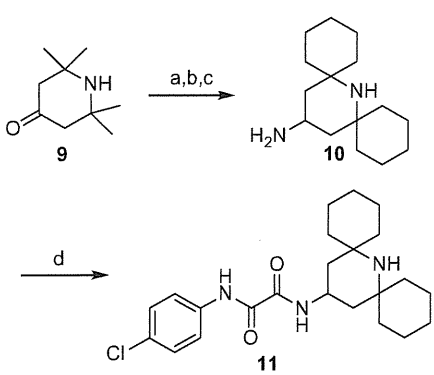


Figure 2. Design strategy for novel CD4 mimics with enhanced electrostatic/hydrophobic interaction.



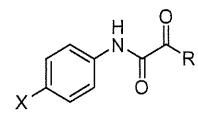
Scheme 1. Synthesis of N-modified piperidine derivatives **4–8**. Reagents and conditions: (a) Ethyl chloroglyoxylate, Et_3N, THF, quant.; (b) 1-benzyl-4-aminopiperidine, Et_3N, EtOH, 150 °C, microwave, 78%; (c) 1-chloroethyl chloroformate, CH_2Cl_2; (d) MeOH, reflux, 64% in two steps; (e) 1*H*-pyrazole-1-carboxamidine hydrochloride, Et_3N, DMF, 61%; (f) (trimethylsilyl)isothiocyanate, $CHCl_3$, 36%; (g) (trimethylsilyl)isocyanate, $CHCl_3$, 30%; (h) phenyl isocyanate, $CHCl_3$, 32%, (i) benzoyl chloride, Et_3N, CH_2Cl_2, 68%.

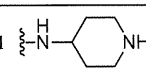
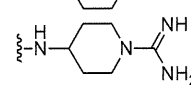
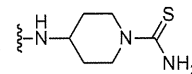
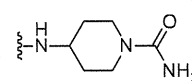
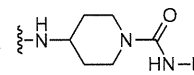
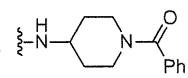
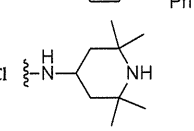
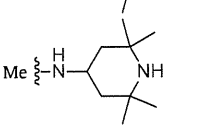


Scheme 2. Synthesis of dicyclohexyl derivative **11**. Reagents and conditions: (a) Cyclohexanone, NH_4Cl, DMSO, 60 °C; (b) benzylamine, $NaBH_4$, MeOH; (c) 10% Pd/C, H_2, MeOH, 7% from **9**; (d) **2**, Et_3N, EtOH, 150 °C, microwave, 17%.

of the compounds based on the viability of mock-infected PM1/CCR5 cells was evaluated using WST-8 method. The assay results for the CD4 mimics **3–8** are shown in Table 1. Compound **12** (NBD-556) showed potent anti-HIV activity; its IC_{50} value was 0.61 μM, and it is thus 13–20-fold more potent than the reported values.[11,12] Although previous studies found that compound **13**, with a methyl group at the *p*-position of the phenyl ring, and compound **3**, with no dimethyl groups on the piperidine ring, showed potent anti-HIV activity, only moderate activities were observed in the current study; this is about 12–14-fold less potency than reported for compound **12** and is probably due to

Table 1
Effects of the nitrogen-substituents on anti-HIV activity and cytotoxicity of CD4 mimic analogs[a]

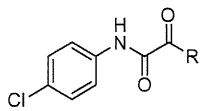


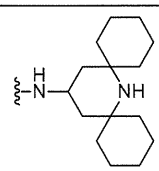
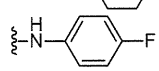
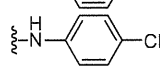
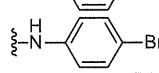
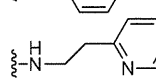
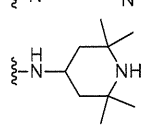
Compd	X	R	IC_{50}[b] (μM)	CC_{50}[c] (μM)	SI (CC_{50}/IC_{50})
			YTA (R5)		
3[d]	Cl		7.0	51	7.3
4[e]	Cl		6.1	72	12
5	Cl		5.5	42	7.6
6	Cl		8.3	310	37
7	Cl		11	6.2	0.56
8	Cl		5.1	ND	–
12 (NBD-556)	Cl		0.61	35	57
13	Me		8.4	260	31

[a] All data with standard deviation are the mean values for at least three independent experiments (ND = not determined)
[b] IC_{50} values are based on the reduction in the β-galactosidase activity in TZM-bl cells.
[c] CC_{50} values are based on the reduction of the viability of mock-infected PM1/CCR5 cells.
[d] Desalted by satd $NaHCO_3$ aq.
[e] TFA salts.

the different assay system. All of the synthesized novel derivatives of compound **12** showed moderate to potent anti-HIV activity. A guanidine derivative **4** and thiourea derivative **5** showed potent anti-HIV activities (IC_{50} of **4** = 6.1 μM and IC_{50} of **5** = 5.5 μM) but their potency was approximately 10-fold lower than that of the parent compound **12**. A urea derivative **6** also showed potent anti-HIV activity (IC_{50} = 8.3 μM) and exhibited lower cytotoxicity (CC_{50} = 310 μM). On the other hand, introduction of a phenyl group in the urea derivative **6**, led to an *N*-phenylurea derivative **7**, with an increase of cytotoxicity (CC_{50} = 6.2 μM). To examine the influence of the N–H group on anti-HIV activity, an *N*-benzoyl derivative **8** was also tested. The IC_{50} value of **8** was 5.1 μM, which is equipotent with the thioamide derivative **5**. The *N*-benzoyl derivative **8** was essentially equipotent with **3** and this result suggests the presence of the hydrogen atom of the N–H group does not contribute to an increase in anti-HIV activity. The thiourea derivative **5** and the *N*-phenylurea derivative **7**, which have more acidic protons (pK_a of thiourea and *N*-phenylurea; 21.0 and 19.5,[16] respectively) than the urea derivative **6** (pK_a of urea; 26.9[16]), were found to exhibit relatively strong cytotoxicity. This observation indicates that

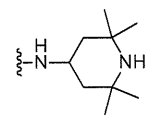
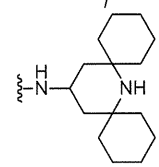
Table 2
Anti-HIV activity and cytotoxicity of CD4 mimic analogs **11**, **12**, and **14–17**[a]



Compd	R	IC_{50}[b] (μM)	CC_{50}[c] (μM)	SI (CC_{50}/IC_{50})
		YTA (R5)		
11		0.68	120	176
14		3.1	>500	>160
15		>100	>500	–
16		>100	>500	–
17		19.8	480	24
12 (NBD-556)		0.61	35	57

[a] All data with standard deviation are the mean values for at least three independent experiments
[b] IC_{50} values are based on the reduction in the β-galactosidase activity in TZM-bl cells.
[c] CC_{50} values are based on the reduction of the viability of mock-infected PM1/CCR5 cells.

Table 3
Anti-HIV activity of CD4 mimic **12** and dicyclohexyl derivative **11**[a]

Compd	R	IC_{50}[b] (μM) Single-round assay	IC_{50}[c] (μM) Multi-round assay
12 (NBD-556)		0.61	0.90
11		0.68	0.56

[a] All data with standard deviation are the mean values for at least three independent experiments.
[b] IC_{50} values of the single-round assay are based on the reduction in the β-galactosidase activity in TZM-bl cells.
[c] IC_{50} values of the multi-round assay are based on the inhibition of HIV-1-induced cytopathogenicity in PM1/CCR5 cells.

substitution on the piperidine moiety of acidic functional groups was unfavorable.

The assay results for CD4 mimics that target hydrophobic interactions are shown in Table 2. Compound **11** showed significant anti-HIV activity (IC_{50} = 0.68 μM) comparable to that of the lead compound **12**, but exhibited lower cytotoxicity. Compound **11** showed approximately four-fold less cytotoxicity than **12**. The SI of **11** is 176, 3 times higher than that of **12** (SI = 57). This result suggests that substitution of bulky hydrophobic groups into the piperidine moiety may be consistent with lower cytotoxicity of CD4 mimics. It is noteworthy that compound **14**, which has a *p*-fluoroanilino group in place of the piperidine ring, exhibits potent anti-HIV activity (IC_{50} = 3.1 μM) without significant cytotoxicity (CC_{50} >500 μM). The SI of compound **14** is >160, which is comparable to that of **11**. However, replacement of the piperidine moiety with a *p*-bromo- or *p*-chloroanilino group resulted in the loss of anti-HIV activity. These results suggest that the introduction of a fluorine atom to the piperidine moiety might be consistent with improvement of the anti-HIV activity. Extension of the alkyl chain by two carbons, as in **17** resulted in a 30-fold loss of anti-HIV activity, indicating that relatively rigid structures are preferable for anti-HIV activity.

The anti-HIV activities of **12** and compound **11**, which has a higher SI than the parent compound **12** were evaluated in a multi-round viral infective assay and the results are shown in Table 3. In this assay, the IC_{50} value of **12** was 0.90 μM, which was slightly larger value than measured in a single-round assay (IC_{50} = 0.61 μM). Compound **11** showed higher anti-HIV activity (IC_{50} = 0.56 μM) than compound **12**, indicating that the introduction of hydrophobic cyclohexyl groups into the piperdine moiety has a positive effect on not only the cytotoxicity but also the anti-HIV activity. This is possibly due to the stability in the assay condition derived from the hydrophobicity of cyclohexyl group(s). These results are consistent with a previous study of the analog with one hydrophobic *gem*-dimethyl group on the piperidine moiety, a compound with potent anti-HIV activity and efficient binding affinity for gp120.[13]

To gain insight into the interactions involved in the binding, molecular modeling of compound **11** docked into gp120 (1RZJ) was carried with Sybyl 7.1 (Fig. 3). The binding mode of compound **11** in the Phe43 cavity suggested that the orientation of the piperidine moiety of **11** is different from that in compound **12**, and that the cyclohexyl group can be positioned near Val430 with whose isopropyl group it can interact hydrophobically.

Fluorescence activated cell sorting (FACS) analysis was performed as previously reported,[11,12] to evaluate the CD4 mimicry effects on conformational changes of gp120 and the results are shown in Figure 4. Comparison of the binding of an anti-envelope CD4-induced monoclonal antibody (4C11) to the cell surface pretreated with the above CD4 mimics was measured in terms of the mean fluorescence intensity (MFI). Our previous studies revealed that the profile of the binding of 4C11 to the Env-expressing cell surface pretreated with compound **12** was entirely similar to that of pretreatment of soluble CD4. In this FACS analysis, the MFI of pretreatment with compound **12** is 23.13. The profiles of the binding of 4C11 to the cell surface pretreated with compounds **3**, **4** and **5** were comparable to that of compound **12** [MFI (**3**) = 20.54, MFI (**4**) = 20.85, MFI (**5**) = 20.24, respectively], suggesting that these derivatives offer a significant enhancement of binding affinity for 4C11. On the other hand, pretreatment with **6** and **8** did not cause significant enhancement of the binding affinity for 4C11, indicating that introduction of a carbonyl group on the piperidine nitrogen is not conducive to CD4 mimicry. The profile of the binding of 4C11 to the Env-expressing cell surface pretreated with compound **11**, which had significant anti-HIV activity and lower cytotoxicity than compound **12**, (MFI (**11**) = 22.17) was similar to that of compound **12**, suggesting that compound **11** offers significant enhancement of binding affinity for 4C11. This result indicates that compound **11** retains the CD4 mimicry on the conformational changes of gp120. Although compound **14** and compound **17** showed potent anti-HIV activity and no significant cytotoxicity, the profiles pretreated with (MFI (**14** and **17**) = 15.20 and 15.38) were similar to that of the control (MFI = 14.94), suggesting that these compounds **14** and **17** failed to produce a significant increase in binding affinity for 4C11. These

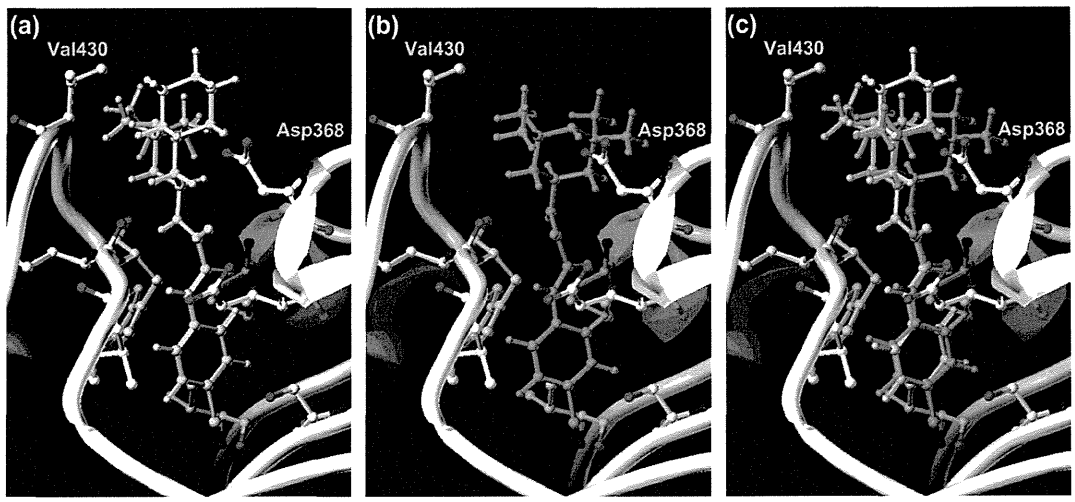


Figure 3. Docking structures of (a) compound **11** and (b) compound **12** bound in the Phe43 cavity of gp120 (1RZJ); (c) merge image of compounds **11** and **12**. Compounds **11** and **12** are represented in yellow and green sticks, respectively. Key residues in the cavity forming interactions with compounds are represented in gray sticks.

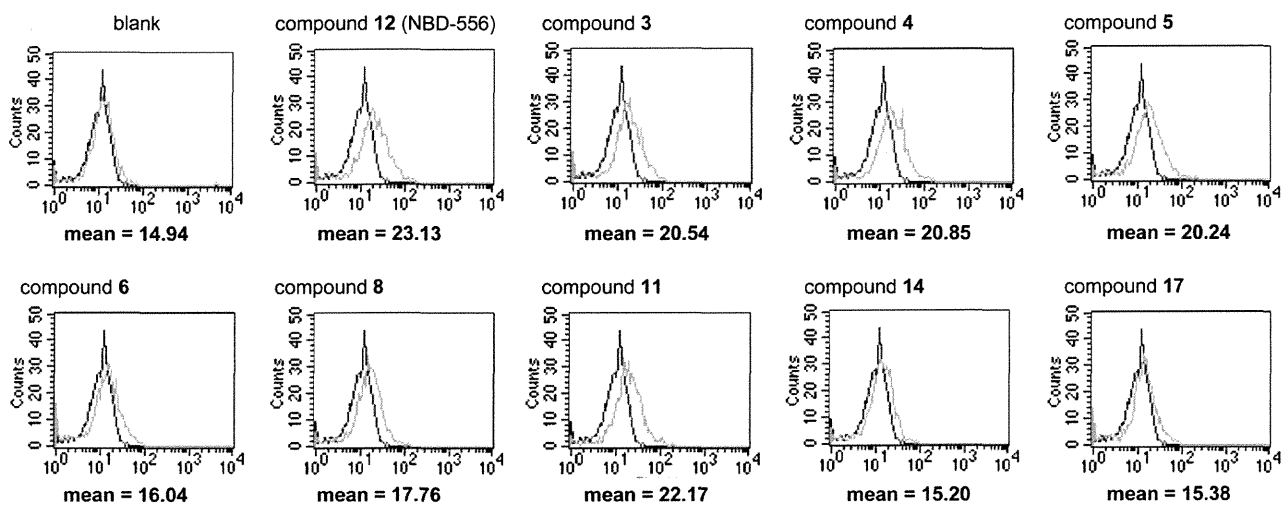


Figure 4. FACS analysis of compounds **12**, **3**–**6**, **8** (Table 1), **11**, **14**, and **17** (Table 2).

results are consistent with our previous finding that the piperidine ring is critical to the CD4 mimicry of the conformational changes in gp120.

3. Conclusion

A series of CD4 mimics were designed and synthesized to interact with the conserved residues in the Phe43 cavity of gp120 to investigate their anti-HIV activity, cytotoxicity, and CD4 mimicry as a function of conformational change of gp120. The biological activities of the synthetic compounds indicate that (1) the hydrogen atom of the piperidine moieties contributes significantly to cytotoxicity, and (2) installation of bulky hydrophobic groups into the piperidine moiety can increase anti-HIV activity and decrease cytotoxicity thus providing a novel compound with higher selective index than those of the original CD4 mimics. Furthermore, this modification has no great influence on the CD4 mimicry on the conformational change of gp120. Thus, compound **11** is promising for further studies. More detailed SAR investigations with respect to the substitution on the piperidine moiety have been ongoing studies.

4. Experimentals

^{1}H NMR and ^{13}C NMR spectra were recorded using a Bruker Avance III spectrometer. Chemical shifts are reported in δ (ppm) relative to Me_4Si (in $CDCl_3$) as internal standard. Low- and high-resolution mass spectra were recorded on a Bruker Daltonics microTOF focus in the positive and negative detection mode. For flash chromatography, Wakogel C-200 (Wako Pure Chemical Industries, Ltd) and silica gel 60 N (Kanto Chemical Co., Inc.) were employed. For analytical HPLC, a Cosmosil 5C_{18}-ARII column (4.6 × 250 mm, Nacalai Tesque, Inc., Kyoto, Japan) was employed with a linear gradient of CH_3CN containing 0.1% (v/v) TFA at a flow rate of 1 cm^3 min^{-1} on a JASCO PU-2089 plus (JASCO Corporation, Ltd., Tokyo, Japan), and eluting products were detected by UV at 220 nm. Preparative HPLC was performed using a Cosmosil 5C_{18}-ARII column (20 × 250 mm, Nacalai Tesque, Inc.) on a JASCO PU-2087 plus (JASCO Corporation, Ltd, Tokyo, Japan) in a suitable

gradient mode of CH_3CN solution containing 0.1% (v/v) TFA at a flow rate of 7 cm^3 min^{-1}. Microwave reactions were performed in Biotage Microwave Reaction Kit (sealed vials) in an Initiator™ (Biotage). The wattage was automatically adjusted to maintain the desired temperature for the desired period of time.

4.1. Chemistry

4.1.1. N^1-(4-Chlorophenyl)-N^2-(piperidin-4-yl)oxalamide (3)

To a stirred solution of *p*-choroaniline (**1**) (14.0 g, 110 mmol) in THF (146 mL) were added ethyl chloroglyoxylate (8.13 mL, 73.2 mmol) and triethylamine (Et_3N) (15.2 mL, 110 mmol) at 0 °C. The mixture was stirred for 6 h at room temperature. After the precipitate was filtrated off, the filtrate solution was concentrated under reduced pressure. The residue was dissolve in EtOAc, and washed with 1 M HCl, saturated $NaHCO_3$ and brine, then dried over $MgSO_4$. Concentration under reduced pressure gave the crude ethyl oxalamate, which was used without further purification. To a solution of the above ethyl oxalamate (1.27 g, 5.25 mmol) in EtOH (13.0 mL) were added Et_3N (1.46 mL, 10.5 mmol) and 4-amino-1-benzylpiperidine (2.97 mL, 15.8 mmol). The reaction mixture was stirred for 3 h at 150 °C under microwave irradiation. After being cooled to room temperature, the crystal was collected and washed with cold EtOH and *n*-hexane, and dried under reduced pressure to provide the corresponding amide (1.58 g, 81% yield) as colorless crystals. To a stirred solution of **S1** (1.46 g, 3.90 mmol) in CH_2Cl_2 (39.0 mL) was added dropwise 1-chloroethyl chloroformate (0.860 mL, 7.80 mmol) at 0 °C. After being stirred at room temperature for 30 min, the mixture was refluxed for 1 h. After concentration under reduced pressure, the residue was dissolved in MeOH and then refluxed for 1 h. After concentration under reduced pressure, the residue was diluted with EtOAc and washed with saturated $NaHCO_3$ and brine, then dried over $MgSO_4$. After concentration under reduced pressure, the residue was washed with cold EtOAc, and dried under reduced pressure to provide the title compound **3** (778 mg, 71% yield) as white powder.

^1H NMR (400 MHz, $CDCl_3$) δ 1.39–1.52 (m, 2H), 1.92–2.01 (m, 2H), 2.67–2.79 (m, 2H), 3.06–3.19 (m, 2H), 3.83–3.95 (m, 1H), 7.34 (d, J = 8.80 Hz, 2H), 7.44 (d, J = 7.64 Hz, 1H), 7.59 (d, J = 8.80 Hz, 2H), 9.28 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 33.0 (2C), 45.2 (2C), 47.9, 21.0 (2C), 129.3 (2C), 130.5, 135.0, 157.6, 158.8; HRMS (ESI), *m/z* calcd for $C_{13}H_{17}ClN_3O_2$ (MH^+) 282.1004, found 282.1002.

4.1.2. N^1-(1-Carbamimidoylpiperidin-4-yl)-N^2-(4-chlorophenyl) oxalamide (4)

To a stirred solution of **3** (50.0 mg, 0.178 mmol) in DMF (20.0 mL) was added 1-aminopyrazole hydrochlride (312 mg, 2.13 mmol) and Et_3N (0.390 mL, 28.1 mmol). The reaction mixture was stirred at room temperature for 24 h. After concentration under reduced pressure, purification by preparative HPLC gave the trifluoroacetate of the title compound **4** as white powder (36.0 mg, 61% yield).

^1H NMR (500 MHz, DMSO) δ 1.41–1.55 (m, 2H), 1.59–1.71 (m, 2H), 2.70–2.74 (m, 2H), 3.74–3.87 (m, 1H), 3.88–4.03 (m, 2H), 5.93 (s, 2H), 7.42 (d, J = 9.00 Hz, 2H), 7.85 (d, J = 9.00 Hz, 2H), 8.95 (d, J = 9.00 Hz, 1H), 10.80 (s, 1H); ^{13}C NMR (125 MHz, DMSO) δ 31.3 (2C), 43.0 (2C), 47.6, 122.4 (2C), 128.6, 129.1 (2C), 137.1, 158.2, 159.3, 159.5; HRMS (ESI), *m/z* calcd for $C_{14}H_{19}ClN_5O_2$ (MH^+) 324.1222, found 324.1213.

4.1.3. N^1-(1-Carbamothioylpiperidin-4-yl)-N^2-(4-chlorophenyl) oxalamide (5)

To a stirred solution of **3** (140 mg, 0.498 mmol) in $CHCl_3$ (5.00 mL) was added trimethylsilyl isothiocyanate (141 mL, 1.00 mmol) and stirred at room temperature for 1 h. The precipitate was collected and washed with cold $CHCl_3$, and dried under reduced pressure to provide the title compound **5** as white powder. (62.0 mg, 36% yield).

^1H NMR (400 MHz, DMSO) δ 1.45–1.69 (m, 2H), 1.69–1.81 (m, 2H), 2.67–2.81 (m, 2H), 3.02–3.16 (m, 2H), 3.75–3.89 (m, 1H), 7.41 (d, J = 9.00 Hz, 2H), 7.85 (d, J = 9.00 Hz, 2H), 9.00 (d, J = 8.50 Hz, 1H), 10.80 (s, 1H); ^{13}C NMR (125 MHz, DMSO) δ 27.8 (2C), 42.3 (2C), 44.4, 122.0 (2C), 128.2, 128.6 (2C), 129.5, 136.6, 158.6, 159.4; Anal. calcd for $C_{14}H_{18}ClN_4O_2S$: C, 49.34; H, 5.03; N, 16.44. Found: C, 49.32; H, 4.76; N, 16.11.

4.1.4. N^1-(1-Carbamoylpiperidin-4-yl)-N^2-(4-chlorophenyl) oxalamide (6)

To a stirred solution of **3** (60.0 mg, 0.213 mmol) in $CHCl_3$ (1.10 mL) was added trimethylsilyl isocyanate (56.0 µL, 0.421 mmol), and the mixture was stirred at room temperature for 1 h. The precipitate was collected and washed with cold $CHCl_3$, and dried under reduced pressure to provide the title compound **6** (20.1 mg, 30% yield) as white powder.

^1H NMR (500 MHz, DMSO) δ 1.44–1.55 (m, 2H), 1.58–1.71 (m, 2H), 2.65–2.78 (m, 2H), 3.76–3.87 (m, 1H), 3.87–4.01 (m, 2H), 5.94 (s, 1H), 7.42 (d, J = 9.00 Hz, 2H), 7.86 (d, J = 9.00 Hz, 2H), 8.95 (d, J = 9.00 Hz, 1H), 10.80 (s, 1H); ^{13}C NMR (125 MHz, DMSO) δ 30.8 (2C), 42.6 (2C), 47.1, 122.0 (2C), 128.1, 128.6 (2C), 136.7, 157.8, 158.8, 159.0; HRMS (ESI), *m/z* calcd for $C_{14}H_{18}ClN_4O_3$ (MH^+) 325.1062, found 325.1060.

4.1.5. N^1-(4-Chlorophenyl)-N^2-(1-(phenylcarbamoyl)piperidin-4-yl)oxalamide (7)

To a stirred solution of **3** (140 mg, 0.498 mmol) in $CHCl_3$ (5.00 mL) was added phenyl isocyanate (54.0 µL, 0.500 mmol) and stirred at room temperature for 1 h. The precipitate was collected and washed with cold $CHCl_3$, and dried under reduced pressure to provide the title compound **7** as white powder. (64.1 mg, 32% yield).

^1H NMR (500 MHz, DMSO) δ 1.52–1.66 (m, 2H), 1.68–1.80 (m, 2H), 2.81–2.95 (m, 2H), 3.84–3.96 (m, 1H), 4.08–4.20 (m, 2H), 6.91–6.94 (m, 2H), 7.21–7.24 (m, 2H), 7.36–7.52 (m, 4H), 7.86 (d, J = 9.00 Hz, 2H), 8.53 (s, 1H), 8.99 (d, J = 8.50 Hz, 2H), 10.81 (s, 1H); ^{13}C NMR (125 MHz, DMSO) δ 31.3 (2C), 43.4 (2C), 47.5, 120.0 (2C), 122.0, 122.4 (2C), 128.6, 128.7 (2C), 129.1 (2C), 137.1, 141.1, 155.2, 159.2, 159.5; HRMS (ESI), *m/z* calcd for $C_{20}H_{22}ClN_4O_3$ (MH^+) 401.1375, found 401.1372.

4.1.6. N^1-(1-Benzoylpiperidin-4-yl)-N^2-(4-chlorophenyl) oxalamide (8)

To a stirred solution of **3** (500 mg, 1.78 mmol) in $CHCl_3$ (17.8 mL) was added benzoyl chloride (307 µL, 2.67 mmol) and the mixture was stirred at room temperature for 1 h. The precipitate was collected and washed with cold EtOAc, and dried under reduced pressure to provide the title compound **8** (232 mg, 34% yield).

^1H NMR (500 MHz, $CDCl_3$) δ 1.21–1.68 (br, 4H), 1.96–2.08 (br, 2H), 3.02–3.16 (br, 2H), 4.04–4.07 (m, 1H), 7.35 (d, J = 9.00 Hz, 2H), 7.41–7.43 (m, 5H), 7.52 (d, J = 8.00 Hz, 1H), 7.59 (d, J = 9.00 Hz, 2H), 9.25 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 31.4 (2C), 41.0 (2C), 47.6, 121.0 (2C), 126.9 (2C), 128.6 (2C), 129.3 (2C), 129.9, 130.6, 134.8, 135.6, 157.2, 159.0, 170.5; HRMS (ESI), *m/z* calcd for $C_{20}H_{21}ClN_3O_3$ (MH^+) 386.1266, found 386.1276.

4.1.7. Amine (10)

To a stirred solution of 2,2,6,6-tetramethylpiperidin-4-one (7.75 g, 50.0 mmol) and cyclohexanone (15.5 mL, 150 mmol) in DMSO (71.0 mL) was added NH_4Cl (16.1 g, 300 mmol) and stirred at 60 °C for 5 h. The reaction mixture was diluted with H_2O

(150 mL), acidified with 7% aq HCl, and extracted with Et_2O (200 mL × 3). The water layer was adjusted to pH 9 using 10% aq K_2CO_3 and then back-extracted with EtOAc. The extract was washed with brine and dried over Na_2SO_4. After concentration under reduced pressure, the residue was dissolve in MeOH (60.0 mL) and benzylamine (10.9 mL, 100 mmol) was added. After being stirred at room temperature for 1 h, sodium cyanoborohydride was added and stirred at room temperature for 6 h. The reaction mixture was poured into saturated $NaHCO_3$ and extracted with EtOAc, then dried over $MgSO_4$. After concentration under reduced pressure, the residue was dissolve in MeOH (150 mL) and 10% Pd/C (5.32 g, 5.00 mmol) was added and stirred at room temperature for 24 h under hydrogen atmosphere. After the reaction mixture was filtered through celite, the filtrate solution was concentrated under reduced pressure followed by flash chromatography over silica gel with EtOAc–EtOH (4:1) to gave the title compound **10** (820 mg, 7 % yield) as a colorless oil.

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.730 (t, J = 12.0 Hz, 2H), 1.15–1.85 (m, 23H), 2.01–3.7 (m, 2H), 2.95–3.05 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 22.2 (2C), 22.8 (2C), 26.2 (2C), 37.3 (2C), 42.3 (2C), 43.6 (2C), 47.0, 53.2 (2C); HRMS (ESI), m/z calcd for $C_{15}H_{29}N_2$ (MH^+) 237.2325, found 237.2321.

4.1.8. N^1-(4-Chlorophenyl)-N^2-(2,6-dicyclohexylpiperidin-4-yl) oxalamide (11)

To a solution of **10** (722 mg, 3.05 mmol) in EtOH (15.0 mL) was added ethyl 2-((4-chlorophenyl)amino)-2-oxoacetate (363 mg, 1.50 mmol) and triethylamine (0.415 mL, 3.00 mmol) and stirred for 3 h at 150 °C under microwave irradiation. The mixture was filtered and the precipitate was collected and washed with cold EtOH, and dried under reduced pressure to provide the compound **11** (108 mg, 17% yield) as white powder.

^{1}H NMR (500 MHz, DMSO) δ 1.12–1.91 (br, 24H), 4.02–4.07 (m, 1H), 7.42 (d, J = 9.00 Hz, 2H), 7.84 (d, J = 9.00 Hz, 2H), 8.76 (br, 1H), 9.25 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 22.1 (2C), 22.7 (2C), 26.0 (2C), 37.2 (2C), 42.5 (2C), 42.9 (2C), 43.6, 52.7 (2C), 120.9 (2C), 129.3 (2C), 130.4, 135.0, 157.6, 158.8; HRMS (ESI), m/z calcd for $C_{23}H_{33}ClN_3O_2$ (MH^+) 418.2256, found 418.2261.

4.1.9. N^1-(4-Chlorophenyl)-N^2-(4-fluorophenyl)oxalamide (14)

To a solution of the ethyl 2-((4-chlorophenyl)amino)-2-oxoacetate (1.21 g, 5.00 mmol) in EtOH (25.0 mL) were added Et_3N (1.38 mL, 10.0 mmol) and 4-fluoroaniline **12** (1.44 mL, 15.0 mmol). The reaction mixture was stirred for 3 h at 150 °C under microwave irradiation. After being cooled to room temperature, the crystal was collected and washed with cold EtOH and n-hexane, and dried under reduced pressure to provide the compound **14** (601 mg, 41% yield) as colorless crystals. Compounds **15** and **16** were similarly synthesized.

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.07–7.14 (m, 2H), 7.35–7.40 (m, 2H), 7.59–7.63 (m, 4H), 9.29 (s, 1H), 9.33 (s, 1H); ^{13}C NMR (125 MHz, DMSO) δ 115.8 (d, J = 22.5 Hz, 2C), 122.5 (2C), 122.8 (d, J = 7.5 Hz, 2C), 128.8, 129.1 (2C), 134.4, 137.1, 158.3, 158.9 (d, J = 42.5 Hz), 160.2; HRMS (ESI), m/z calcd for $C_{14}H_{11}ClFN_2O_2$ (MH^+) 293.0488, found 293.0485.

4.1.10. N^1-(4-Chlorophenyl)-N^2-(2-(pyridin-2-yl)ethyl) oxalamide (17)

To a solution of the ethyl 2-((4-chlorophenyl)amino)-2-oxoacetate (726.3 mg, 3.00 mmol) in EtOH (10.0 mL) were added Et_3N (0.831 mL, 6.00 mmol) and 2-(pyridin-2-yl)ethanamine 14 (1.07 mL, 9.00 mmol). The reaction mixture was stirred for 3 h at 150 °C under microwave irradiation. After being cooled to room temperature, the crystal was collected and washed with cold EtOH and n-hexane, and dried under reduced pressure to provide the title compound **17** (336 mg, 37% yield) as colorless crystals.

^{1}H NMR (500 MHz, $CDCl_3$) δ 3.08 (t, J = 6.50 Hz, 2H), 3.82 (q, J = 6.50 Hz, 2H), 7.12–7.21 (m, 2H), 7.30–7.37 (m, 2H),7.54–7.66 (m 3H), 8.40 (s, 1H), 8.60 (d, J = 5.00 Hz, 1H), 9.26 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 36.5, 39.0, 121.0 (2C), 121.8, 123.4, 129.2 (2C), 130.3, 135.1, 136.7, 149.5, 157.5, 158.6, 159.6; HRMS (ESI), m/z calcd for $C_{15}H_{15}ClN_3O_2$ (MH^+) 304.0847, found 304.0850.

4.2. Molecular modeling

The structures of compounds **11** and **12** were built in Sybyl and minimized with the MMFF94 force field and partial charges.[17] Dockings were then performed using FlexSIS through its SYBYL module, into the crystal structure of gp120 (PDB: 1RZJ).

4.3. FACS analysis

JR-FL (R5, Sub B) chronically infected PM1 cells were pre-incubated with 100 μM of a CD4 mimic for 15 min, and then incubated with an anti-HIV-1 mAb, 4C11, at 4 °C for 15 min. The cells were washed with PBS, and fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG antibody was used for antibody-staining. Flow cytometry data for the binding of 4C11 (green lines, Fig. 4) to the Env-expressing cell surface in the presence of a CD4 mimic are shown among gated PM1 cells along with a control antibody (anti-human CD19: black lines, Fig. 4). Data are representative of the results from a minimum of two independent experiments. The number at the bottom of each graph in Figure 4 shows the mean fluorescence intensity (MFI) of the antibody 4C11.

Acknowledgments

This work was supported in part by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and Health and Labour Sciences Research Grants from Japanese Ministry of Health, Labor, and Welfare.

Supplementary data

Supplementary data (NMR charts of compounds) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.09.045.

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