Bioorganic & Medicinal Chemistry Letters 20 (2010) 5853–5858

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

# CD4 mimics targeting the HIV entry mechanism and their hybrid molecules with a CXCR4 antagonist

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ARTICLE INFO

*Article history:*
Received 14 June 2010
Revised 22 July 2010
Accepted 26 July 2010
Available online 3 August 2010

*Keywords:*
CD4
HIV entry
Hybrid molecule
gp120

ABSTRACT

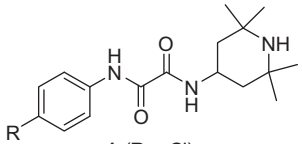
Small molecules behaving as CD4 mimics were previously reported as HIV-1 entry inhibitors that block the gp120–CD4 interaction and induce a conformational change in gp120, exposing its co-receptor-binding site. A structure–activity relationship (SAR) study of a series of CD4 mimic analogs was conducted to investigate the contribution from the piperidine moiety of CD4 mimic **1** to anti-HIV activity, cytotoxicity, and CD4 mimicry effects on conformational changes of gp120. In addition, several hybrid molecules based on conjugation of a CD4 mimic analog with a selective CXCR4 antagonist were also synthesized and their utility evaluated.



The infection of host cells by HIV-1 takes place in multiple steps via a dynamic supramolecular mechanism mediated by two viral envelope glycoproteins (gp41, gp120) and several cell surface proteins (CD4, CCR5/CXCR4).[1] Cell penetration begins with the interaction of gp120 with the primary receptor CD4. This induces conformational changes in gp120, leading to the exposure of its V3 loop allowing the subsequent binding of gp120 to a co-receptor, CCR5[2] or CXCR4.[3]

*N*-(4-Chlorophenyl)-*N*′-(2,2,6,6-tetramethyl-piperidin-4-yl)oxalamide (NBD-556: **1**) and the related compounds NBD-557 (**2**) and YYA-021 (**3**) have been identified as a novel class of HIV-1 entry inhibitors, which exert potent cell fusion and virus cell fusion inhibitory activity at low micromolar levels (Fig. 1).[4] Furthermore, compound **1** can also induce thermodynamically favored conformational changes in gp120 similar to those caused by CD4 binding. The X-ray crystal structure of gp120 complexed with CD4 revealed the presence of a hydrophobic cavity, the Phe43 cavity, which is penetrated by the aromatic ring of $Phe^{43}$ of CD4.[5] Molecular modeling revealed that compound **1** is also inserted into the Phe43 cavity, the *para*-chlorophenyl group of **1** entering more deeply than the phenyl ring of $Phe^{43}$ of CD4 and interacting with the conserved gp120 residues such as $Trp^{427}$, $Phe^{382}$, and $Trp^{112}$.[4c] The modeling also suggested that an oxalamide linker forms hydrogen bonds with carbonyl groups of the gp120 backbone peptide bonds. Our model of **1** docked into gp120 revealed that eight other gp120 residues, $Val^{255}$, $Asp^{368}$, $Glu^{370}$, $Ser^{375}$, $Ile^{424}$, $Trp^{427}$, $Val^{430}$, and $Val^{475}$ are located within a 4.4 Å-radius of **1** and that a large cavity exists around the *p*-position of the aromatic ring of **1**.[4e] Based on these observations, we conducted a structure–activity relationship (SAR) study of a series of analogs of CD4 mimics with substituents at the *p*-position of the aromatic ring. This study revealed that a certain size and electron-withdrawing ability of the substituents are indispensable for potent anti-HIV activity.[4e]

Although several reported SAR studies of **1** have revealed the contributions of the phenyl ring and the oxalamide linker of **1** to the binding affinity with gp120, the anti-HIV activity and the CD4 mimicry on conformational changes of gp120,[4] there has been, to the best of our knowledge, no prior report describing SAR studies of the piperidine ring of **1**. In this paper, the contributions of the piperidine ring of **1** to the anti-HIV activity, CD4 mimicry and cytotoxicity were investigated through the SAR studies focused on the piperidine ring of **1**. Furthermore, to apply the utility of CD4 mimics to the development of potent anti-HIV agents, a series of the




**1** (R = Cl)
**2** (R = Br)
**3** (R = Me)

**Figure 1.** NBD-556 (**1**) and related compounds.

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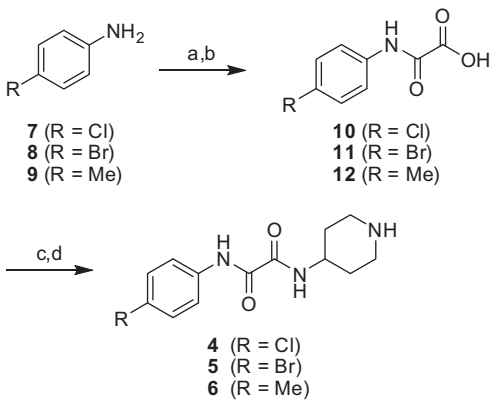
doi:10.1016/j.bmcl.2010.07.106

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-

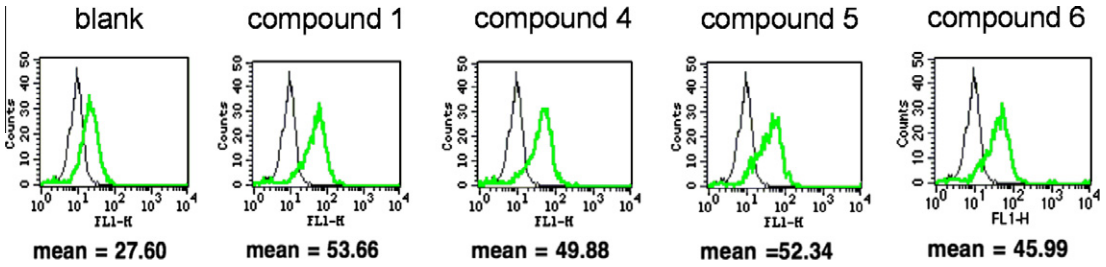
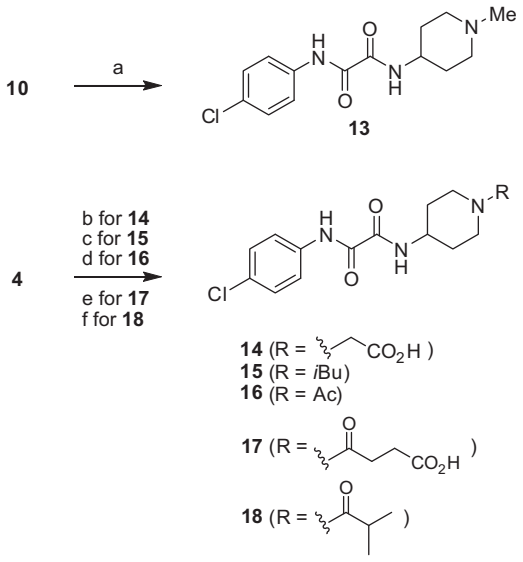
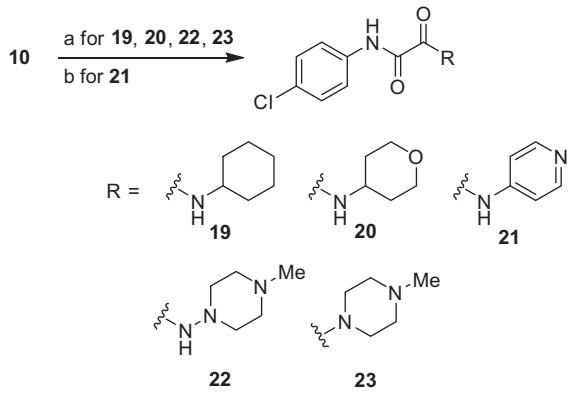



**Figure 2.** FACS analysis of compounds **1** and **4**–**6**. JR-FL (R5, Sub B) chronically infected PM1 cells were preincubated with 100 μM of a CD4 mimic for 15 min, and then incubated with an anti-HIV-1 mAb, 4C11, at 4 °C for 15 min. The cells were washed with PBS, and fluorescein isothiocyanate (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.

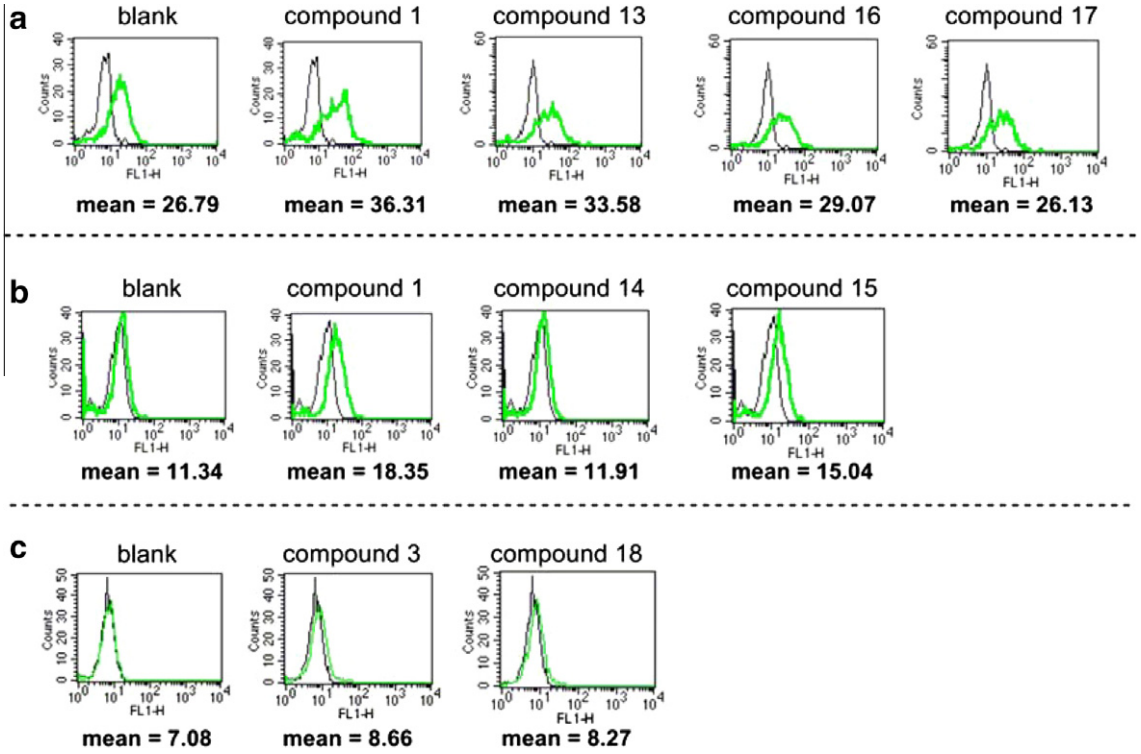



**Figure 3.** FACS analysis of compounds **1**, **3**, and **13–18**. The experimental procedures are described in Figure 2. The lanes of (a), (b) and (c) show independent experiments.

**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 |

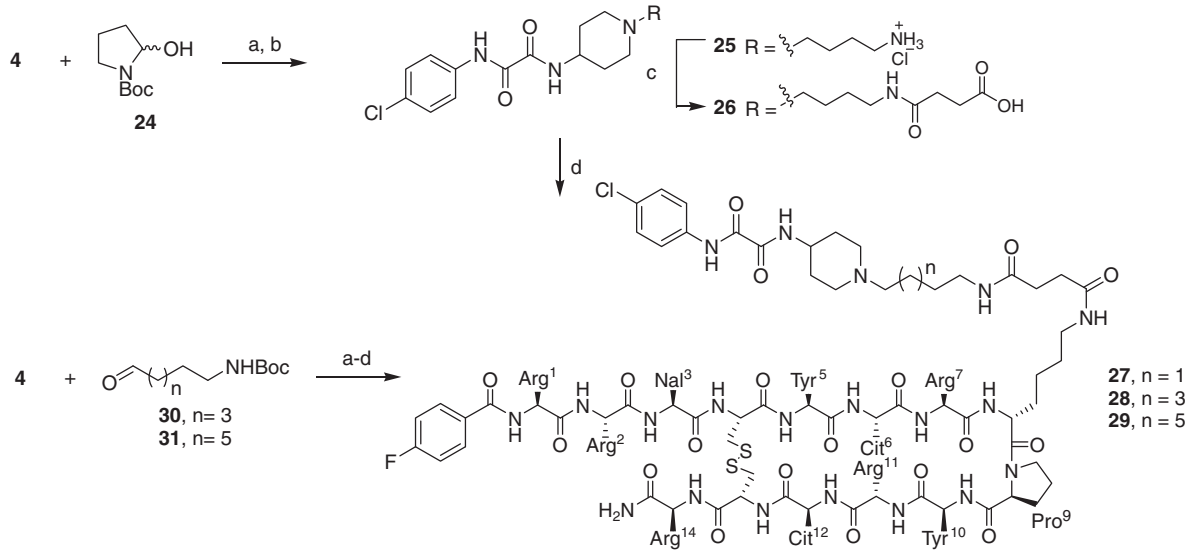
[a] All data with standard deviation are the mean values for at least three independent experiments.

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 $N^{\alpha}$-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), $HOBt{\cdot}H_2O$ (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). $\delta_H$(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), $HOBt{\cdot}H_2O$ (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.