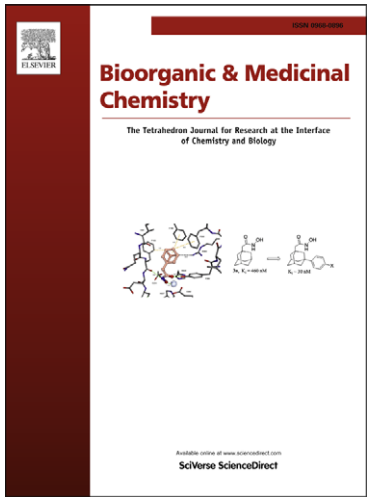

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CD4 mimics as HIV entry inhibitors: lead optimization studies of the aromatic substituents

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Abstract

Several CD4 mimics have been reported as HIV-1 entry inhibitors that can intervene in the interaction between a viral envelope glycoprotein gp120 and a cell surface protein CD4. Our previous SAR studies led to a finding of a highly potent analogue **3** with bulky hydrophobic groups on a piperidine moiety. In the present study, the aromatic ring of **3** was modified systematically in an attempt to improve its antiviral activity and CD4 mimicry which induces the conformational changes in gp120 that can render the envelope more sensitive to neutralizing antibodies. Biological assays of the synthetic compounds revealed that the introduction of a fluorine group as a *meta*-substituent of the aromatic ring caused an increase of anti-HIV activity and an enhancement of a CD4 mimicry, and led to a novel compound **13a** that showed twice as potent anti-HIV activity compared to **3** and a substantial increase in a CD4 mimicry even at lower concentrations.

Keywords

CD4 mimicry

conformational change in gp120

HIV entry inhibitor

envelope protein opener

1. Introduction

The first step of HIV entry into host cells is the interaction of a viral envelope glycoprotein gp120 with the cell surface protein CD4.[1] Such a viral attachment process is an attractive target for the development of the drugs to prevent the HIV-1 infection of its target cells.[2] Several small molecules including BMS-806,[3] IC-9564[4] and NBDs[5] have been identified that inhibit the viral attachment process by binding to gp120. Recently, we and others have been exploring the potentials of NBDs-derived CD4 mimics as a novel class of HIV entry inhibitors (Figure 1).[6-8]

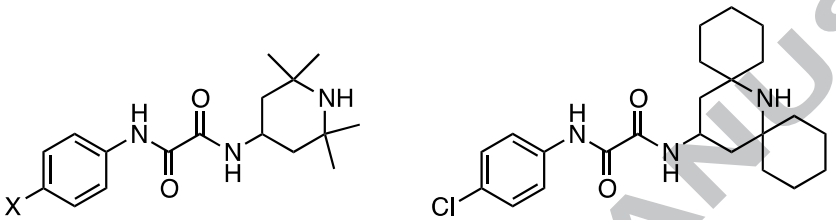


Figure 1. Structures of NBD-556 (**1**), YYA-021 (**2**) and HAR-171 (**3**)

Small molecular CD4 mimics identified by an HIV syncytium formation assay showed potent cell fusion and virus cell fusion inhibitory activity against several HIV-1 laboratory and primary isolates.[5] Furthermore, the interaction of CD4 mimics with a highly conserved and functionally important pocket on gp120, known as the "Phe43 cavity", induces conformational changes in gp120,[9] a process which occurs with unfavorable binding entropy, leading to a favorable enthalpy change similar to those caused by binding of the soluble CD4 binding to gp120. These unique properties render CD4 mimics valuable not only for the development of entry inhibitors, but which also, when combined with neutralizing antibodies function as envelope protein openers-putatively, stimulants.[10]

The structure of the complex formed by NBD-556 (**1**) bound to the gp120 core from an HIV-1 clade C strain (C1086) was recently determined by X-ray analysis (PDB: 3TGS).[11] As expected with molecular modeling by us[8a] and others,[6a] NBD-556 binds with Phe43 cavity with its *p*-chlorophenyl

ring inserted into the cavity, and in addition multiple contacts were observed, with Trp112, Val255, Phe382, Ile424, Asn425, Trp427, Gly473, and Val430 of gp120 were observed (Figure 2). However, no obvious interaction with Arg59 of CD4 was observed, although the salt bridge formation between Arg59 of CD4 and Asp368 of gp120 is a critical interaction of the viral attachment.[12] Based on this binding model, several potent compounds were recently identified.[6c,7]

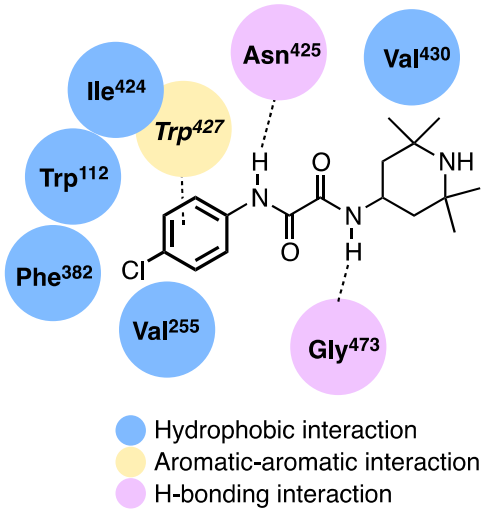


Figure 2. Major interactions between NBD-556 and Phe43 cavity of gp120

Prior to those studies, we performed structure-activity relationship (SAR) studies based on the modification of the piperidine moiety of CD4 mimics to interact with Val430 and/or Asp368. These resulted in the discovery of a potent compound **3** which has bulky hydrophobic groups on its piperidine ring, and shows significant anti-HIV activity and lower cytotoxicity than other known CD4 mimics.[8c] Our study of the docking of **3** into the Phe43 cavity of gp120 suggests that the cyclohexyl group of **3** can interact hydrophobically with the isopropyl group of Val430.

We hypothesized that the optimization of the aromatic ring of **3** would lead to an increase of antiviral activity and CD4 mimicry, the latter inducing the conformational changes in gp120. Here, we describe the systematic modification of the aromatic ring of **3** for further optimization to evaluate substituent effects on anti-HIV activity, cytotoxicity and CD4 mimicry.

2. Results and Discussion

The co-crystal structure of **1** with the gp120 core revealed that the aromatic group of **1** binds to gp120 by several aromatic-aromatic and hydrophobic interactions (Figure 2). In particular, hydrophobic space surrounded by the hydrophobic amino acid residues Trp112, Val255, Phe382, and Ile424 is likely to be affected by substituents at the *meta*- and *para*-positions of the aromatic ring, and consequently we decided to investigate substituents at these positions (Figure 3).

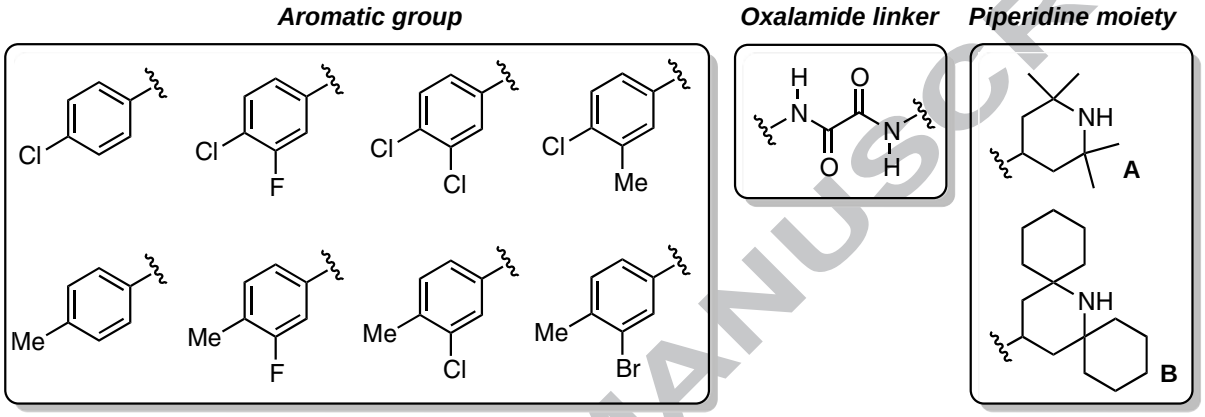


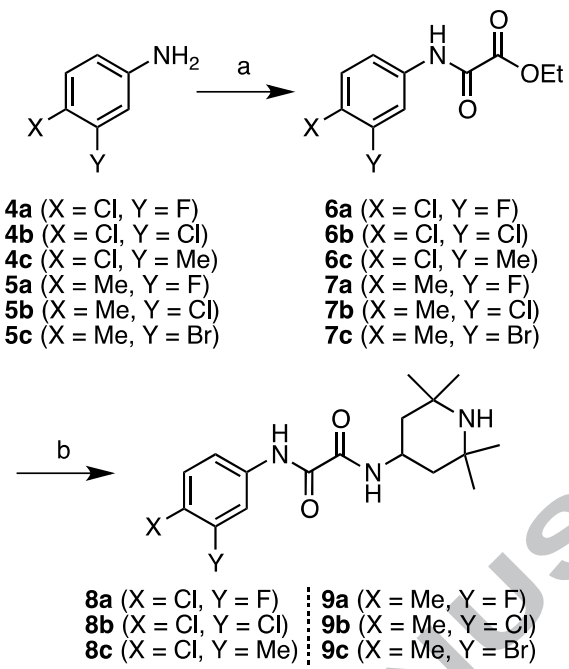
Figure 3. The structures of scaffolds in the design of novel CD4 mimics.

Initially, we selected a chlorine or a methyl group to serve as the *para*-substituent of the aromatic group because CD4 mimic compounds such as **1** (NBD-556) with a *p*-chloro substituent, and because **3** showed significant anti-HIV activity compared to other substituents. Further, CD4 mimic structures such as **2** with a *p*-methyl substituent also showed potent anti-HIV activity and exhibits lower cytotoxicity than those with the *p*-chlorophenyl derivatives.[8a] Next, we chose several halogens including F, Cl and Br, to be the *meta*-substituent on the aromatic group since previous SAR studies revealed that the introduction of an appropriate group with an electron-withdrawing ability at the *meta*-position leads to an increase of binding affinity and antiviral activity.[6a] Furthermore, to investigate whether electron withdrawal and hydrophobicity of the *meta*-position are appropriate, the CD4 mimics with a *meta*-methyl substituent, which has electron-donating properties and is similar in size to bromine, were also synthesized. Finally, two piperidine scaffolds (the

2,2,6,6-tetramethylpiperidine **A** and the dicyclohexylpiperidine **B**) were combined with these aromatics *via* the oxalamide linker.

2.1 Chemistry

The syntheses of novel compounds are depicted in Schemes 1 and 2. Starting from the appropriate aniline with *m*- and *p*-substituents, coupling with ethyl chloroglyoxylate in the presence of Et_3N gave the corresponding amidoesters **6a-c** and **7a-c**. Subsequently, microwave-assisted aminolysis[13] of **6a-c** and **7a-c** with commercially available 4-amino-2,2,6,6-tetramethylpiperidines afforded the desired compounds **8a-c** and **9a-c** (Scheme 1). A series of CD4 mimics with two cyclohexyl groups **13a-c** and **14a-c** were prepared from 2,2,6,6-tetramethylpiperidin-4-one **10** by the method previously reported,[8c] with slight modification (Scheme 2). Briefly, treatment of **10** with cyclohexanone in the presence of ammonium chloride gave a 2,6-substituted piperidin-4-one **11** *via* Grob fragmentation followed by intramolecular cyclization.[14] Reductive amination with *p*-methoxybenzyl amine, acidic treatment with TMSBr/TFA, and oxidative cleavage of *p*-methoxybenzyl group with cerium(IV) ammonium nitrates (CAN) furnished the corresponding 4-aminopiperidines **(12**) with higher yields and less burdensome purifications than the previous method. Finally, coupling of **12** with the corresponding esters **6a-c** and **7a-c** under microwave irradiation provided the desired compounds **13a-c** and **14a-c**.



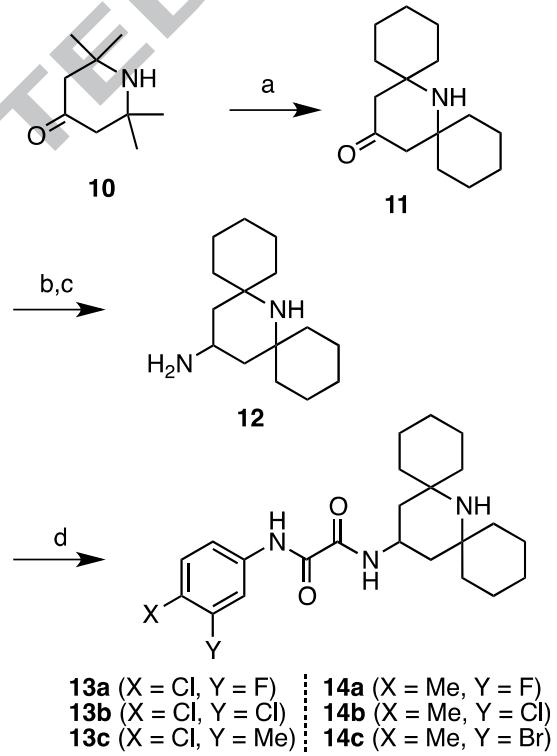
4a (X = Cl, Y = F)
4b (X = Cl, Y = Cl)
4c (X = Cl, Y = Me)
5a (X = Me, Y = F)
5b (X = Me, Y = Cl)
5c (X = Me, Y = Br)

6a (X = Cl, Y = F)
6b (X = Cl, Y = Cl)
6c (X = Cl, Y = Me)
7a (X = Me, Y = F)
7b (X = Me, Y = Cl)
7c (X = Me, Y = Br)

8a (X = Cl, Y = F)
8b (X = Cl, Y = Cl)
8c (X = Cl, Y = Me)

9a (X = Me, Y = F)
9b (X = Me, Y = Cl)
9c (X = Me, Y = Br)

Scheme 1. *Reagents and conditions*: (a) ethyl chloroglyoxylate, Et_3N, THF; (b) 4-amino-2,2,6,6-tetramethylpiperidine, Et_3N, EtOH, 150 °C, microwave.



10

11

12

13a (X = Cl, Y = F)
13b (X = Cl, Y = Cl)
13c (X = Cl, Y = Me)

14a (X = Me, Y = F)
14b (X = Me, Y = Cl)
14c (X = Me, Y = Br)

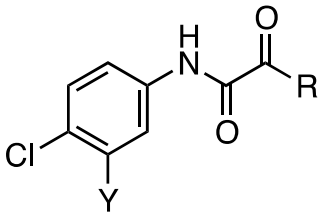
Scheme 2. *Reagents and conditions*: (a) cyclohexanone, NH_4Cl, DMSO, 60 °C; (b) *p*-methoxybenzylamine, $NaBH_3CN$, MeOH, then 1 M TMSBr in TFA; (c) CAN, CH_3CN/H_2O (v:v =2:1); (d) **6** or **7**, Et_3N, EtOH, 150 °C, microwave.

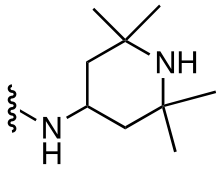
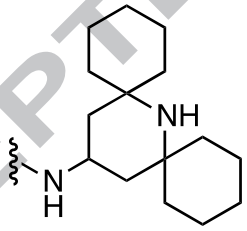
2.2 Biological evaluation

The anti-HIV activity of the synthetic compounds was evaluated against an R5 primary isolate YTA strain. IC_{50} values were determined by the WST-8 method as the concentrations of the compounds that 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 WST-8 method. The assay results for compounds **8a-c** and **13a-c** with a *p*-chlorophenyl group are shown in Table 1. The parent compound **1** and compound **8a**,[6a] known as JRC-II-191, showed significant anti-HIV activities (IC_{50} of **1** = 0.61 M and IC_{50} of **8a** = 0.32 M). Compound **8b**[6a] having a *m,p*-dichlorophenyl group and compound **8c**[6a] (JRC-II-193) having a *p*-chloro-*m*-tolyl group showed moderate anti-HIV activity (IC_{50} of **8b** = 4.1 M and IC_{50} of **8c** = 3.3 M) but their potency was approximately 10-fold lower than that of compound **8a**. The cytotoxicity of **8b** and **8c** is relatively stronger than that of **8a** (CC_{50} of **8b** = 36 M and CC_{50} of **8c** = 38 M). Compounds **13a-c** with hydrophobic cyclohexyl groups in the piperidine moiety showed more potent anti-HIV activity than the corresponding compounds **8a-c**, confirming the contribution of the bulky hydrophobic group(s) to an increase of antiviral activity. Our lead compound **3** showed significant anti-HIV activity comparable to that of compound **8a** (IC_{50} = 0.43 M) but, consistent with previous results, exhibited lower cytotoxicity. In particular, compound **13a** with a *m*-fluoro-*p*-chlorophenyl group exhibited the highest anti-HIV activity. The IC_{50} value of **13a** was 0.23 M, whose potency was approximately twice as high as that of compound **3**. Notably, compound **13b** with a *m,p*-dichlorophenyl group showed 7-fold more potent anti-HIV activity than the corresponding compound **8b**. Compound **13c**, which has a *p*-chloro-*m*-tolyl group, showed potent anti-HIV activity comparable to that of the corresponding compound **8c** and an increase of cytotoxicity (CC_{50} = 15 M). We observed a tendency for compounds **13a-c** with both hydrophobic cyclohexyl groups and a *m,p*-disubstituted phenyl group to exhibit higher cytotoxicity than the corresponding tetramethyl-type compounds **8a-c**. No clear reason for an increase of cytotoxicity in

the *m*,*p*-disubstituted phenyl group-containing compounds is apparent.

Table 1. Anti-HIV activity and cytotoxicity of compounds **8a-c** and **13a-c** containing a *p*-chlorophenyl group[a]



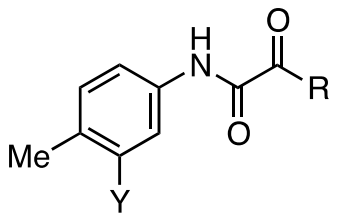
Compd	R	Y	IC_{50} (M)[b]	CC_{50} (M)[c]
			YTA48P	
1	 **A**	H	0.61	110
8a	**A**	F	0.32	94
8b	**A**	Cl	4.1	36
8c	**A**	Me	3.3	38
3	 **B**	H	0.43	120
13a	**B**	F	0.23	11
13b	**B**	Cl	0.62	11
13c	**B**	Me	2.6	15

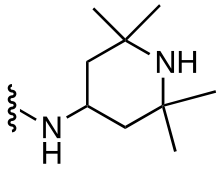
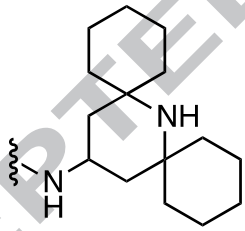
[a]All data are the mean values from three of more independent experiments.
[b]IC_{50} values of the multi-round assay are based on the inhibition of HIV-1-induced cytopathogenicity in PM1/CCR5 cells.
[c]CC_{50} values are based on the reduction of the viability of mock-infected PM1/CCR5 cells.

Assay results for the compounds **9a-c** and **14a-c** with a *p*-tolyl group are shown in Table 2. As

expected, replacement of the *p*-chloro substituent with a *p*-methyl group resulted in somewhat reduction of anti-HIV activity. Compound **2**, YYA-021 has significant anti-HIV activity (IC_{50} = 9.0 M) and exhibits the lowest cytotoxicity among all of the compounds tested (CC_{50} = 260 M). These results are consistent with our previous SAR studies involving the aromatic ring. Introduction of a fluorine at the *meta*-position of the *p*-tolyl group, e.g. in compound **9a** and **14a**, improved the antiviral activity, as observed with **8a** and **13a** and a similar tendency was observed for compound **9b** with a *m*-chloro-*p*-tolyl group. In particular, compound **14a** with cyclohexyl groups and a *m*-fluoro-*p*-tolyl group showed slightly higher anti-HIV activity than the parent compound **1**. Among the compounds with *m*-bromo-*p*-tolyl groups, it was found that compound **9c**, with a 2,2,6,6-tetramethylpiperidine group, showed no anti-HIV activity at a concentration below 10 M, whereas compound **14c** with hydrophobic cyclohexyl groups attached to the piperidine moiety, showed moderate activity (IC_{50} = 3.2 M), indicating that the hydrophobic modification of piperidine ring can contribute to an increase in anti-HIV activity.

Table 2. Anti-HIV activity and cytotoxicity of compounds **9a-c** and **14a-c** containing a *p*-tolyl group[a]



Compd	R	Y	IC_{50} (M)[b]	CC_{50} (M)[c]
			YTA48P	
2	 **A**	H	9.0	260
9a	**A**	F	2.8	110
9b	**A**	Cl	3.2	62
9c	**A**	Br	>10	32
14a	 **B**	F	0.54	91
14b	**B**	Cl	6.2	11
14c	**B**	Br	3.2	11

[a]All data are the mean values from three of more independent experiments.
[b]IC_{50} values of the multi-round assay are based on the inhibition of HIV-1-induced cytopathogenicity in PM1/CCR5 cells.
[c]CC_{50} values are based on the reduction of the viability of mock-infected PM1/CCR5 cells.

All the synthetic compounds were evaluated for their CD4 mimicry on the conformational changes in gp120 by fluorescence activated cell sorting (FACS) analysis, and the results are shown in Figure 4. The profile of binding of a CD4-induced (CD4i) monoclonal antibody (4C11) to the

Env-expressing cell surface pretreated with the synthetic compounds was assessed in terms of the mean fluorescence intensity (MFI). The increase in binding affinity for 4C11 (by the pretreatment with synthetic compounds) suggests that those compounds can reflect the CD4 mimicry as a consequence of the conformational changes in gp120. Our previous studies disclosed that the profiles of the binding to the cell surface pretreated with **1**, **2**, or **3** were similar to those observed in pretreatment with soluble CD4, indicating that these compounds offer a significant enhancement of binding affinity for 4C11.[8] As shown in Figure 4, similar results were obtained with those compounds in this FACS analysis (MFI of **1**, **2**, and **3** = 34.94, 25.97, and 24.17, respectively). A notable increase in binding affinity for 4C11 was observed in essentially all the synthetic compounds. The compounds **8a**, **9a**, **13a** and **14a** with a *meta*-fluorine in the aromatic ring, showed significant anti-HIV activity, and produced a substantial increase in binding affinity for 4C11. These results suggested that the introduction of a fluorine group at the meta position of the aromatic ring is significant not only for the increase of anti-HIV activity, but also for the enhancement of a CD4 mimicry. In particular, a remarkable improvement in binding affinity for 4C11 was observed with **13a** (MFI = 51.39) which has 2-fold more potent anti-HIV activity than the lead compound **3** (HAR-171), and is the most active compound in terms of both anti-HIV activity and the CD4 mimicry resulting from the conformational change in gp120. The profiles of pretreatment of the cell surface with compounds **8b** and **13b** having a *m,p*-dichlorophenyl group, compounds **8c** and **13c** having a *p*-chloro-*m*-tolyl group, and compounds **9b** and **14b** with a *m*-chloro-*p*-tolyl group were similar to results obtained for **3**, suggesting that these compounds produced slightly lower enhancement compared to those of compounds **8a**, **9a**, **13a** and **14a** but significant levels of binding affinity for 4C11. On the other hand, pretreatment with compounds **9c**, which failed to show significant anti-HIV activity and **14c**, which had moderate anti-HIV activity resulted in a slight decrease of binding affinity for 4C11, suggesting that the introduction of a Br group at the *meta*-position of *p*-tolyl group is not advantageous to a CD4 mimicry, possibly due to the steric

hindrance caused by the two bulky substituents. These results are consistent with previous observations that a limited size and electron-withdrawing ability of the aromatic substituents are required for potent anti-HIV activity and CD4 mimicry.[8a]

Since **13a** showed higher CD4 mimicry than the other compounds tested, the effect of the solution concentration of **13a** on the binding affinity for 4C11 was investigated. As shown in Figure 5, pretreatment of the cell surface with a 100 M solution of **13a** produced a higher increase in the binding affinity for 4C11 than pretreatment with the same concentration of compound **3**. Interestingly, the profile pretreated with a 50 M solution of **13a** was similar to that with a 100 μM of compound **3**, and even with a 25 μM solution of **13a** a potent enhancement of the binding affinity for 4C11 was observed: MFI of **13a** at concentrations of 50 M and 25μM = 19.07 and 16.06 respectively. This observation suggests that **13a** could serve as a novel lead compound for the development of envelope protein openers for the use combined with neutralizing antibodies because of its effectiveness at low concentrations.

The substantial increase in the CD4 mimicry of **13a** even at a low concentration is not easily explained because HAR-171 (**3**) and **13a** would be expected to form the similar binding modes with gp120. A probable contribution of **13a** is suggested by modeling studies docked into the Phe43 cavity in gp120 (3TGS) in which the depth and direction of the aromatic ring of **13a** is slightly different from those in compound **3** (Figure 6), leading to the possible formation of appropriate interactions with the hydrophobic amino acid residues such as Val255 and Phe382, and therefore explaining the increased potency observed in the anti-HIV activity and CD4 mimicry of **13a**.

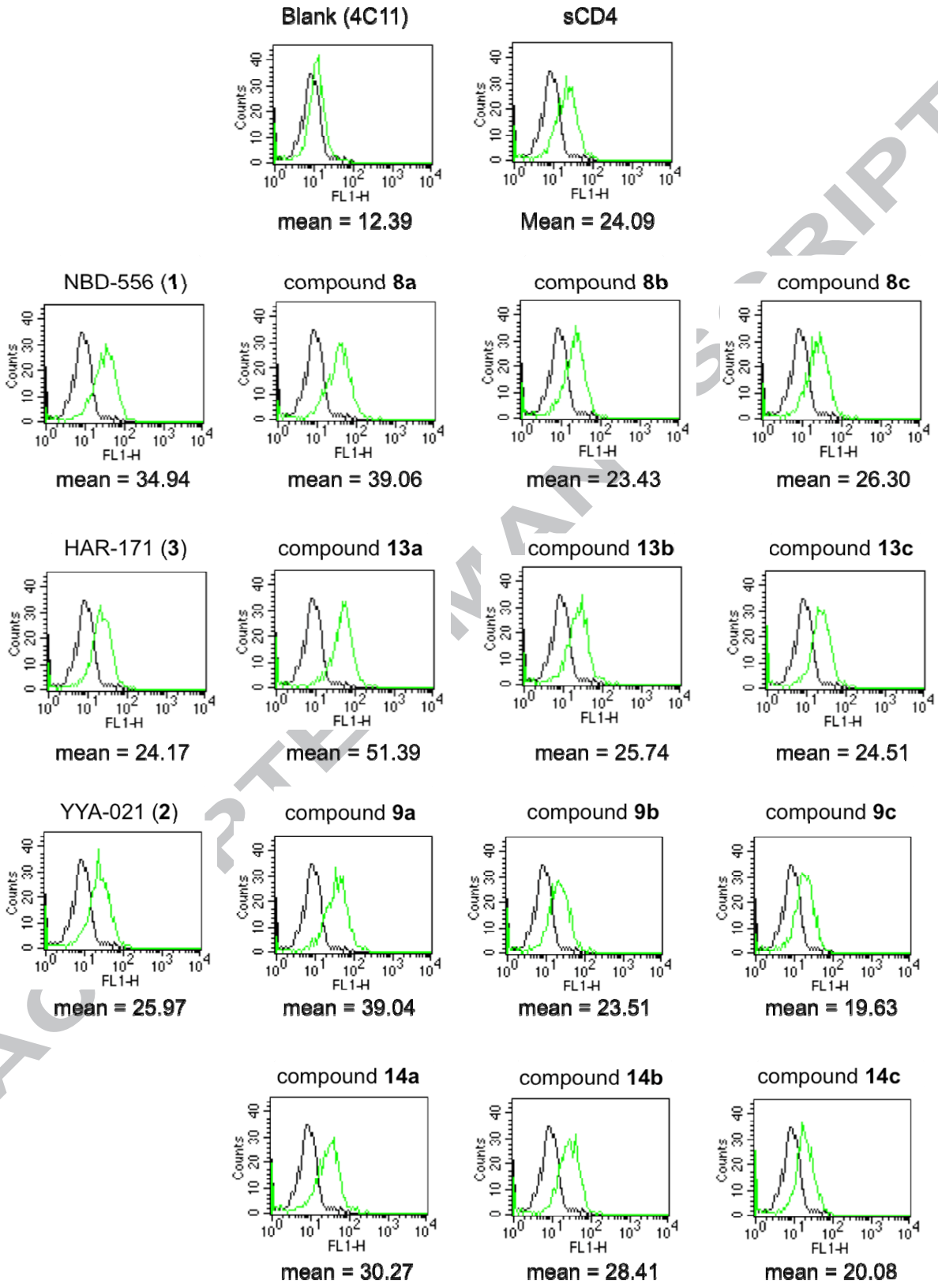


Figure 4. FACS analysis of synthetic compounds **8**,**9**,**13** and **14**.

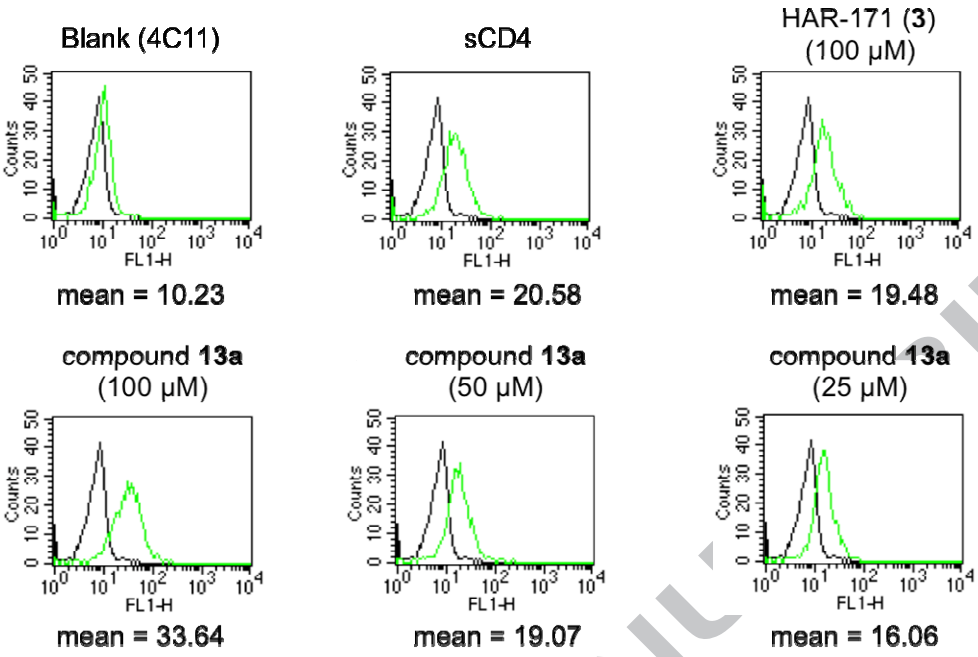


Figure 5. FACS analysis of **3** and **13a** in different concentrations.

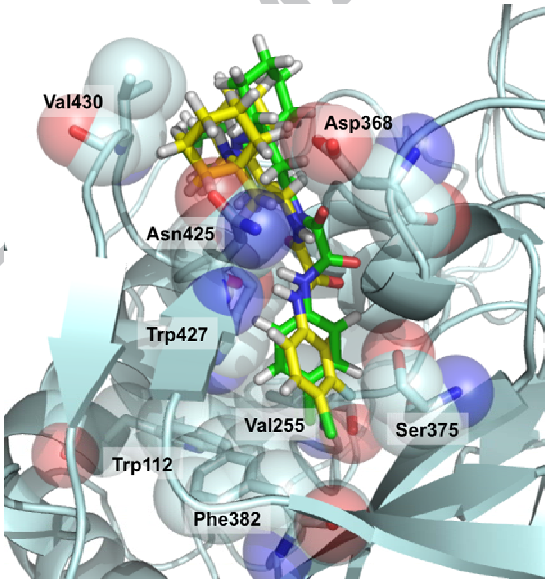


Figure 6. The modeled structure of **13a** (yellow carbon atoms) in the complex with the Phe43 cavity in gp120 (3TGS) overlaid with the modeled structure of **3** (green carbon atoms).

3. Conclusion

CD4 mimics are attractive agents not only for the development of a novel class of HIV entry inhibitors but also as possible cooperating agents for the neutralizing antibodies – i.e. envelope protein openers. In the present study, a structure-activity relationship study of a series of CD4 mimic

compounds was performed with a view to improving the biological activity of HAR-171 (**3**), which was identified in our previous studies as a promising lead compound with anti-HIV activity, cytotoxicity and CD4 mimicry resulting from the conformational change in gp120. Systematic modification of the *meta*- and *para*- substituents of the aromatic ring of **3** led to some potent compounds. In particular, **13a**, which has a bulky hydrophobic group on its piperidine ring and a *m*-fluoro-*p*-chlorophenyl group, demonstrated 2-fold more potent anti-HIV activity and much higher CD4 mimicry than **2** following the conformational changes in gp120, although the cytotoxicity of **13a** is relatively high. Further structural modification studies of the aromatic ring and the oxalamide linker to improve pharmaceutical profiles will be the subject of future reports.

4. Experimentals

^{1}H-NMR and ^{13}C-NMR spectra were recorded using a Bruker Avance III spectrometer. Chemical shifts are reported in (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, silica gel 60 N (Kanto Chemical Co., Inc.) was employed. Microwave reactions were performed in Biotage Microwave Reaction Kit (sealed vials) in an Initiator^{TM} (Biotage). The wattage was automatically adjusted to maintain the desired temperature for the desired period of time.

4.1. Chemistry

Ethyl 2-((4-chloro-3-fluorophenyl)amino)-2-oxoacetate (6a): To a stirred solution of 3-fluoroaniline (1.11 g, 10.0 mmol) in $CHCl_3$ (30.0 mL) was added dropwise N-chlorosuccinimide (NCS) in $CHCl_3$ (20.0 mL) at 0 °C. The mixture was stirred at 0 °C for 42 h. After the reaction mixture was concentrated under reduced pressure, the residue was dissolved in Et_2O. The mixture was washed with water, and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc/n-hexane gave 4-chloro-3-fluoroaniline (259.4 g, 18% yield) as crystalline solids. To a stirred solution of the above aniline (259.4 mg, 1.78 mmol) in

THF (8.9 mL) were added at 0 °C ethyl chloroglyoxylate (237.3 μL, 2.14 mmol) and Et_3N (296.6 μL, 2.14 mmol). The mixture was stirred at room temperature for 12 h. After the precipitate was filtrated off, the filtrate solution was concentrated under reduced pressure. The residue was dissolved in EtOAc, and washed with 1.0 M HCl, saturated $NaHCO_3$ and brine, then dried over $MgSO_4$. Concentration under reduced pressure to provide the title compound **6a** (435.2 mg, 99% yield) as brown crystals, which was used without further purification.

^{1}H NMR (500 MHz, $CDCl_3$) 1.44 (t, *J* = 7.50 Hz, 3H), 4.43 (q, *J* = 7.50 Hz, 2H), 7.24-7.25 (m, 1H), 7.35-7.40 (m, 1H), 7.70-7.75 (m, 1H), 8.93 (br, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) 13.0, 64.1, 108.5 (d, *J* = 26.3 Hz), 115.9 (d, *J* = 3.75 Hz), 117.3 (d, *J* = 18.8 Hz), 130.9 (d, *J* = 10.0 Hz), 135.9, 153.9, 158.1 (d, *J* = 246.3 Hz), 160.5; HRMS (ESI), m/z calcd for $C_{10}H_{10}ClFNO_3$ (MH^-) 244.0182, found 244.0183.

Ethyl 2-((3,4-dichlorophenyl)amino)-2-oxoacetate (6b): To a stirred solution of 3,4-dichloroaniline **4b** (1.94 g, 12.0 mmol) in THF (20.0 mL) were added ethyl chloroglyoxylate (1.11 mL, 10.0 mmol) and Et_3N (15.2 mL, 11.0 mmol) at 0 °C. The mixture was stirred at room temperature for 6 h. After the precipitate was filtrated off, the filtrate solution was concentrated under reduced pressure. The residue was dissolved in EtOAc, and washed with 1.0 M HCl, saturated $NaHCO_3$ and brine, then dried over $MgSO_4$. Concentration under reduced pressure to provide the title compound **6b** (1.58 g, 95% yield) as white powder, which was used without further purification.

^{1}H NMR (500 MHz, $CDCl_3$) 1.44 (t, *J* = 7.00 Hz, 3H), 4.43 (q, *J* = 7.00 Hz, 2H), 7.44 (d, *J* = 8.50 Hz, 1H), 7.49-7.51 (m, 1H), 7.87 2.35 (d, *J* = 2.50 Hz, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) 14.0, 64.0, 119.0, 121.5, 129.0, 130.8, 133.2, 135.7, 153.9, 160.5; HRMS (ESI), m/z calcd for $C_{10}H_{10}Cl_2NO_3$ (MH^+) 262.0038, found 262.0031.

Ethyl 2-((4-chloro-3-methylphenyl)amino)-2-oxoacetate (6c): By use of a procedure similar to that described for the preparation of compound **6b**, the aniline **4c** (3.34 g, 24.0 mmol) was converted into the title compound **6c** (4.63 g, 96% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) 1.43 (t, *J* = 7.00 Hz, 3H), 2.38 (s, 3H), 4.42 (q, *J* = 7.00 Hz, 2H), 7.33 (d, *J* = 8.50 Hz, 1H), 7.43-7.46 (m, 1H), 7.51-7.54 (m, 1H), 8.82 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) ; HRMS (ESI), m/z calcd for $C_{11}H_{13}ClNO_3$ (MH^+) 242.0578, found 242.0568.

Ethyl 2-((3-fluoro-4-methylphenyl)amino)-2-oxoacetate (7a): By use of a procedure similar to that described for the preparation of compound **6b**, the aniline **5a** (3.00 g, 24.0 mmol) was converted into the title compound **7a** (4.24 g, 94% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) 1.43 (t, *J* = 7.20 Hz, 3H), 2.25 (s, 3H), 4.42 (q, *J* = 6.80 Hz, 2H), 7.12-7.21 (m, 2H), 7.48-7.56 (m, 1H), 8.83 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) 14.2 (2C), 63.8, 107.1 (d, *J* = 27.5 Hz), 115.0 (d, *J* = 10.0 Hz), 122.3 (d, *J* = 17.5 Hz), 131.6 (d, *J* = 6.25 Hz), 135.3

(d, *J* = 13.8 Hz), 153.8, 160.8, 161.1 (d, *J* = 243.8 Hz); HRMS (ESI), m/z calcd for $C_{11}H_{13}FNO_3$ (MH^+) 226.0879, found 226.0878.

Ethyl 2-((3-chloro-4-methylphenyl)amino)-2-oxoacetate (7b): By use of a procedure similar to that described for the preparation of compound **6b**, the aniline **5b** (3.40 g, 24.0 mmol) was converted into the title compound **7b** (5.19 g, 94% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) 1.43 (t, *J* = 7.00 Hz, 3H), 2.35 (s, 3H), 4.42 (q, *J* = 7.00 Hz, 2H), 7.22 (d, *J* = 8.50 Hz, 1H), 7.41-7.43 (m, 1H), 7.71 (d, *J* = 2.00 Hz, 1H), 8.83 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) ; HRMS (ESI), m/z calcd for $C_{11}H_{13}ClNO_3$ (MH^+) 242.0584, found 242.0573.

Ethyl 2-((3-bromo-4-methylphenyl)amino)-2-oxoacetate (7c): By use of a procedure similar to that described for the preparation of compound **6b**, the aniline **5c** (4.47 g, 27.0 mmol) was converted into the title compound **7c** (6.24 g, 96% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) 1.43 (t, *J* = 7.00 Hz, 3H), 2.38 (s, 3H), 4.42 (q, *J* = 7.00 Hz, 2H), 7.23 (t, *J* = 8.50 Hz, 1H), 7.48-7.53 (m, 1H), 7.83-7.90 (m, 1H), 8.80 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) 14.0, 22.4, 63.9, 118.7, 123.4, 125.0, 131.0, 135.0, 135.2, 153.7, 160.8; HRMS (ESI), m/z calcd for $C_{11}H_{13}BrNO_3$ (MH^+) 286.0079, found 286.0068.

***N*^{1}-(4-chloro-3-fluorophenyl)-*N*^{2}-(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (8a):** To a solution of compound **6a** (70.0 mg, 0.286) in EtOH (2.9 mL) were added Et_3N (0.200 mL, 1.45 mmol) and 2,2,6,6-tetramethylpiperidin-4-amine (0.150 mL, 0.870 mmol). The reaction mixture was stirred for 3 h at 150 °C under microwave irradiation. After being concentrated in vacuo, the residue was extracted with $CHCl_3$, and washed with saturated $NaHCO_3$ and brine, then dried over $MgSO_4$. Concentration under reduced pressure to provide the title compound **8a** (34.6 mg, 34% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) 0.99-1.50 (m, 15H), 1.92 (dd, *J* = 3.50, 9.00 Hz, 2H), 4.20-4.32 (m, 1H), 7.21-7.25 (m, 1H), 7.34-7.41 (m, 1H), 7.69-7.73 (m 1H), 9.31 (br, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) 28.4, 34.8, 43.8, 44.5, 51.0, 108.3 (d, *J* = 26.3 Hz), 115.8 (d, *J* = 3.75 Hz), 117.1 (d, *J* = 17.5 Hz), 130.8, 136.2 (d, *J* = 8.75 Hz), 157.6, 158.1 (d, *J* = 247.5 Hz), 158.4; HRMS (ESI), m/z calcd for $C_{17}H_{24}ClFN_3O_2$ (MH^+) 356,1536, found 356.1548.

***N*^{1}-(3,4-dichlorophenyl)-*N*^{2}-(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (8b):** By use of a procedure similar to that described for the preparation of compound **8a**, the compound **6b** (261.0 mg, 1.00 mmol) was converted into the title compound **8b** (520.0 mg, 70% yield) as white powder.

^{1}H NMR (500 MHz, $CDCl_3$) 1.07 (t, *J* = 12.0 Hz, 2H), 1.16 (s, 6H), 1.28 (s, 6H), 1.90-1.93 (m, 2H), 4.20-4.32 (m, 1H), 7.26 (m, 1H), 7.40-7.48 (m, 2H), 7.88 (s, 1H), 9.33 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) 28.5 (2C), 34.9 (2C), 43.8, 44.6 (2C), 50.9 (2C), 119.0, 121.4, 128.7, 130.8, 133.1, 135.8,

157.7, 158.5; HRMS (ESI), m/z calcd for $C_{17}H_{22}Cl_2N_3O_2$ (MH^-) 370.1095, found 370.1105.

***N*^{1}-(4-chloro-3-methylphenyl)-*N*^{2}-(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (8c):** By use of a procedure similar to that described for the preparation of compound **8a**, the compound **6c** (482.0 mg, 2.00 mmol) was converted into the title compound **8c** (364.0 mg, 49% yield) as white powder. ^{1}H NMR (500 MHz, $CDCl_3$) 1.07 (t, *J* = 12.0 Hz, 2H), 1.15 (s, 6H), 1.28 (s, 6H), 1.86-1.94 (m, 2H), 4.15-4.31 (m, 1H), 7.21-7.24 (m, 1H), 7.32-7.38 (m, 2H), 7.74 (s, 1H), 9.24 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) 19.6, 28.5 (2C), 34.9 (2C), 43.7, 44.7 (2C), 50.9 (2C), 117.9, 120.2, 131.2, 133.1, 134.7, 135.1, 157.5, 158.8; HRMS (ESI), m/z calcd for $C_{18}H_{25}ClN_3O_2$ (MH^-) 350.1641, found 350.1656.

***N*^{1}-(3-fluoro-4-methylphenyl)-*N*^{2}-(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (9a):** By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7a** (225.0 mg, 1.00 mmol) was converted into the title compound **9a** (161.0 mg, 48% yield) as white powder. ^{1}H NMR (500 MHz, $CDCl_3$) 1.07 (t, *J* = 12.5 Hz, 2H), 1.15 (s, 6H), 1.28 (s, 6H), 1.92 (dd, *J* = 12.5, 3.50 Hz, 2H), 2.26 (s, 3H), 4.12-4.32 (m, 1H), 7.12-7.20 (m, 2H), 7.30-7.37 (m, 1H), 7.48-7.54 (m, 1H), 9.27 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) 14.2, 28.5 (2C), 34.9 (2C), 43.7, 44.7 (2C), 50.9 (2C), 107.1 (d, *J* = 26.3 Hz), 115.0, 121.8, (d, *J* = 17.5 Hz) 131.6, 135.4(d, *J* = 15.0 Hz), 157.5, 158.8, 161.1 (d, *J* = 242.5 Hz); HRMS (ESI), m/z calcd for $C_{18}H_{25}FN_3O_2$ (MH^-) 334.1936, found 334.1942.

***N*^{1}-(3-chloro-4-methylphenyl)-*N*^{2}-(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (9b):** By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7b** (482.0 mg, 1.00 mmol) was converted into the title compound **9b** (448.0 mg, 48% yield) as white powder. ^{1}H NMR (500 MHz, $CDCl_3$) 1.09 (t, *J* = 12.5 Hz, 3H), 1.18 (s, 6H), 1.30 (s, 6H), 1.93-1.95 (m, 2H), 2.41 (s, 3H), 4.20-4.34 (m, 1H), 7.30-7.37 (m, 2H), 7.44-7.46 (m, 1H), 7.53 (d, *J* = 2.50 Hz, 1H), 9.25 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) 20.3, 28.5 (2C), 34.9 (2C), 43.7, 44.7 (2C), 50.9 (2C), 118.5, 122.0, 130.0, 130.7, 134.8, 137.1, 157.5, 158.8; HRMS (ESI), m/z calcd for $C_{18}H_{25}ClN_3O_2$ (MH^-) 350.1641, found 350.1636.

***N*^{1}-(3-bromo-4-methylphenyl)-*N*^{2}-(2,2,6,6-tetramethylpiperidin-4-yl)oxalamide (9c):** By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7c** (285.0 mg, 1.00 mmol) was converted into the title compound **9c** (157.0 mg, 40% yield) as white powder. ^{1}H NMR (500 MHz, $CDCl_3$) 1.07 (t, *J* = 12.5 Hz, 3H), 1.15 (s, 6H), 1.28 (s, 6H), 1.91 (dd, *J* = 8.00, 4.00 Hz, 2H), 2.38 (s, 3H), 3.70-3.75 (m, 1H), 7.22 (d, *J* = 8.50 Hz, 1H), 7.30-7.37 (m, 1H), 7.43-7.45 (m, 1H), 7.90 (d, *J* = 2.50 Hz, 1H), 9.25 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) 22.4, 28.5 (2C), 34.9 (2C), 43.7, 44.7 (2C), 50.9 (2C), 118.6, 123.4, 125.0, 131.0, 134.9 (2C), 157.5, 158.8; HRMS (ESI), m/z calcd for $C_{18}H_{25}BrN_3O_2$ (M^-) 394.1136, found 394.1158.

Amine (12): the compound **11** was prepared according to the reported procedure.[14] To a stirred solution of piridone **11** (247.8 mg, 1.05 mmol) in MeOH (2.10 mL) was added *p*-methoxybenzylamine (0.41 mL, 3.15 mmol). After being stirred at room temperature for 23 h, sodium cyanoborohydride was added and stirred at room temperature for 48 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 treated with 1 M TMS in THF (4.8 mL). The mixture was stirred at 0℃ for 14 h. Concentration under reduced pressure followed by short chromatography with $CHCl_3$/MeOH gave the PMB-protected amine. To a solution of the above amine (584.0 mg, 1.64 mmol) in CH_3CN/H_2O (13.1 mL, v:v = 2:1) was added CAN (2.74 g, 8.2 mmol). The mixture was stirred at room temperature for 14 h. The reaction mixture was diluted with 0.5 M HCl and washed with CH_2Cl_2. The water layer was alkalized and extracted with EtOAc, then dried over Na_2SO_4. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc-EtOH (4:1) to gave the title compound **12** (175.5 mg, 71% yield) as a yellow oil.

^{1}H NMR (500 MHz, $CDCl_3$) 1.15-1.85 (m, 24H), 2.95-3.05 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) 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.

***N*^{1}-((4-chloro-3-fluorophenyl)-*N*^{2}-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (13a):** By use of a procedure similar to that described for the preparation of compound **8a**, the compound **6a** (36.8 mg, 0.150 mmol) was converted into the title compound **13a** (7.6 mg, 12% yield) as yellow powder.

^{1}H NMR (400 MHz, $CDCl_3$) 0.71-2.28 (m, 24H), 2.03-2.20 (m, 2H), 4.02-4.16 (m, 1H), 7.13-7.18 (m, 1H), 7.27-7.33 (m, 1H), 7.62-7.66 (m, 1H), 9.25 (br, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) 14.1, 22.0 (2C), 22.6 (2C), 25.8 (2C), 29.3, 29.7 (2C), 31.9, 70.5, 108.3 (d, J = 26.3 Hz), 115.8, 117.1 (d, J = 18.8 Hz), 130.8, 136.2 (d, J = 10.0 Hz), 157.6, 158.1 (d, J = 247.5 Hz), 158.6; HRMS (ESI), m/z calcd for $C_{23}H_{32}ClFN_3O_2$ (MH^+) 436.2162, found 436.2156.

***N*^{1}-(4-chlorophenyl)-*N*^{2}-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (13b):** By use of a procedure similar to that described for the preparation of compound **8a**, the compound **6b** (31.3 mg, 0.120 mmol) was converted into the title compound **13b** (28.0 mg, 52% yield) as white powder.

^{1}H NMR (400 MHz, $CDCl_3$) 0.96 (t, J = 12.5 Hz, 2H), 1.10-1.84 (br, 20H), 2.05-2.19 (m, 2H), 4.08-4.21 (m, 1H), 7.23-7.33 (br, 1H), 7.39-7.46 (m, 2H), 7.88 (t, J = 1.00 Hz, 1H), 9.34 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) 14.1, 22.1 (2C), 22.7 (2C), 26.1 (2C), 31.6, 37.2 (2C), 42.6, 43.0, 43.6, 52.6 (2C), 119.0, 121.4, 128.7, 130.8, 133.1, 135.8, 157.7, 158.5; HRMS (ESI), m/z calcd for $C_{23}H_{32}Cl_2N_3O_2$ (MH^+) 452.1872, found 452.1865.

***N*^{1}-((4-chloro-3-methylphenyl)-*N*^{2}-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (13c):** By use of a procedure similar to that described for the preparation of compound **8a**, the compound **6c** (121.0 mg, 0.500 mmol) was converted into the title compound **13c** (15.1 mg, 7% yield) as white powder.
^{1}H NMR (500 MHz, $CDCl_3$) 0.87-1.88 (br, 22H), 2.09-2.20 (m, 2H), 2.38 (s, 3H), 4.09-4.22 (m, 1H), 7.32-7.33 (m, 1H), 7.41-7.43 (m, 1H), 7.51 (d, *J* = 2.00 Hz, 1H), 7.73 (m, 1H), 9.24 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) 20.2, 22.1 (2C), 22.7 (2C), 26.0 (2C), 29.7, 37.0, 42.3 (2C), 42.8 (2C), 43.4, 52.9 (2C), 118.4, 122.0, 130.0, 130.6, 134.8, 137.1, 157.5, 158.9; HRMS (ESI), m/z calcd for $C_{24}H_{35}ClN_3O_2$ (MH^+) 430.2267, found 430.2264.

***N*^{1}-(3-fluoro-4-methylphenyl)-*N*^{2}-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (14a):** By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7a** (225.0 mg, 1.00 mmol) was converted into the title compound **14a** (27.5 mg, 7% yield) as white powder.
^{1}H NMR (500 MHz, $CDCl_3$) 0.971 (t, *J* = 12.5 Hz, 2H), 1.18-1.86 (m, 20H), 2.13-2.16 (m, 2H), 2.26 (s, 3H), 4.09-4.21 (m, 1H), 7.13-7.18 (m, 2H), 7.33 (d, *J* = 8.00 Hz, 1H), 7.50-7.53 (m, 1H), 9.27 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) 14.2, 22.2 (2C), 22.8 (2C), 26.1 (2C), 37.2 (2C), 42.2 (2C), 43.3 (2C), 43.5, 52.6 (m, 2C), 107.0 (d, *J* = 27.5 Hz), 115.0 (d, *J* = 3.75 Hz), 121.8 (d, *J* = 17.5 Hz), 131.6 (d, *J* = 6.25 Hz), 135.4 (d, *J* = 10.0 Hz), 157.5, 158.9,161.3 (d, *J* = 242.5 Hz); HRMS (ESI), m/z calcd for $C_{24}H_{33}FN_3O_2$ (M^-) 414.2554, found 414.2562.

***N*^{1}-(3-chloro-4-methylphenyl)-*N*^{2}-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (14b):** By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7b** (120.5 mg, 0.500 mmol) was converted into the title compound **14b** (12.9 mg, 6% yield) as white powder.
^{1}H NMR (500 MHz, $CDCl_3$) 0.973 (t, *J* = 12.5 Hz, 2H), 1.18-1.86 (br, 20H), 2.11-2.19 (m, 2H), 2.35 (s, 3H), 4.09-4.21 (m, 1H), 7.20-7.22 (m, 1H), 7.30-7.32 (m, 1H), 7.35-7.37 (d, *J* = 2.50 Hz, 1H), 7.73 (m, 1H), 9.22 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) 19.6, 22.1 (2C), 22.7 (2C), 26.0 (2C), 29.7, 37.0, 42.1 (2C), 42.7 (2C), 43.2, 53.3 (2C), 118.0, 120.3, 131.2, 133.0, 134.7, 135.1, 157.5, 158.8; HRMS (ESI), m/z calcd for $C_{24}H_{33}ClN_3O_2$ (MH^+) 430.2267, found 430.2257.

***N*^{1}-(3-bromo-4-methylphenyl)-*N*^{2}-(2,6-dicyclohexylpiperidin-4-yl)oxalamide (14c):** By use of a procedure similar to that described for the preparation of compound **8a**, the compound **7c** (142.0 mg, 0.500 mmol) was converted into the title compound **14c** (11.5 mg, 5% yield) as white powder.
^{1}H NMR (500 MHz, $CDCl_3$) 0.67-2.07 (br, 22H), 2.28 (br, 2H), 2.38 (s, 3H), 4.09-4.21 (m, 1H), 7.22 (d, *J* = 8.00 Hz, 1H), 7.28-7.38 (br, 1H), 7.43 (dd, *J* = 4.50, 2.50 Hz, 1H), 7.90 (d, *J* = 2.50 Hz, 1H), 9.21 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) 14.1, 22.1 (2C), 22.4 (2C), 22.7 (2C), 25.9, 30.0, 31.6, 36.9 (2C), 42.7 (3C), 52.7, 52.9, 118.6, 123.4, 125.0, 131.0, 134.9, 135.1, 157.4, 158.8; HRMS (ESI), m/z calcd for $C_{24}H_{33}BrN_3O_2$ (MH^+) 474.1762, found 474.1746.

4.2 Antiviral assay and cytotoxicity assay

Anti-HIV activity and cytotoxicity measurements in PM1/CCR5 cells (Yoshimura et al., 2010) were based on viability of cells that had been infected or not infected with 100 TCID50 of an R5 primary isolate YTA48P exposed to various concentrations of the test compound. After the PM1/CCR5 cells were incubated at 37 ºC for 7 days. The 50% inhibitory concentration (IC_{50}) values and the 50% cytotoxic concentration (CC_{50}) were then determined using the Cell Counting Kit-8 assay (Dojindo Laboratories). All assays were performed in duplicate or triplicate.

4.3 FACS analysis

JR-FL (R5, Sub B) chronically infected PM1 cells were pre-incubated with 0.5 μg/mL of sCD4 or 100 M 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 mouse 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.

4.4. Molecular modeling

Dockings of compounds **3** and **13a** were performed using Molecular Operating Environment modeling package (MOE 2008. 10, Canada), into the crystal structure of gp120 (PDB, entry 3TGS).

Supplementary data

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

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CD4 mimics as HIV entry inhibitors: lead optimization studies of the aromatic substituents

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