

- the human immune response to code for antibodies against the V3 domain of HIV-1. *Mol. Immunol.* 46:917–926.
65. Yoshimura K, Harada S, Shibata J, Hatada M, Yamada Y, Ochiai C, Tamamura H, Matsushita S. 2010. Enhanced exposure of human immunodeficiency virus type 1 primary isolate neutralization epitopes through binding of CD4 mimetic compounds. *J. Virol.* 84:7558–7568.
 66. Cole KS, Alvarez M, Elliott DH, Lam H, Martin E, Chau T, Micken K, Rowles JL, Clements JE, Murphey-Corb M, Montelaro RC, Robinson JE. 2001. Characterization of neutralization epitopes of simian immunodeficiency virus (SIV) recognized by rhesus monoclonal antibodies derived from monkeys infected with an attenuated SIV strain. *Virology* 290: 59–73.
 67. Glamann J, Burton DR, Parren PW, Ditzel HJ, Kent KA, Arnold C, Montefiori D, Hirsch VM. 1998. Simian immunodeficiency virus (SIV) envelope-specific Fabs with high-level homologous neutralizing activity: recovery from a long-term-nonprogressor SIV-infected macaque. *J. Virol.* 72:585–592.
 68. Edinger AL, Ahuja M, Sung T, Baxter KC, Haggarty B, Doms RW, Hoxie JA. 2000. Characterization and epitope mapping of neutralizing monoclonal antibodies produced by immunization with oligomeric simian immunodeficiency virus envelope protein. *J. Virol.* 74:7922–7935.
 69. Zhang PF, Cham F, Dong M, Choudhary A, Bouma P, Zhang Z, Shao Y, Feng YR, Wang L, Mathy N, Voss G, Broder CC, Quinnan GV, Jr. 2007. Extensively cross-reactive anti-HIV-1 neutralizing antibodies induced by gp140 immunization. *Proc. Natl. Acad. Sci. U. S. A.* 104:10193–10198.
 70. Huang C-c, Venturi M, Majeed S, Moore MJ, Phogat S, Zhang M-Y, Dimitrov DS, Hendrickson WA, Robinson J, Sodroski J, Wyatt R, Choe H, Farzan M, Kwong PD. 2004. Structural basis of tyrosine sulfation and VH-gene usage in antibodies that recognize the HIV type 1 coreceptor-binding site on gp120. *Proc. Natl. Acad. Sci. U. S. A.* 101:2706–2711.
 71. Xiao X, Chen W, Feng Y, Zhu Z, Prabakaran P, Wang Y, Zhang MY, Longo NS, Dimitrov DS. 2009. Germline-like predecessors of broadly neutralizing antibodies lack measurable binding to HIV-1 envelope glycoproteins: implications for evasion of immune responses and design of vaccine immunogens. *Biochem. Biophys. Res. Commun.* 390:404–409.
 72. Chen W, Streaker ED, Russ DE, Feng Y, Prabakaran P, Dimitrov DS. 2012. Characterization of germline antibody libraries from human umbilical cord blood and selection of monoclonal antibodies to viral envelope glycoproteins: implications for mechanisms of immune evasion and design of vaccine immunogens. *Biochem. Biophys. Res. Commun.* 417: 1164–1169.
 73. Robinson JE, Franco K, Elliott DH, Maher MJ, Reyna A, Montefiori DC, Zolla-Pazner S, Gorny MK, Kraft Z, Stamatatos L. 2010. Quaternary epitope specificities of anti-HIV-1 neutralizing antibodies generated in rhesus macaques infected by the simian/human immunodeficiency virus SHIVSF162P4. *J. Virol.* 84:3443–3453.
 74. Yuan T, Li J, Zhang Y, Wang Y, Streaker E, Dimitrov DS, Zhang M-Y. 2011. Putative rhesus macaque germline predecessors of human broadly HIV-neutralizing antibodies: differences from the human counterparts and implications for HIV-1 vaccine development. *Vaccine* 29:6903–6910.
 75. Link JM, Larson JE, Schroeder HW. 2005. Despite extensive similarity in germline DH and JH sequence, the adult rhesus macaque CDR-H3 repertoire differs from human. *Mol. Immunol.* 42:943–955.
 76. Labrijn AF, Poignard P, Raja A, Zwick MB, Delgado K, Franti M, Binley J, Vivona V, Grundner C, Huang Venturi C-CM, Petropoulos CJ, Wrinn T, Dimitrov DS, Robinson J, Kwong PD, Wyatt RT, Sodroski J, Burton DR. 2003. Access of antibody molecules to the conserved coreceptor binding site on glycoprotein gp120 is sterically restricted on primary human immunodeficiency virus type 1. *J. Virol.* 77:10557–10565.
 77. Thenin S, Roch E, Samleerat T, Moreau T, Chaillon A, Moreau A, Barin F, Braibant M. 2012. Naturally occurring substitutions of conserved residues in human immunodeficiency virus type 1 variants of different clades are involved in PG9 and PG16 resistance to neutralization. *J. Virol.* 93: 1495–1505.
 78. Utachee P, Nakamura S, Isarangkura-Na-Ayuthaya P, Tokunaga K, Sawanpanyalert P, Ikuta K, Auwanit W, Kameoka M. 2010. Two N-linked glycosylation sites in the V2 and C2 regions of human immunodeficiency virus type 1 CRF01_AE envelope glycoprotein gp120 regulate viral neutralization susceptibility to the human monoclonal antibody specific for the CD4 binding domain. *J. Virol.* 84:4311–4320.
 79. Henzler-Wildman K, Kern D. 2007. Dynamic personalities of proteins. *Nature* 450:964–972.
 80. Ode H, Nakashima M, Kitamura S, Sugiura W, Sato H. 2012. Molecular dynamics simulation in virus research. *Front. Microbiol.* 3:258.
 81. Klein F, Gaebler C, Mouquet H, Sather DN, Lehmann C, Scheid JF, Kraft Z, Liu Y, Pietzsch J, Hurley A, Poignard P, Feizi T, Morris L, Walker BD, Fatkenheuer G, Seaman MS, Stamatatos L, Nussenzweig MC. 2012. Broad neutralization by a combination of antibodies recognizing the CD4 binding site and a new conformational epitope on the HIV-1 envelope protein. *J. Exp. Med.* 209:1469–1479.
 82. Poignard P, Saphire EO, Parren PW, Burton DR. 2001. GP120: biologic aspects of structural features. *Annu. Rev. Immunol.* 19:253–274.
 83. Sekaly RP. 2008. The failed HIV Merck vaccine study: a step back or a launching point for future vaccine development? *J. Exp. Med.* 205:7–12.
 84. Watkins DI, Burton DR, Kallas EG, Moore JP, Koff WC. 2008. Non-human primate models and the failure of the Merck HIV-1 vaccine in humans. *Nat. Med.* 14:617–621.



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



Tetsuo Narumi^a, Hiroshi Arai^a, Kazuhisa Yoshimura^{b,c}, Shigeyoshi Harada^{b,c}, Yuki Hirota^a, Nami Ohashi^a, Chie Hashimoto^a, Wataru Nomura^a, Shuzo Matsushita^b, Hirokazu Tamamura^{a,*}

^a Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101-0062, Japan

^b Center for AIDS Research, Kumamoto University, Kumamoto 860-0811, Japan

^c AIDS Research Center, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan

ARTICLE INFO

Article history:

Received 22 January 2013

Revised 25 February 2013

Accepted 26 February 2013

Available online 7 March 2013

Keywords:

CD4 mimicry

Conformational change in gp120

HIV entry inhibitor

Envelope protein opener

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 anti-viral 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.

© 2013 Published by Elsevier Ltd.

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-9564⁴ and NBDs⁵ 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 (Fig. 1).^{6–8}

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 anti-

bodies 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 (Fig. 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}

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 de-

* Corresponding author. Tel.: +81 3 5280 8036; fax: +81 3 5280 8039.

E-mail address: tamamura.mr@tmd.ac.jp (H. Tamamura).

scribe 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 (Fig. 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 (Fig. 3).

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*-

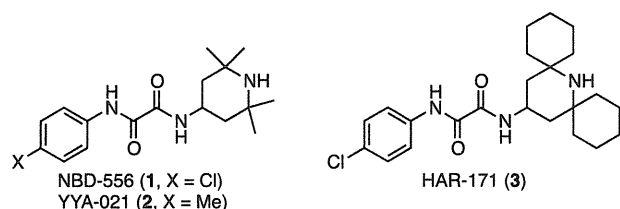


Figure 1. Structures of NBD-556 (**1**), YYA-021 (**2**) and HAR-171 (**3**).

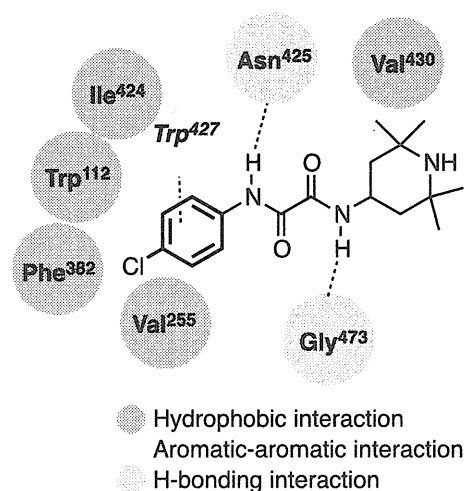


Figure 2. Major interactions between NBD-556 and Phe43 cavity of gp120.

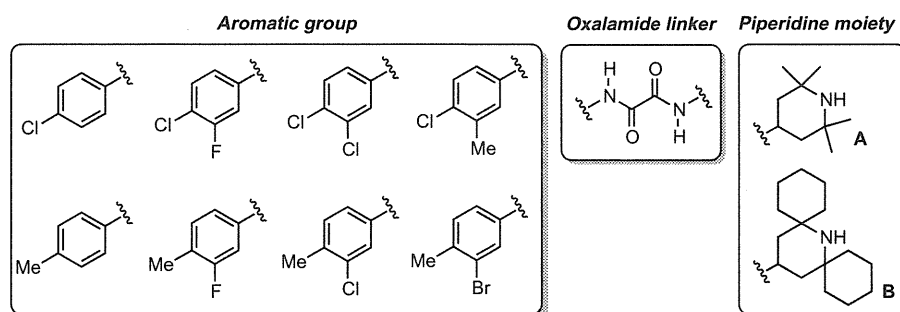


Figure 3. The structures of scaffolds in the design of novel CD4 mimics.

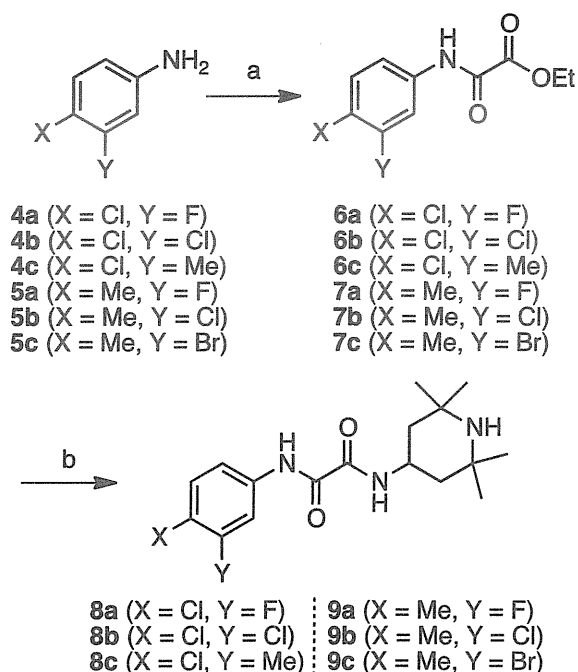
methyl substituent also showed potent anti-HIV activity and exhibits lower cytotoxicity than those with the *p*-chlorophenyl derivatives.^{8a} Next, we chose several halogens including F, Cl and Br, to be the *meta*-substituent on the aromatic group since previous SAR studies revealed that the introduction 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

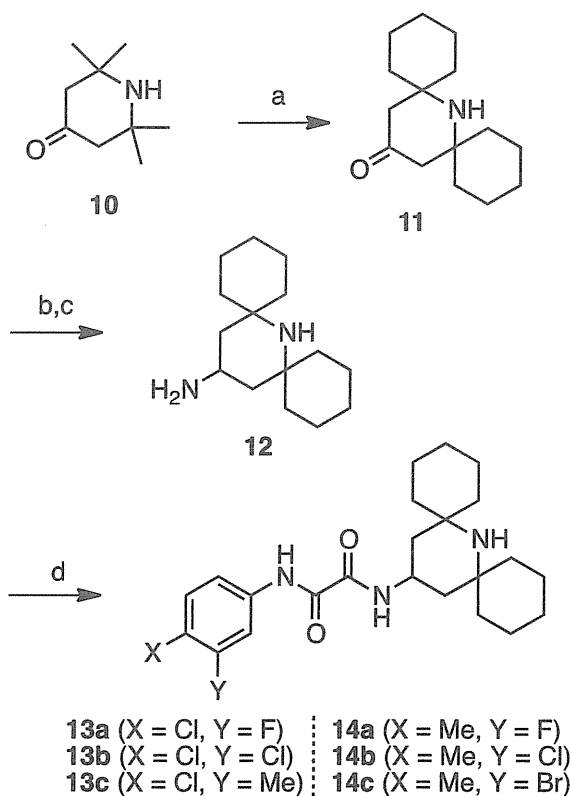
The syntheses of novel compounds are depicted in Schemes 1 and 2. Starting from the appropriate aniline with *m*- and *p*-substituents, coupling with ethyl chloroglyoxylate in the presence of Et₃N gave the corresponding amidoesters **6a–c** and **7a–c**. Subsequently, microwave-assisted aminolysis¹³ 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**.

2.2. Biological evaluation

The anti-HIV activity of the synthetic compounds was evaluated against an R5 primary isolate YTA strain. IC₅₀ 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 (IC₅₀



Scheme 1. Reagents and conditions: (a) ethyl chloroglyoxylate, Et₃N, THF; (b) 4-amino-2,2,6,6-tetramethylpiperidine, Et₃N, EtOH, 150 °C, microwave.



Scheme 2. Reagents and conditions: (a) cyclohexanone, NH₄Cl, DMSO, 60 °C; (b) *p*-methoxybenzylamine, NaBH₃CN, MeOH, then 1 M TMSBr in TFA; (c) CAN, CH₃CN/H₂O (v:v = 2:1); (d) **6** or **7**, Et₃N, EtOH, 150 °C, microwave.

of **1** = 0.61 μM and IC₅₀ of **8a** = 0.32 μM). 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 (IC₅₀ of **8b** = 4.1 μM and IC₅₀ of **8c** = 3.3 μM) but their potency was

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 ^b (μM) YTA48P	CC ₅₀ ^c (μM)
1		H	0.61	110
8a	A	F	0.32	94
8b	A	Cl	4.1	36
8c	A	Me	3.3	38
3		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 or more independent experiments.

^b IC₅₀ values of the multi-round assay are based on the inhibition of HIV-1-induced cytopathogenicity in PM1/CCR5 cells.

^c CC₅₀ values are based on the reduction of the viability of mock-infected PM1/CCR5 cells.

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 ^b (μM) YTA48P	CC ₅₀ ^c (μM)
2		H	9.0	260
9a	A	F	2.8	110
9b	A	Cl	3.2	62
9c	A	Br	>10	32
14a		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 or more independent experiments.

^b IC₅₀ values of the multi-round assay are based on the inhibition of HIV-1-induced cytopathogenicity in PM1/CCR5 cells.

^c CC₅₀ values are based on the reduction of the viability of mock-infected PM1/CCR5 cells.

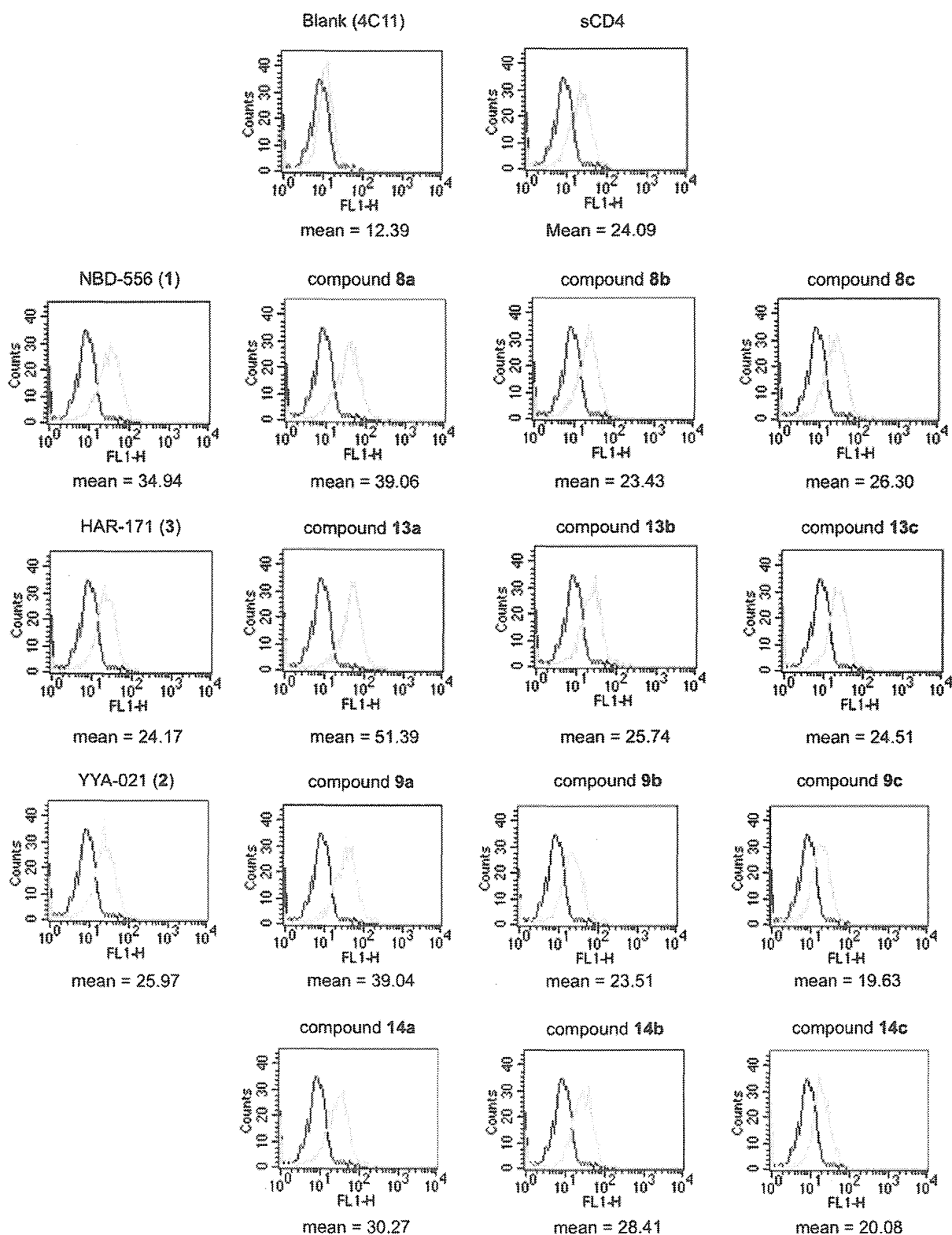


Figure 4. FACS analysis of synthetic compounds 8, 9, 13 and 14.

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 μ M and CC_{50} of **8c** = 38 μ 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 μ 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 μ M, whose potency was

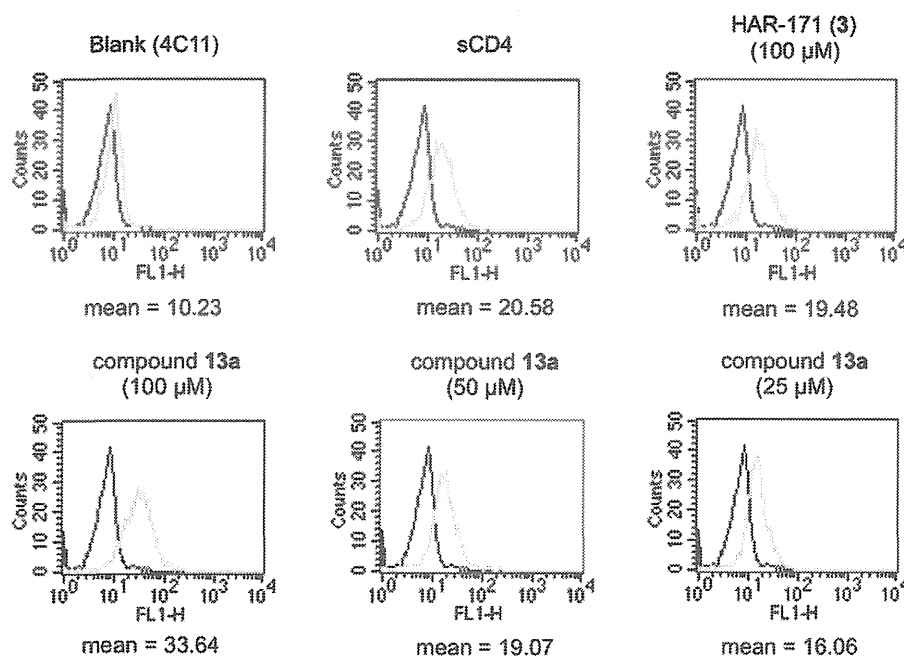


Figure 5. FACS analysis of 3 and 13a in different concentrations.

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 ($CC_{50} = 15 \mu\text{M}$). 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.

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 ($IC_{50} = 9.0 \mu\text{M}$) and exhibits the lowest cytotoxicity among all of the compounds tested ($CC_{50} = 260 \mu\text{M}$). 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 $10 \mu\text{M}$, whereas compound 14c with hydrophobic cyclohexyl groups attached to the piperidine moiety, showed moderate activity ($IC_{50} = 3.2 \mu\text{M}$), indicating that the hydrophobic modification of piperidine ring can contribute to an increase in anti-HIV activity.

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 twofold 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}

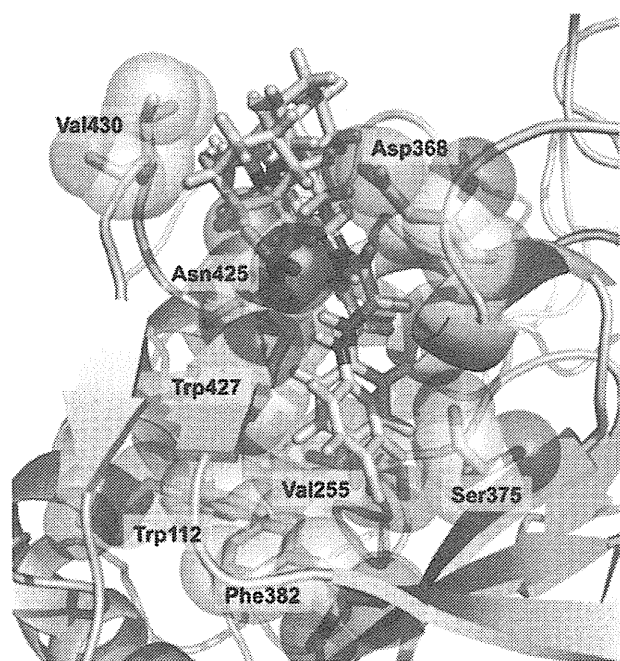


Figure 6. The modeled structure of **13a** (yellow carbon atoms) in the complex with the Phe43 cavity in gp120 (3TGS) overlaid with the modeled structure of **3** (green carbon atoms).

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 μM 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 μM 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 μM and 25 μM = 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** (Fig. 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**.

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—that is, 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 result-

ing 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 twofold 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

^1H 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, silica gel 60 N (Kanto Chemical Co., Inc.) was employed. 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. 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.

^1H NMR (500 MHz, CDCl_3) δ 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) δ 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 $\text{C}_{10}\text{H}_{10}\text{ClFNO}_3$ (MH^-) 244.0182, found 244.0183.

4.1.2. 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.

^1H NMR (500 MHz, CDCl_3) δ 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) δ 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 $\text{C}_{10}\text{H}_{10}\text{Cl}_2\text{NO}_3$ (MH^+) 262.0038, found 262.0031.

4.1.3. 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.

^1H NMR (500 MHz, CDCl_3) δ 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) δ 14.0, 20.2, 63.8, 118.5, 122.0, 129.7, 130.9, 134.8, 137.1, 153.8, 160.9; HRMS (ESI), m/z calcd for $\text{C}_{11}\text{H}_{13}\text{ClNO}_3$ (MH^+) 242.0578, found 242.0568.

4.1.4. 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.

^1H NMR (500 MHz, CDCl_3) δ 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) δ 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 $\text{C}_{11}\text{H}_{13}\text{FNO}_3$ (MH^+) 226.0879, found 226.0878.

4.1.5. 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.

^1H NMR (500 MHz, CDCl_3) δ 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) δ 14.0, 20.0, 63.8, 118.0, 120.3, 131.2, 133.3, 134.7, 135.0, 153.8, 160.8; HRMS (ESI), m/z calcd for $\text{C}_{11}\text{H}_{13}\text{ClNO}_3$ (MH^+) 242.0584, found 242.0573.

4.1.6. 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.

^1H NMR (500 MHz, CDCl_3) δ 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) δ 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 $\text{C}_{11}\text{H}_{13}\text{BrNO}_3$ (MH^+) 286.0079, found 286.0068.

4.1.7. 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.

^1H NMR (500 MHz, CDCl_3) δ 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) δ 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 $\text{C}_{17}\text{H}_{24}\text{ClFN}_3\text{O}_2$ (MH^+) 356.1536, found 356.1548.

4.1.8. 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.

^1H NMR (500 MHz, CDCl_3) δ 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) δ 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 $\text{C}_{17}\text{H}_{22}\text{Cl}_2\text{N}_3\text{O}_2$ (MH^-) 370.1095, found 370.1105.

4.1.9. 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.

^1H NMR (500 MHz, CDCl_3) δ 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) δ 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 $\text{C}_{18}\text{H}_{25}\text{ClN}_3\text{O}_2$ (MH^-) 350.1641, found 350.1656.

4.1.10. 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.

^1H NMR (500 MHz, CDCl_3) δ 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) δ 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 $\text{C}_{18}\text{H}_{25}\text{FN}_3\text{O}_2$ (MH^-) 334.1936, found 334.1942.

4.1.11. 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.

^1H NMR (500 MHz, CDCl_3) δ 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) δ 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 $\text{C}_{18}\text{H}_{25}\text{ClN}_3\text{O}_2$ (MH^-) 350.1641, found 350.1636.

4.1.12. *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.

¹H NMR (500 MHz, CDCl₃) δ 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); ¹³C NMR (125 MHz, CDCl₃) δ 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 C₁₈H₂₅BrN₃O₂ (MH⁺) 394.1136, found 394.1158.

4.1.13. Amine (12)

The compound **11** was prepared according to the reported procedure.¹⁴ To a stirred solution of piperidone **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 NaHCO₃ and extracted with EtOAc, then dried over MgSO₄. After concentration under reduced pressure, the residue was treated with 1 M TMS in THF (4.8 mL). The mixture was stirred at 0 °C for 14 h. Concentration under reduced pressure followed by short chromatography with CHCl₃/MeOH gave the PMB-protected amine. To a solution of the above amine (584.0 mg, 1.64 mmol) in CH₃CN/H₂O (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 CH₂Cl₂. The water layer was alkalized and extracted with EtOAc, then dried over Na₂SO₄. Concentration under reduced pressure followed by flash chromatography over silica gel with EtOAc–EtOH (4:1) to give the title compound **12** (175.5 mg, 71% yield) as a yellow oil.

¹H NMR (500 MHz, CDCl₃) δ 1.15–1.85 (m, 24H), 2.95–3.05 (m, 1H); ¹³C NMR (125 MHz, CDCl₃) 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 C₁₅H₂₉N₂ (MH⁺) 237.2325, found 237.2321.

4.1.14. *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.

¹H NMR (400 MHz, CDCl₃) δ 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); ¹³C NMR (125 MHz, CDCl₃) δ 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 C₂₃H₃₂ClFN₃O₂ (MH⁺) 436.2162, found 436.2156.

4.1.15. *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.

¹H NMR (400 MHz, CDCl₃) δ 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); ¹³C NMR (125 MHz, CDCl₃) δ 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 C₂₃H₃₂Cl₂N₃O₂ (MH⁺) 452.1872, found 452.1865.

4.1.16. *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.

¹H NMR (500 MHz, CDCl₃) δ 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); ¹³C NMR (125 MHz, CDCl₃) δ 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 C₂₄H₃₅ClN₃O₂ (MH⁺) 430.2267, found 430.2264.

4.1.17. *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.

¹H NMR (500 MHz, CDCl₃) δ 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); ¹³C NMR (125 MHz, CDCl₃) δ 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 C₂₄H₃₃FN₃O₂ (MH⁺) 414.2554, found 414.2562.

4.1.18. *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.

¹H NMR (500 MHz, CDCl₃) δ 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); ¹³C NMR (125 MHz, CDCl₃) δ 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 C₂₄H₃₃ClN₃O₂ (MH⁺) 430.2267, found 430.2257.

4.1.19. *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.

¹H NMR (500 MHz, CDCl₃) δ 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); ¹³C NMR (125 MHz, CDCl₃) δ 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 C₂₄H₃₃BrN₃O₂ (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 °C for 7 days. The 50% inhibitory concentration (IC₅₀) values and the 50% cytotoxic concentration (CC₅₀) 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 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 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).

Acknowledgements

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. We are grateful to Professor Yoshio Hayashi and Dr. Fumika Yakushiji, Tokyo University of Pharmacy and Life Sciences for their assistance in the molecular modelings.

Supplementary data

Supplementary data (NMR charts of compounds) associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bmc.2013.02.041>.

References and notes

- Chan, D. C.; Kim, P. S. *Cell* **1998**, *93*, 681.
- (a) Kadow, J.; Wang, H.-G.; Lin, P.-F. *Curr. Opin. Investig. Dugs* **2006**, *7*, 721; (b) Repik, A.; Clapham, P. R. *Structure* **2008**, *16*, 1603.
- Holz-Smith, S.; Sun, I. C.; Jin, L.; Matthews, T. J.; Lee, K. H.; Chen, C. H. *Antimicrob. Agents Chemoth.* **2001**, *45*, 60.
- Lin, P.-F.; Blair, W.; Wang, T.; Spicer, T.; Guo, Q.; Zhou, N.; Gong, Y.-F.; Wang, H.-F. H.; Rose, R.; Yamanaka, G.; Robinson, B.; Li, C.-B.; Fridell, R.; Deminie, C.; Demers, G.; Yang, Z.; Zadjura, L.; Meanwell, N.; Colonna, R. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 11013.
- Zhao, Q.; Ma, L.; Jiang, S.; Lu, H.; Liu, S.; He, Y.; Strick, N.; Neamati, N.; Debnath, A. K. *Virology* **2005**, *339*, 213.
- (a) Madani, N.; Schön, A.; Princiotta, A. M.; LaLonde, J. M.; Courter, J. R.; Soeta, T.; Ng, D.; Wang, L.; Brower, E. T.; Xiang, S.-H.; Do Kwon, Y.; Huang, C.-C.; Wyatt, R.; Kwong, P. D.; Freire, E.; Smith, A. B., III; Sodroski, J. *Structure* **2008**, *16*, 1689; (b) LaLonde, J. M.; Elban, M. A.; Courter, J. R.; Sugawara, A.; Soeta, T.; Madani, N.; Princiotta, A. M.; Kwon, Y. D.; Kwong, P. D.; Schön, A.; Freire, E.; Sodroski, J.; Smith, A. B., III *Bioorg. Med. Chem. Lett.* **2011**, *20*, 354; (c) LaLonde, J. M.; Kwon, Y. D.; Jones, D. M.; Sun, A. W.; Courter, J. R.; Soeta, T.; Kobayashi, T.; Princiotta, A. M.; Wu, X.; Schön, A.; Freire, E.; Kwong, P. D.; Mascola, J. R.; Sodroski, J.; Madani, N.; Smith, A. B., III *J. Med. Chem.* **2012**, *55*, 4382.
- Curreli, F.; Choudhury, S.; Pyatkin, I.; Zagorodnikov, V. P.; Bulay, A. K.; Altieri, A.; Kwon, Y. D.; Kwon, P. D.; Debnath, A. K. *J. Med. Chem.* **2012**, *55*, 4764.
- (a) Yamada, Y.; Ochiai, C.; Yoshimura, K.; Tanaka, T.; Ohashi, N.; Narumi, T.; Nomura, W.; Harada, S.; Matsushita, S.; Tamamura, H. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 354; (b) Narumi, T.; Ochiai, C.; Yoshimura, K.; Harada, S.; Tanaka, T.; Nomura, W.; Arai, H.; Ozaki, T.; Ohashi, N.; Matsushita, S.; Tamamura, H. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 5853; (c) Narumi, T.; Arai, H.; Yoshimura, K.; Harada, S.; Nomura, W.; Matsushita, S.; Tamamura, H. *Bioorg. Med. Chem.* **2011**, *19*, 6735.
- (a) Schön, A.; Madani, N.; Klein, J. C.; Hubicki, A.; Ng, D.; Yang, X.; Smith, A. B., III; Sodroski, J.; Freire, E. *Biochemistry* **2006**, *45*, 10973; (b) Schön, A.; Lam, S. Y.; Freire, E. *Future Med. Chem.* **2011**, *3*, 1129.
- Yoshimura, K.; Harada, S.; Shibata, J.; Hatada, M.; Yamada, Y.; Ochiai, C.; Tamamura, H.; Matsushita, S. *J. Virol.* **2010**, *84*, 7558.
- Kwon, Y. D.; Finzi, A.; Wu, X.; Dogo-Isonagie, C.; Lee, L. K.; Moore, L. R.; Schmidt, S. D.; Stuckey, J.; Yang, Y.; Zhou, T.; Zhu, J.; Vivic, D. A.; Debnath, A. K.; Shapiro, L.; Bewley, C. A.; Mascola, J. R.; Sodroski, J. G.; Kwong, P. D. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 5663.
- (a) Kwong, P. D.; Wyatt, R.; Robinson, J.; Sweet, R. W.; Sodroski, J.; Hendrickson, W. A. *Nature* **1998**, *393*, 648; (b) Kwong, P. D.; Wyatt, R.; Mcajeeed, S.; Robinson, J.; Sweet, R. W.; Sodroski, J.; Hendrickson, W. A. *Structure* **2000**, *8*, 1329.
- McFarland, C.; Vivic, D. A.; Debnath, A. K. *Synthesis* **2006**, 807.
- Sakai, K.; Yamada, K.; Yamasaki, T.; Kinoshita, Y.; Mito, F.; Utsumi, H. *Tetrahedron* **2010**, *66*, 2311.



Increased infectivity in human cells and resistance to antibody-mediated neutralization by truncation of the SIV gp41 cytoplasmic tail

Takeo Kuwata¹, Kaori Takaki¹, Ikumi Enomoto¹, Kazuhisa Yoshimura² and Shuzo Matsushita^{1*}

¹ Center for AIDS Research, Kumamoto University, Kumamoto, Japan

² AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan

Edited by:

Akio Adachi, The University of Tokushima Graduate School, Japan

Reviewed by:

Tetsuro Matano, University of Tokyo, Japan

Tsutomu Murakami, National Institute of Infectious Diseases, Japan
Hirofumi Akari, Kyoto University, Japan

*Correspondence:

Shuzo Matsushita, Center for AIDS Research, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan.
e-mail: shuzo@kumamoto-u.ac.jp

The role of antibodies in protecting the host from human immunodeficiency virus type 1 (HIV-1) infection is of considerable interest, particularly because the RV144 trial results suggest that antibodies contribute to protection. Although infection of non-human primates with simian immunodeficiency virus (SIV) is commonly used as an animal model of HIV-1 infection, the viral epitopes that elicit potent and broad neutralizing antibodies to SIV have not been identified. We isolated a monoclonal antibody (MAb) B404 that potently and broadly neutralizes various SIV strains. B404 targets a conformational epitope comprising the V3 and V4 loops of Env that intensely exposed when Env binds CD4. B404-resistant variants were obtained by passaging viruses in the presence of increasing concentration of B404 in PM1/CCR5 cells. Genetic analysis revealed that the Q733stop mutation, which truncates the cytoplasmic tail of gp41, was the first major substitution in Env during passage. The maximal inhibition by B404 and other MAbs were significantly decreased against a recombinant virus with a gp41 truncation compared with the parental SIVmac316. This indicates that the gp41 truncation was associated with resistance to antibody-mediated neutralization. The infectivities of the recombinant virus with the gp41 truncation were 7,900-, 1,000-, and 140-fold higher than those of SIVmac316 in PM1, PM1/CCR5, and TZM-bl cells, respectively. Immunoblotting analysis revealed that the gp41 truncation enhanced the incorporation of Env into virions. The effect of the gp41 truncation on infectivity was not obvious in the HSC-F macaque cell line, although the resistance of viruses harboring the gp41 truncation to neutralization was maintained. These results suggest that viruses with a truncated gp41 cytoplasmic tail were selected by increased infectivity in human cells and by acquiring resistance to neutralizing antibody.

Keywords: SIV, gp41, truncation, infectivity, resistance, neutralization, antibody

INTRODUCTION

The RV144 trial demonstrated 31% vaccine efficacy for preventing human immunodeficiency virus type 1 (HIV-1) infection (Rerks-Ngarm et al., 2009). Antibodies against the HIV-1, particularly against the V1/V2 loops, correlate inversely with infection risk (Haynes et al., 2012). Further recent isolation of monoclonal antibodies (MAbs) that neutralize a broad range of HIV-1 strains suggest the possibility for developing a vaccine that can induce cross-neutralizing antibodies effective for various HIV-1 strains (Kwong and Mascola, 2012). Although non-human primate models of simian immunodeficiency virus (SIV) infection can facilitate the evaluation of immunogens, epitopes and immune correlates, no potent and broad neutralizing MAb against SIV had been available.

To understand the mechanisms involved in neutralization of infectivity by antibodies in an SIV model, we recently isolated MAb B404 from a SIVsmH635FC-infected rhesus macaque, which potently and broadly neutralizes various SIV strains, such as SIVsmE543-3, SIVsmE660 and the neutralization-resistant variants, genetically diverse SIVmac316, and highly

neutralization-resistant SIVmac239 (Kuwata et al., 2011). The B404 epitope, which comprises the V3 and V4 loops of Env and is intensely exposed by ligation of Env to CD4, is the target for potent and broad neutralization of SIV (Kuwata et al., 2013). Vigorous induction of B404-like neutralizing antibodies using the specific VH3 gene with a long complementarity-determining region 3 loop and λ light chain was observed in four SIVsmH635FC-infected macaques. The B404-resistant variants were induced by passaging viruses in the presence of increasing concentrations of B404. Genetical analysis of the gp120 region of B404-resistant variants revealed that the mutations in the C2 region of Env were important for the resistance to antibody-mediated neutralization (Kuwata et al., 2013).

In the present study, we further analyzed B404-resistant variants and determined the precise region responsible for the resistance to antibody-mediated neutralization. Genetic analysis of viruses during passage in the presence of B404 as well as phenotypic analysis using recombinant viruses revealed that a truncation of the gp41 cytoplasmic tail was the primary step leading to escape from neutralization.

MATERIALS AND METHODS

CELLS

PM1 (Lusso et al., 1995), PM1/CCR5 (Yusa et al., 2005), and HSC-F (Akari et al., 1999) cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% fetal bovine serum (FBS). TZM-bl (Platt et al., 1998; Derdeyn et al., 2000; Wei et al., 2002; Takeuchi et al., 2008) and 293T (DuBridge et al., 1987) cells were maintained in Dulbecco's modified Eagle's medium containing 10% FBS.

GENETIC ANALYSIS OF B404-RESISTANT VARIANTS

The induction of variants resistant to Fab-B404 (Kuwata et al., 2011) from SIVmac316 (Mori et al., 1992) harboring full-length gp41 was performed as described previously (Yoshimura et al., 2006; Hatada et al., 2010; Kuwata et al., 2013). Briefly, 5,000 TCID₅₀ (50% tissue culture infectious dose) SIVmac316 was incubated with 5 ng/ml Fab-B404 for 30 min at 37°C. Then, 5 × 10⁴ PM1/CCR5 cells were added to the virus–Fab mixture. After incubation for 5 h, cells were washed with phosphate-buffered saline (PBS) and resuspended in RPMI 1640 supplemented with 10% FBS without Fab-B404. The culture supernatant was harvested 7 days later and used to infect fresh PM1/CCR5 cells for the next round of culture in the presence of increasing concentrations of Fab-B404. Proviral DNA samples were extracted from cells using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) after 8, 17, 20, 23, and 26 passages as well as from P26C cells obtained after 26 passages in the absence of Fab-B404. The gp120 region was amplified using Platinum Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA) with primers SEnv-F (5'-ATG GGA TGT CTT GGG AAT CAG C-3') and SER1 (5'-CCA AGA ACC CTA GCA CAA AGA CCC-3'). The whole *env* gene was amplified with primers SRev-F (5'-GGT TTG GGA ATA TGC TAT GAG-3') and SEnv-R (5'-CCT ACT AAG TCA TCA TCT T-3'). The polymerase chain reaction (PCR) products were cloned using a TA cloning kit (Invitrogen), and subjected to sequencing. Nucleotide sequences were aligned and analyzed phylogenetically using Molecular Evolutionary Genetics Analysis version 5 (MEGA5) (Tamura et al., 2011).

CONSTRUCTION OF INFECTIOUS MOLECULAR CLONES WITH THE Env REGION FROM B404-RESISTANT VARIANTS

One of the clones from passage 26, P26B404 clone 26, was selected for construction of recombinant viruses, because this clone had mutations typical of the major population of P26B404 variants. Infectious molecular clones SS, SN, and NS were generated by replacing fragments *SphI*–*SacI* [nucleotides (nt) 6,446–9,226], *SphI*–*NheI* (nt 6,446–8,742), and *NheI*–*SacI* (nt 8,742–9,226) with the corresponding regions of SIVmac316, respectively. Mutants F277V and N295S, which have point mutations at amino acid residues 277 and 295 of Env, respectively, were constructed by PCR mutagenesis using the SIVmac316 plasmid as template. The changes from phenylalanine (TTC) to valine (GTC) in F277V and asparagine (AAT) to serine (AGT) in N295S were introduced using primers F277VfW (5'-TTG GTT TGG CGT CAA TGG TAC TAG GGC-3'), F277Vrv (5'-GTA CCA TTG ACG CCA AAC CAA G-3'), N295SFw (5'-GGC AAT AGT AGT AGA ACC ATA ATT AG-3'), and N295SRv (5'-AAT TAT GGT TCT ACT ACT ATT GCC-3').

Mutant and parental SIVmac316 plasmids were transfected into 293T cells using X-tremeGENE 9 DNA Transfection Reagent (Roche Molecular Biochemicals, Mannheim, Germany). After 2 days, the supernatants containing viruses were filtered (0.45 μm) and stored at –80°C.

ANALYSIS OF VIRAL INFECTIVITY

For determination of TCID₅₀ in PM1 and PM1/CCR5 cells, 5 × 10⁴ cells in 50 μl were inoculated with 50 μl serially diluted virus stocks in a 96-well plate and cultured for 2 weeks. Virus replication was judged by observation of cytopathic effects (CPE) by light microscopy. The TCID₅₀ in TZM-bl cells was determined by measuring luciferase activities. Briefly, 100 μl medium, 50 μl serially diluted virus stock, and 50 μl 1 × 10⁴ cells containing 37.5 μg/ml diethylaminoethyl (DEAE) dextran were added to the wells of a 96-well plate. The plate was then incubated at 37°C for 2 days. After washing with PBS, cells were lysed with 30 μl cell lysing buffer (Promega, Madison, WI, USA) for 15 min at room temperature (RT) and then 10 μl of cell lysate was transferred to a 96-well white solid plate (Coster, Cambridge, MA, USA). Luciferase activity was measured using a Centro XS3 LB960 microplate luminometer (Berthold Technologies, Bad Wildbad, Germany) and a luciferase assay system (Promega). The TCID₅₀ was calculated according to the formula of Reed and Muench (1938).

Infectivity of viruses in PM1, PM1/CCR5, and HSC-F cells was evaluated by detecting infected cells using flow cytometry as described previously (Kuwata et al., 2011). Briefly, PM1 and PM1/CCR5 cells were adjusted to 1 × 10⁶ cells/ml and HSC-F cells were adjusted to 5 × 10⁶ cells/ml. Aliquots of 100 μl cells per well in a 24-well plate were inoculated with 100 μl of diluted virus stocks. After incubation for 6 h, 800 μl fresh medium was added to wells. One-half of the cells in each well were collected at 4, 7, and 10 days post-inoculation. Cells were washed with PBS and fixed with IC Fixation Buffer (eBioscience, San Diego, CA, USA). After washing with Permeabilization Buffer (eBioscience) twice, the cells were intracellularly stained with 4 μg/ml (50 μl) anti-p27 Fab, B450 (Kuwata et al., 2011) by incubation for 20 min at RT. The cells were then incubated with 50 μl anti-HA antibody (1:200; 3F10, Roche Molecular Biochemicals) for 20 min at RT followed by incubation with 50 μl of anti-rat-FITC (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 20 min at RT. The stained cells were analyzed using a FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA). Frequencies of infected cells were determined by comparison with an uninfected control. Data analysis was performed using FlowJo (TreeStar, San Carlos, CA, USA).

All infectivity experiments were performed at least twice and the representative results are shown.

ANALYSIS OF NEUTRALIZING ACTIVITIES

The Fab clones B404 and K8, isolated from an SIV-infected macaque (Kuwata et al., 2011), and murine MAb M318T (Matsumi et al., 1995) were used to examine the sensitivity of viruses to antibody-mediated neutralization in TZM-bl cells as described previously (Kuwata et al., 2011). Briefly, 100 μl serially diluted antibodies in duplicate were incubated with 200 TCID₅₀ (50 μl) of virus in a 96-well plate. After incubation for 1 h at 37°C, 100 μl

of 1×10^5 TZM-bl cells/ml containing 37.5 $\mu\text{g/ml}$ DEAE dextran were added. After incubation for 2 days, luciferase activities were measured as described above for the analysis of viral infectivity. The 50% inhibitory concentrations (IC_{50}) and maximal percent of inhibition (MPI) were calculated from the average values by non-linear regression using Prism5 (GraphPad Software, San Diego, CA, USA).

Sensitivity to neutralization by B404 in macaque cells was analyzed using HSC-F cells, a cynomolgus macaque cell line immortalized by infection with *Herpesvirus saimiri* (Akari et al., 1999). Fab-B404 was serially diluted and 50 μl aliquots were mixed with 50 μl undiluted or 10-fold diluted virus in a 96-well plate. After 1 h incubation at 37°C, 2×10^5 cells in 100 μl were added to each well and cultured for 1 day. The infected cells were washed twice with PBS, resuspended in 200 μl fresh medium, and cultured in a new 96-well plate. Viral infection was examined 4 days post-inoculation by intracellular staining of p27, as described above for the analysis of viral infectivity. Infectivity was determined in duplicate and the average value was used for the analysis of neutralization.

All neutralizing assays were performed at least twice and the representative results are shown.

WESTERN BLOTTING ANALYSIS OF VIRAL PROTEINS

Cells and supernatants were collected from six-well plate 2 days after transfection of 293T cells with infectious molecular clones, as previously described (Yuste et al., 2005). Supernatants were filtered (0.45 μm) and clarified by centrifugation for 10 min at 3,000 rpm. The clarified supernatants were centrifuged at 13,200 rpm for 90 min at 4°C, and the viral pellets were resuspended in 1 ml PBS and centrifuged again. Pellets were then dissolved in 80 μl sample buffer [62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 25% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue]. Cells were washed with PBS and lysed in 300 μl sample buffer. Samples of virions and cell lysates were boiled for 5 min, and the proteins were separated by SDS-polyacrylamide gel electrophoresis using SuperSep Ace 5–20% (Wako Pure Chemical Industries, Osaka, Japan). Proteins were transferred to an Immun-Blot PVDF Membrane (Bio-Rad Laboratories, Hercules, CA, USA). The membrane was blocked with 5% skim milk TBS-T (Tris-buffered saline containing 0.1% Tween 20) for 1 h at RT, and then washed three times with TBS-T. For the detection of gp120, the membrane was incubated overnight at 4°C with 1 $\mu\text{g/ml}$ M318T (Matsumi et al., 1995) in 5% skim milk TBS-T. After washing three times with TBS-T, the membrane was incubated with anti-mouse immunoglobulin G (IgG) peroxidase (1:4,000, Santa Cruz Biotechnology) for 1 h at RT. The membrane was washed three times with TBS-T and once with TBS, and then TMB solution (KPL, Gaithersburg, MD, USA) was added to develop color. Viral proteins gp41 and p26 were similarly examined using crude supernatants from bacterial culture producing B408 and B450 (Kuwata et al., 2011), which were mixed with the same amount of 5% skim milk TBS-T. The membrane was incubated with anti-HA-HRP antibody (1:1,000; Roche Molecular Biochemicals) and Chemi-Lumi One L (Nacalai Tesque, Kyoto, Japan), and viral proteins were visualized using ImageQuant LAS 4000 (GE Healthcare, Piscataway, NJ, USA).

RESULTS

EVOLUTION OF VIRUSES DURING PASSAGE UNDER THE PRESSURE OF Fab-B404

To select for variants resistant to MAb B404, an antibody that targets a conformational epitope comprising the gp120 V3 and V4 loops, we passaged SIVmac316 that possesses a full-length gp41 in PM1/CCR5 cells in the presence of increasing concentrations of Fab-B404. The virus recovered at passage 26 (P26B404) was resistant to neutralization by B404 (V3/V4) and other antibodies, MAb K8 (CD4i) and M318T (V2), that target epitopes other than that recognized by B404 (Kuwata et al., 2013). The region covering the whole *env* gene were amplified by PCR and cloned from viruses at passage 8, 17, 20, 23, and 26. The nucleotide sequences were phylogenetically analyzed to show the evolution of B404-resistant variants (Figure 1). The first major mutation was a change from glutamine (CAG) to a stop codon (TAG) at 733rd amino acid residue of Env. The Q733stop substitution in the gp41 cytoplasmic domain was observed in 12 of 14 clones at passage 8 and in all clones thereafter. Another stop codon (W782stop) was the second

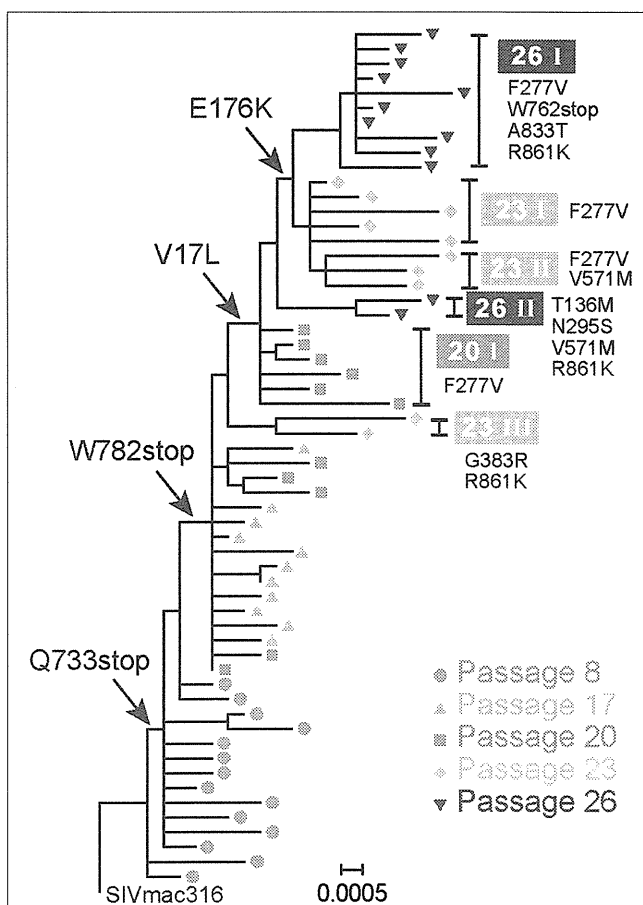


FIGURE 1 | Evolution of B404-resistant variants. Nucleotide sequences of the *env* gene, which were derived from proviruses at passages 8, 17, 20, 23, and 26, were phylogenetically analyzed using MEGA5. Arrows indicate major substitutions as follows: Q733stop, W782stop, V17L, and E176K. Clones with representative amino acid substitutions were separated into groups, which are shown on the right side along with the corresponding substitutions.

major mutation, which was detected after 17 passages. Substitutions V17L in the signal peptide and E176K in the V2 loop emerged after 20 and 23 passages, respectively, although the E176K substitution was also observed in P26C, control viruses after 26 passages in the absence of B404 (Table 1). In addition to these substitutions, most of clones acquired the F277V substitution in the late stage of evolution, except for one group at passage 26 which has the N295S substitution (see Figure 1, group 26II). Group 26II was clearly distinguished from group 26I by amino acid substitutions, such as T136M, N295S, and D571M/E (Table 1), suggesting two lineages of variants in P26B404.

These results demonstrated that the first step in acquiring resistance to B404 was the truncation of gp41. Although substitutions

in gp120, represented by F277V, might contribute to the resistance to a high concentration of B404, 20 passages were required for the emergence of these substitutions.

TRUNCATION OF gp41 CONFERRED RESISTANCE TO ANTIBODY-MEDIATED NEUTRALIZATION

To analyze effect of substitutions in B404-resistant variants on resistance to neutralization, recombinant viruses were constructed (Figure 2). The env region of SIVmac316 was replaced by that of P26B404 clone 26, which had substitutions typical to the P26I group. The resultant molecular clones SS, SN, and NS had substitutions in the entire env region, gp120 and gp41 from P26B404I, respectively. SS and NS were predicted to have a truncated gp41 with no other mutation in gp41, because the Q733stop substitution was the first substitution in gp41. Point mutants with substitutions F277V and N295S, which were representative mutations at late passages, were also constructed by PCR mutagenesis.

These mutant viruses were examined for their sensitivity to neutralization by three MAbs B404 (V3/V4 conformational), K8 (CD4i), and M318T (V2). The neutralization of SS that contain the entire env region from P26B404I was similar to those of P26B404, indicating that the env region is responsible for the

Table 1 | Frequency* of amino acid substitutions in Env clones from B404-resistant variants after 26 passages.

Substitution	Region	P26B404		P26C
		I	II	
	gp120	(n = 22)	(n = 8)	(n = 14)
V17L	Signal peptide	100%	100%	0.0%
G62S	C1	0.0%	0.0%	21.4%
M67V/L/T	C1	4.5%	0.0%	21.4%
A68T	C1	0.0%	0.0%	92.9%
T136M	V1	4.5%	87.5%	0.0%
T137I	V1	0.0%	0.0%	14.3%
K141E/R	V1	0.0%	12.5%	7.1%
E176K	V2	90.9%	12.5%	35.7%
F277V	C2	100%	0.0%	0.0%
N295S	C2	0.0%	100%	0.0%
Q341H	V3	13.6%	12.5%	14.3%
D374N	C3	0.0%	0.0%	28.6%
K403R	V4	0.0%	12.5%	7.1%
W441R	C4	4.5%	0.0%	7.1%
	gp41	(n = 10)	(n = 2)	(n = 7)
F528S/L	Extracellular	20.0%	0.0%	0.0%
D571M/E	Extracellular	10.0%	100%	0.0%
Q733stop	Cytoplasmic	100%	100%	0.0%
W762stop	Cytoplasmic	100%	0.0%	0.0%
W782stop	Cytoplasmic	100%	0.0%	0.0%
A833T	Cytoplasmic	90.0%	0.0%	0.0%
R839K	Cytoplasmic	0.0%	0.0%	57.1%
R861K	Cytoplasmic	100%	100%	0.0%

*Percentages of substitutions in populations P26B404 and P26C, which were obtained after 26 passages in the presence and absence of B404, respectively, are shown. The P26B404 population is separated into two subpopulations according to the phylogenetic analysis in Figure 1. All the substitutions that are observed in more than one clone are shown here. Boldface indicates substitutions dominant (>50%) in each population. The numbers of clones analyzed for the gp120 and gp41 regions are shown in parentheses.

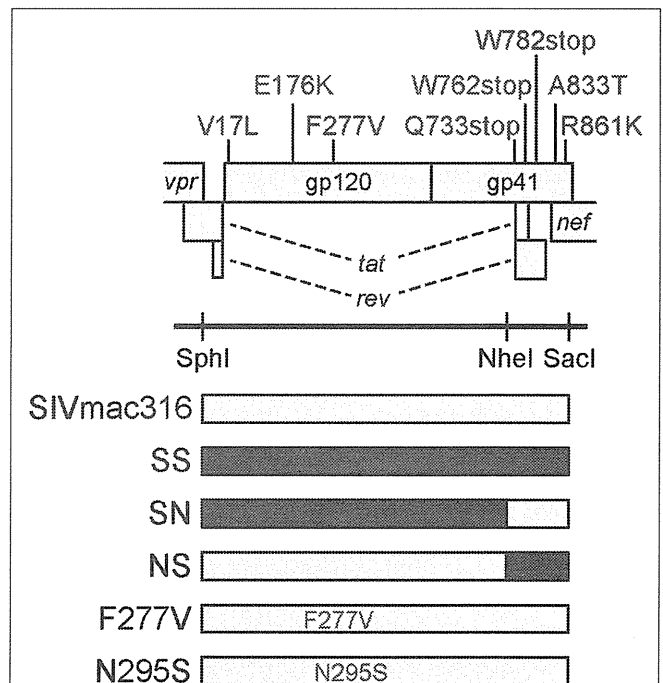


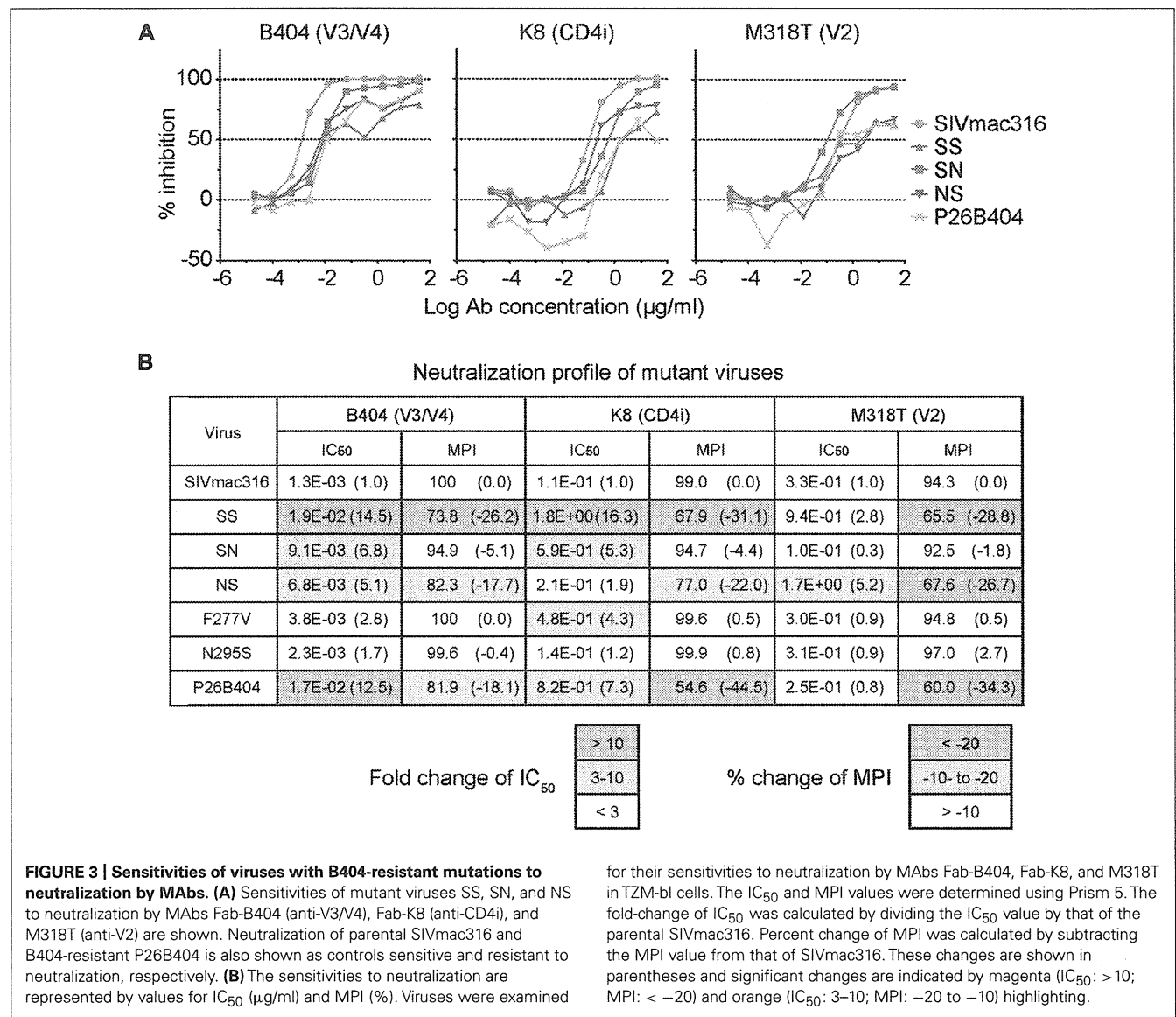
FIGURE 2 | Construction of infectious SIV clones with B404-resistant mutations. The open reading frames of the SIV genome are shown along with the Env substitutions in the B404-resistant variant typical to P26B404I. Full-length SIV clones were constructed from parental SIVmac316 by replacing the env region with that of the B404-resistant variant (blue) using SphI, NheI, and SacI sites. The resultant virus SS contains all of the Env substitutions present in P26B404I. Viruses SN and NS contain substitutions in gp120 and gp41, respectively. Point mutants F277V and N295S were constructed by inserting the substitutions F277V and N295S into SIVmac316, respectively.

resistance to neutralization (Figure 3A). Recombinants SN and NS, which have substitutions in gp120 and gp41 from P26B404I, respectively, showed varying degrees of resistance. The IC₅₀ values of SN and NS against B404 were intermediates between the parental SIVmac316 and the neutralization-resistant P26B404. Maximal inhibition reached a plateau at 73.8, 82.3, and 81.9% in SS, NS, and P26B404, respectively, but the MPI value of SN (94.9%) was close to that of SIVmac316 (100%; Figure 3B). Neutralization resistance to anti-CD4i MAb K8 was characterized by decreases in the IC₅₀ value of SN and the MPI of NS. Neutralization by anti-V2 MAb M318T was even enhanced in SN, although NS showed the resistance comparable to those of SS and P26B404. The decreases in MPI values were commonly observed for the neutralization of NS by the three MAbs (Figure 3B). Resistance to neutralization was not significantly detected by the point mutants F277V and N295S, except for the neutralization of F277V by K8 (4.3-fold decrease of IC₅₀ value). These results indicated that the

entire *env* region, including substitutions in both gp120 and gp41, was responsible for the full-resistance of P26B404 to neutralization. The decrease of MPI values for NS suggested that truncation of gp41 by the Q733stop substitution, the first major substitution in viral evolution, was important to escape from the neutralizing antibodies.

INCREASED INFECTIVITY FOR HUMAN CELLS BY SIV WITH A TRUNCATED gp41

Truncation of gp41 in SIV is associated with the adaptation to human cells (Hirsch et al., 1989; Kodama et al., 1989), which may partially contribute to neutralization resistance (Yuste et al., 2005). To explore the mechanism of neutralization resistance of P26B404, the infectivity of recombinant viruses was analyzed by determining the TCID₅₀ values of virus stocks prepared by transfection of 293T cells (Table 2). The TCID₅₀ values in all the human cells tested were significantly higher for SS and NS viruses with truncated gp41 than



parental SIVmac316 and SN, in which gp41 is intact. In particular, NS showed a striking increase in TCID₅₀ values, which were 7,100-, 1,000-, and 140-fold higher than those of parental SIVmac316 in PM1, PM1/CCR5, and TZM-bl cells, respectively. These results indicate that truncation of gp41 caused by the Q733stop substitution increases viral infectivity for human cells.

To compare viral infectivity in human and macaque cells, viral infection was monitored after inoculation of PM1 and PM1/CCR5 human cells and the HSC-F cynomolgus macaque cell line with varying dilutions of virus stocks (Figure 4). Consistent with the TCID₅₀ analysis, a higher frequency of infected cells was detected earlier in PM1 and PM1/CCR5 cells inoculated with NS than the parental SIVmac316. In contrast, SN showed decreased infectivity in PM1 and PM1/CCR5 cells, apparently because PM1 cells were not infected by a 1,000-fold diluted SN stock. Although the TCID₅₀ values of SS were much higher than those of SIVmac316, the replication kinetics of SS were similar to those of SIVmac316 in PM1 and PM1/CCR5 cells. These results suggest

that gp41 truncation increases infectivity for human cells and that the substitutions in gp120 of P26B404I are associated with slow and poor replication compared with that of SIVmac316.

Infectivity for macaque cells was more significantly affected than that for human cells by the substitutions in gp120 of P26B404I (Figure 4, lower panels). Infected cells were detected in HSC-F cells inoculated with 1,000-fold diluted virus stocks of SIVmac316 and NS, but viral infection in HSC-F cells was limited to a low frequency even by inoculation with 10-fold diluted virus stocks of SS and SN. Truncation of gp41 did not significantly affect replication in HSC-F macaque cells, although truncation of gp41 was disadvantageous for replication in primary T cell cultures from macaques (Hirsch et al., 1989; Kodama et al., 1989).

These results demonstrate that gp41 truncation strikingly increases infectivity for human cells, but not for macaque cells, and that the substitutions in gp120 decrease infectivity in human and macaque cells. Truncation of gp41, which conferred extremely high infectivity for PM1/CCR5 cells, may be the first step to escape from neutralization and the substitutions in gp120 may be the second step to replicate in the presence of high concentration of B404.

Table 2 | Infectivity* of viruses with substitutions from P26B404.

Viruses	PM1	PM1/CCR5	TZM-bl
SIVmac316	4.2E+02 (1.0)	1.4E+03 (1.0)	9.6E+04 (1.0)
SS	2.9E+05 (710)	4.7E+05 (350)	6.3E+06 (66)
SN	2.0E+03 (4.8)	8.4E+03 (6.2)	2.9E+05 (3.1)
NS	2.9E+06 (7,100)	1.4E+06 (1,000)	1.4E+07 (140)

*Infectivity is shown by the TCID₅₀/ml values of the viruses, which were prepared by transfection of 293T cells, in PM1, PM1/CCR5, and TZM-bl cells. The fold-change, which was calculated by dividing the mutant TCID₅₀/ml value by that of the parental SIVmac316, is shown in the parentheses.

INCREASED INCORPORATION OF Env INTO VIRIONS IN SIV WITH TRUNCATED gp41

Incorporation of Env into virions was examined using these recombinant viruses, because increased infectivity by gp41 truncation was suggested to be associated with the Env content of virions (Manrique et al., 2001; Zhu et al., 2003, 2006; Yuste et al., 2004, 2005). Analysis of viral proteins in cells and supernatants from transfected 293T cells revealed that incorporation of Env into virions was significantly high in SS and NS viruses with the Q733stop substitution (Figure 5). MAb to gp120 showed a higher amount

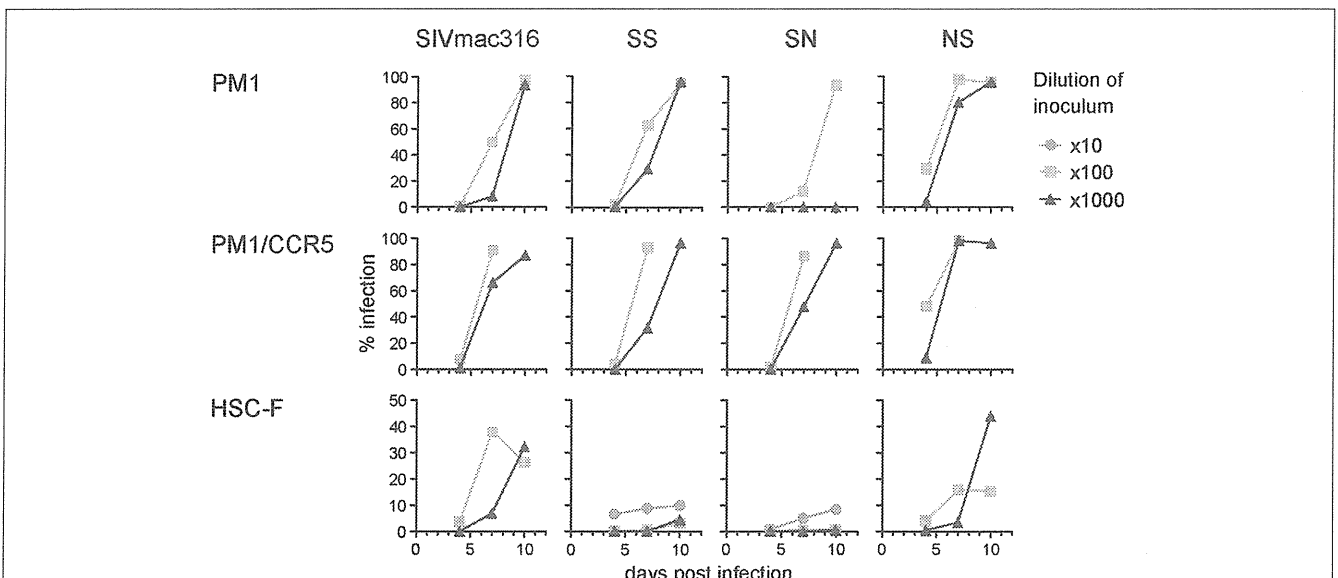


FIGURE 4 | Replication of B404-resistant viruses in human and macaque cells. Frequencies of infected cells at 4, 7, and 10 days post-infection are shown. PM1 and PM1/CCR5 human T cells and HSC-F cynomolgus macaque

T cells were infected with the various dilutions of SIVmac316, SS, SN, and NS. Infected cells were detected using flow cytometry after intracellular staining by B450 (anti-p27 Fab).

of gp120 and gp160 in virions from SS and NS than those from SN and the parental SIVmac316, although the production of Env proteins in the transfected cells was at the same level among all the viruses (Figure 5A). MAb to gp41 also demonstrated that truncated gp41 was more abundant in virions compared with

full-length gp41 (Figure 5B). Semi-quantification by densitometric scanning of gp41 and p26 images suggested that the levels of gp41 amount per virion in SS and NS were 12- and 44-fold higher than that of SIVmac316, respectively, after adjusting virion numbers using the p26 amounts. In contrast to the increased amount of Env proteins in virions from viruses with truncated gp41, the level of Gag p27 in virions was low in SS and NS compared with those in SN and SIVmac316 (Figure 5C). This indicates that the Env content per virion, which was normalized by the amount of p27, was significantly high in viruses with truncated gp41. These results suggest that truncation of gp41 by the Q733stop substitution enhances incorporation of Env into virions.

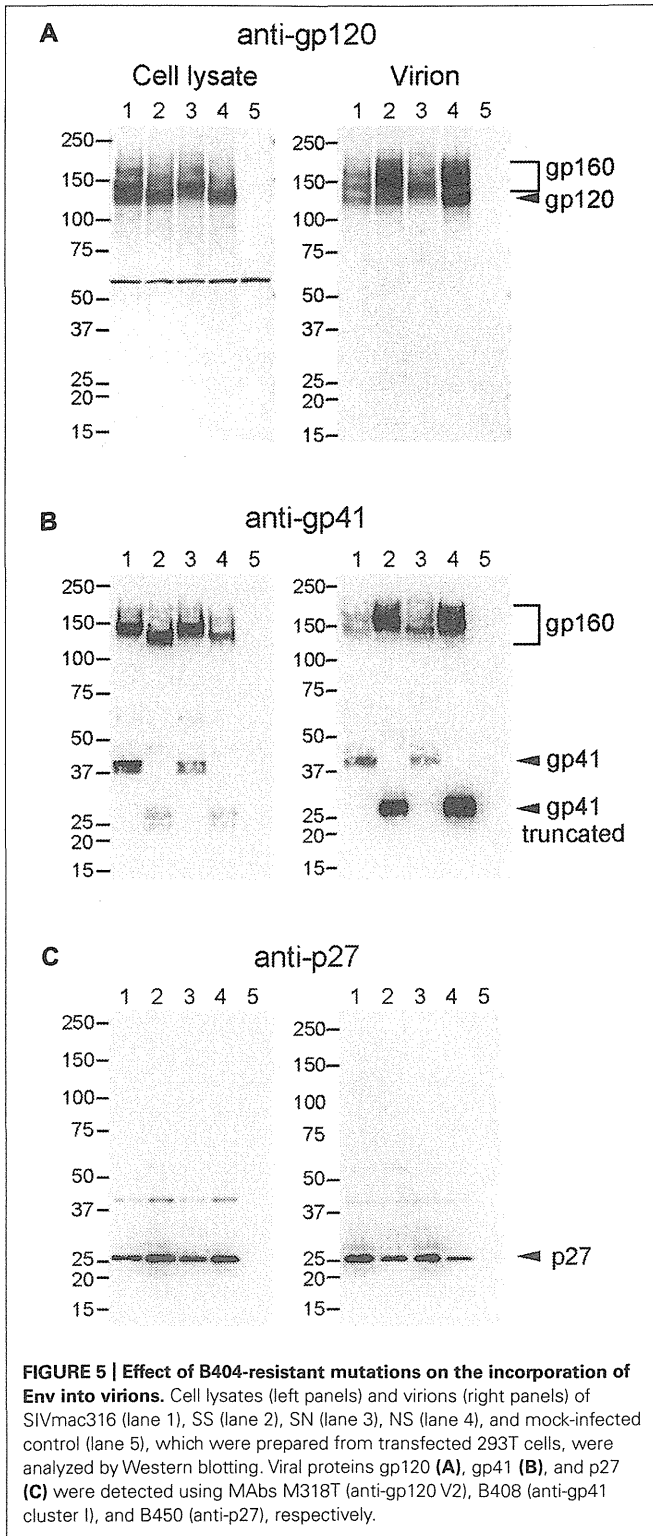
NEUTRALIZATION RESISTANCE OF SIV WITH TRUNCATED gp41 IN MACAQUE CELLS

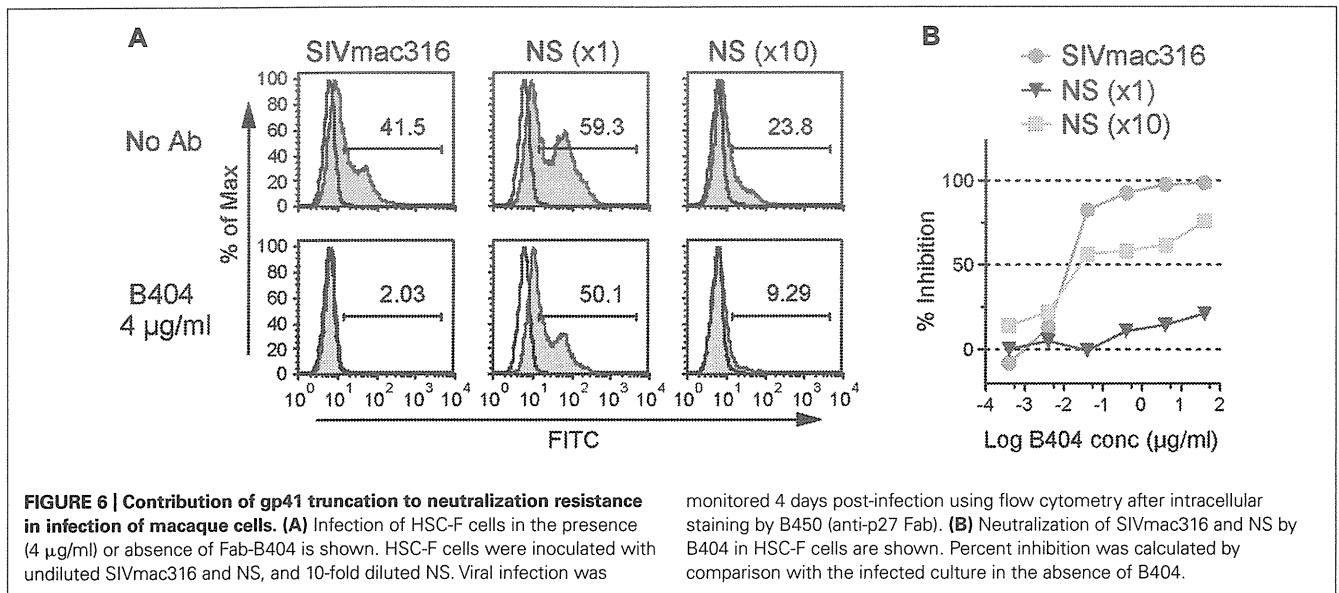
The analysis of infectivity of recombinant viruses suggested that the resistance to neutralization by truncation of gp41 might be due to adaptation to human cells. To examine this hypothesis, sensitivity to neutralization by B404 was determined in HSC-F macaque cells using SIVmac316 and NS, which showed similar infectivity for HSC-F cells (Figure 4). In flow cytometric analysis, infection in the presence or absence of B404 demonstrated that the high sensitivity of SIVmac316 and resistance of NS to neutralization were maintained in HSC-F cells (Figure 6). The frequency of infected cells decreased from 41.5% to the background level (2.03%) in inoculation with the undiluted stock of SIVmac316. In contrast, infection with NS, even with a 10-fold diluted virus stock, was significant in HSC-F cells in the presence of B404 (Figure 6A). Neutralization of NS in HSC-F cells was characterized by a decrease in maximal inhibition (Figure 6B), which was also observed in TZM-bl cells (Figure 3A). The magnitude of resistance of NS to B404 was greater when infection was performed using the undiluted stock compared with the 10-fold diluted stock, raising the possibility that B404 did not inhibit infection with a high titer of viruses. However, the resistance of NS was shown by infection with a low titer of NS, in which the frequency of infected cells in the absence of B404 (23.8%) was lower than infection with undiluted SIVmac316 (41.5%). Further, immunoblotting analysis revealed that the amount of virions was higher in the virus stock of SIVmac316 than that of NS (Figure 5).

These results indicate that gp41 truncation by the Q733stop substitution contributes to neutralization resistance of viruses in macaque cells. This suggests that the resistance to neutralization by truncation of gp41 is not due to the adaptation to human cells. The Q733stop substitution, the first major mutation during passages in the presence of B404, might be selected because it facilitates adaptation of virus to human cells and imparts resistance to antibody.

DISCUSSION

In the present study, truncation of the cytoplasmic tail of gp41, which was caused by the Q733stop substitution in Env, was the first major mutation detected during passage of SIV in the presence of the neutralizing antibody B404. Analysis of recombinant viruses suggested that the gp41 truncation was selected by their resistance to neutralizing antibody, which was characterized by the decrease of maximal inhibition compared with viruses with intact gp41, and





increased infectivity for human cells. The premature stop codon in the gp41 cytoplasmic region was frequently detected in SIV strains propagated in human cell culture *in vitro*, such as the original SIVmac316 clone, SIVmac1A11 and 17E-Fr (Hirsch et al., 1989; Kodama et al., 1989; Mori et al., 1992; Bonavia et al., 2005; Vzorov et al., 2005). The truncation of gp41 is considered as an adaptation of SIV to replication in human cell culture, because the premature stop codon rapidly reverted to express full-length gp41 after infection of rhesus primary cell culture *in vitro* and rhesus macaques *in vivo* (Hirsch et al., 1989; Kodama et al., 1989). Mutant viruses harboring the gp41 truncation showed increased infectivity for human cells, although the effects on infectivity varied depending on the SIV strain and the length of the gp41 truncation (Manrique et al., 2001; Yuste et al., 2004, 2005; Vzorov et al., 2005, 2007). The enhancement effect of gp41 truncation on incorporation of Env into virions, which were demonstrated by quantification of viral proteins in virions (Yuste et al., 2004) and electron tomography analysis of Env trimers on virions (Zhu et al., 2003, 2006), was partly associated with the increased infectivity caused by gp41 truncation (Manrique et al., 2001; Yuste et al., 2004, 2005). Because expression of Env on the cell surface is regulated by the cytoplasmic domain of gp41, truncation of gp41 may increase Env density on both cells and virions (LaBranche et al., 1995; Berlioz-Torrent et al., 1999; Postler and Desrosiers, 2013). Consistent with these studies, infectivity for human cells and Env incorporation into virions was enhanced by gp41 truncation in the present study. Although the mechanism responsible for increasing viral infectivity caused by gp41 truncation remains unclear, the high virion Env content may contribute to the efficient replication of viruses with truncated gp41 in human cells.

The effect of gp41 truncation on susceptibility to antibody-mediated neutralization is controversial, perhaps due to the SIV strains used for the analyses. Because most of prototypic SIV clones with truncated gp41 were macrophage-tropic, CD4-independent, and neutralization-sensitive (Mori et al., 1992; Bonavia et al., 2005; Vzorov et al., 2005), the truncation of gp41 was assumed

responsible for the high sensitivity to neutralization. However, the resistance to neutralization by gp41 truncation was shown using the E767stop mutant of SIVmac316 (Yuste et al., 2005). This is consistent with our results using SIVmac316 harboring the Q733stop substitution, indicating that gp41 truncation contributes to resistance of SIVmac316 to neutralization. The increased infectivity of viruses with gp41 truncation in human cells may partially play a role in resistance by overcoming antibody-mediated neutralization via efficient attachment and entry of viruses to cells. However, we showed that gp41 truncation was also associated with neutralization resistance in macaque cells, in which gp41 truncation did not significantly affect infectivity. This suggests that the increased infectivity in human cells does not significantly affect the neutralization resistance of viruses with truncated gp41. As shown by provision of excess Env *in trans*, high Env content in virions may be critical for antibody-mediated neutralization (Yuste et al., 2005). Further studies will be required to understand the mechanism of resistance to neutralization conferred by gp41 truncation.

In the present study, we demonstrated that truncation of the cytoplasmic tail of gp41 contributes to resistance to antibody-mediated neutralization. Although non-human primate models of SIV infection are commonly used to estimate vaccine efficacy, the lack of broadly neutralizing MAbs has hampered development of antibody-based vaccine candidates in an SIV-macaque model. The broadly neutralizing MAb B404, which neutralizes multiple, diverse SIV isolates (Kuwata et al., 2013), is a useful tool for understanding the mechanism of neutralization in an SIV-macaque model and will contribute to the development of HIV-1 vaccines.

ACKNOWLEDGMENTS

We thank Dr. Hirofumi Akari for providing HSC-F cells. TZM-bl cells were obtained from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc. through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. This work

was supported by MEXT KAKENHI Grant Number 10839786, the Program of Founding Research Centres for Emerging and Re-emerging Infectious Diseases, the Global COE program Global Education and Research Centre Aiming at the Control of AIDS and

a grant-in-aid for scientific research (C-24591484) from the Ministry of Education, Culture, Sport, Science and Technology, Japan and a grant from the Ministry of Health, Welfare and Labour of Japan (H24-AIDS-007).

REFERENCES

- Akari, H., Nam, K. H., Mori, K., Otani, I., Shibata, H., Adachi, A., et al. (1999). Effects of SIVmac infection on peripheral blood CD4+CD8+ T lymphocytes in cynomolgus macaques. *Clin. Immunol.* 91, 321–329.
- Berlioz-Torrent, C., Shacklett, B. L., Erdtmann, L., Delamarre, L., Bouchaert, I., Sonigo, P., et al. (1999). Interactions of the cytoplasmic domains of human and simian retroviral transmembrane proteins with components of the clathrin adaptor complexes modulate intracellular and cell surface expression of envelope glycoproteins. *J. Virol.* 73, 1350–1361.
- Bonavia, A., Bullock, B. T., Gisselman, K. M., Margulies, B. J., and Clements, J. E. (2005). A single amino acid change and truncated TM are sufficient for simian immunodeficiency virus to enter cells using CCR5 in a CD4-independent pathway. *Virology* 341, 12–23.
- Derdeyn, C. A., Decker, J. M., Sfakianos, J. N., Wu, X., O'Brien, W. A., Ratner, L., et al. (2000). Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. *J. Virol.* 74, 8358–8367.
- DuBridge, R. B., Tang, P., Hsia, H. C., Leong, P. M., Miller, J. H., and Calos, M. P. (1987). Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. *Mol. Cell. Biol.* 7, 379–387.
- Hatada, M., Yoshimura, K., Harada, S., Kawanami, Y., Shibata, J., and Matsushita, S. (2010). Human immunodeficiency virus type 1 evasion of a neutralizing anti-V3 antibody involves acquisition of a potential glycosylation site in V2. *J. Gen. Virol.* 91, 1335–1345.
- Haynes, B. F., Gilbert, P. B., Mcelrath, M. J., Zolla-Pazner, S., Tomaras, G. D., Alam, S. M., et al. (2012). Immune-correlates analysis of an HIV-1 vaccine efficacy trial. *N. Engl. J. Med.* 366, 1275–1286.
- Hirsch, V. M., Edmondson, P., Murphey-Corb, M., Arbelite, B., Johnson, P. R., and Mullins, J. I. (1989). SIV adaptation to human cells. *Nature* 341, 573–574.
- Kodama, T., Wooley, D. P., Naidu, Y. M., Kestler, H. W. III, Daniel, M. D., Li, Y., et al. (1989). Significance of premature stop codons in env of simian immunodeficiency virus. *J. Virol.* 63, 4709–4714.
- Kuwata, T., Katsumata, Y., Takaki, K., Miura, T., and Igarashi, T. (2011). Isolation of potent neutralizing monoclonal antibodies from an SIV-Infected rhesus macaque by phage display. *AIDS Res. Hum. Retroviruses* 27, 487–500.
- Kuwata, T., Takaki, K., Yoshimura, K., Enomoto, I., Wu, F., Ourmanov, I., et al. (2013). Conformational epitope consisting of the V3 and V4 loops as a target for potent and broad neutralization of simian immunodeficiency viruses. *J. Virol.* 87, 5424–5436.
- Kwong, P. D., and Mascola, J. R. (2012). Human antibodies that neutralize HIV-1: identification, structures, and B cell ontogenies. *Immunity* 37, 412–425.
- LaBranche, C. C., Sauter, M. M., Haggarty, B. S., Vance, P. J., Romano, J., Hart, T. K., et al. (1995). A single amino acid change in the cytoplasmic domain of the simian immunodeficiency virus transmembrane molecule increases envelope glycoprotein expression on infected cells. *J. Virol.* 69, 5217–5227.
- Lusso, P., Cocchi, F., Balotta, C., Markham, P. D., Louie, A., Farci, P., et al. (1995). Growth of macrophage-tropic and primary human immunodeficiency virus type 1 (HIV-1) isolates in a unique CD4+ T-cell clone (PM1): failure to downregulate CD4 and to interfere with cell-line-tropic HIV-1. *J. Virol.* 69, 3712–3720.
- Manrique, J. M., Celma, C. C., Affranchino, J. L., Hunter, E., and Gonzalez, S. A. (2001). Small variations in the length of the cytoplasmic domain of the simian immunodeficiency virus transmembrane protein drastically affect envelope incorporation and virus entry. *AIDS Res. Hum. Retroviruses* 17, 1615–1624.
- Matsumi, S., Matsushita, S., Yoshimura, K., Javaherian, K., and Takatsuki, K. (1995). Neutralizing monoclonal antibody against an external envelope glycoprotein (gp110) of SIVmac251. *AIDS Res. Hum. Retroviruses* 11, 501–508.
- Mori, K., Ringler, D. J., Kodama, T., and Desrosiers, R. C. (1992). Complex determinants of macrophage tropism in env of simian immunodeficiency virus. *J. Virol.* 66, 2067–2075.
- Platt, E. J., Wehrly, K., Kuhmann, S. E., Chesebro, B., and Kabat, D. (1998). Effects of CCR5 and CD4 cell surface concentrations on infections by macrophage-tropic isolates of human immunodeficiency virus type 1. *J. Virol.* 72, 2855–2864.
- Postler, T. S., and Desrosiers, R. C. (2013). The tale of the long tail: the cytoplasmic domain of HIV-1 gp41. *J. Virol.* 87, 2–15.
- Reed, L. J., and Muench, H. (1938). A simple method of estimating fifty per cent endpoints. *Am. J. Epidemiol.* 27, 493–497.
- Reks-Ngarm, S., Pitisuttithum, P., Nitayaphan, S., Kaewkungwal, J., Chiu, J., Paris, R., et al. (2009). Vaccination with ALVAC and AIDSVAX to prevent HIV-1 infection in Thailand. *N. Engl. J. Med.* 361, 2209–2220.
- Takeuchi, Y., McClure, M. O., and Pizzato, M. (2008). Identification of gammaretroviruses constitutively released from cell lines used for human immunodeficiency virus research. *J. Virol.* 82, 12585–12588.
- Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739.
- Vzorov, A. N., Gernert, K. M., and Compans, R. W. (2005). Multiple domains of the SIV Env protein determine virus replication efficiency and neutralization sensitivity. *Virology* 332, 89–101.
- Vzorov, A. N., Weidmann, A., Kozyr, N. L., Khaoustov, V., Yoffe, B., and Compans, R. W. (2007). Role of the long cytoplasmic domain of the SIV Env glycoprotein in early and late stages of infection. *Retrovirology* 4, 94.
- Wei, X., Decker, J. M., Liu, H., Zhang, Z., Arani, R. B., Kilby, J. M., et al. (2002). Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. *Antimicrob. Agents Chemother.* 46, 1896–1905.
- Yoshimura, K., Shibata, J., Kimura, T., Honda, A., Maeda, Y., Koito, A., et al. (2006). Resistance profile of a neutralizing anti-HIV monoclonal antibody, KD-247, that shows favourable synergism with anti-CCR5 inhibitors. *AIDS* 20, 2065–2073.
- Yusa, K., Maeda, Y., Fujioka, A., Monde, K., and Harada, S. (2005). Isolation of TAK-779-resistant HIV-1 from an R5 HIV-1 GP120 V3 loop library. *J. Biol. Chem.* 280, 30083–30090.
- Yuste, E., Johnson, W., Pavlakis, G. N., and Desrosiers, R. C. (2005). Virion envelope content, infectivity, and neutralization sensitivity of simian immunodeficiency virus. *J. Virol.* 79, 12455–12463.
- Yuste, E., Reeves, J. D., Doms, R. W., and Desrosiers, R. C. (2004). Modulation of Env content in virions of simian immunodeficiency virus: correlation with cell surface expression and virion infectivity. *J. Virol.* 78, 6775–6785.
- Zhu, P., Chertova, E., Bess, J., Lifson, J. D., Arthur, L. O., Liu, J., et al. (2003). Electron tomography analysis of envelope glycoprotein trimers on HIV and simian immunodeficiency virus virions. *Proc. Natl. Acad. Sci. U.S.A.* 100, 15812–15817.
- Zhu, P., Liu, J., Bess, J., Chertova, E., Lifson, J. D., Grisé, H., et al. (2006). Distribution and three-dimensional structure of AIDS virus envelope spikes. *Nature* 441, 847–852.

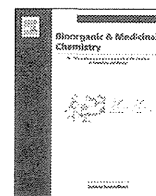
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 22 March 2013; accepted: 25 April 2013; published online: 14 May 2013.

Citation: Kuwata T, Takaki K, Enomoto I, Yoshimura K and Matsushita S (2013) Increased infectivity in human cells and resistance to antibody-mediated neutralization by truncation of the SIV gp41 cytoplasmic tail. *Front. Microbiol.* 4:117. doi: 10.3389/fmicb.2013.00117

This article was submitted to *Frontiers in Virology*, a specialty of *Frontiers in Microbiology*.

Copyright © 2013 Kuwata, Takaki, Enomoto, Yoshimura and Matsushita. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in other forums, provided the original authors and source are credited and subject to any copyright notices concerning any third-party graphics etc.



A CD4 mimic as an HIV entry inhibitor: Pharmacokinetics



Chie Hashimoto^a, Tetsuo Narumi^a, Hiroyuki Otsuki^b, Yuki Hirota^a, Hiroshi Arai^a, Kazuhisa Yoshimura^{c,d}, Shigeyoshi Harada^{c,d}, Nami Ohashi^a, Wataru Nomura^a, Tomoyuki Miura^b, Tatsuhiko Igarashi^b, Shuzo Matsushita^d, Hirokazu Tamamura^{a,*}

^aInstitute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo 101-0062, Japan

^bInstitute for Virus Research, Kyoto University, Kyoto 606-8507, Japan

^cAIDS Research Center, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo 162-8640, Japan

^dCenter for AIDS Research, Kumamoto University, Kumamoto 860-0811, Japan

ARTICLE INFO

Article history:

Received 17 September 2013

Revised 5 October 2013

Accepted 5 October 2013

Available online 17 October 2013

Keywords:

CD4 mimic

HIV entry inhibitor

Intravenous administration

Pharmacokinetics

ABSTRACT

To date, several small molecules of CD4 mimics, which can suppress competitively the interaction between an HIV-1 envelope glycoprotein gp120 and a cellular surface protein CD4, have been reported as viral entry inhibitors. A lead compound **2** (YYA-021) with relatively high potency and low cytotoxicity has been identified previously by SAR studies. In the present study, the pharmacokinetics of the intravenous administration of compound **2** in rats and rhesus macaques is reported. The half-lives of compound **2** in blood in rats and rhesus macaques suggest that compound **2** shows wide tissue distribution and relatively high distribution volumes. A few hours after the injection, both plasma concentrations of compound **2** maintained micromolar levels, indicating it might have promise for intravenous administration when used combinatorially with anti-gp120 monoclonal antibodies.

© 2013 Elsevier Ltd. All rights reserved.

1. Introduction

Several anti-HIV-1 drugs, including protease inhibitors and integrase inhibitors have been developed and have contributed to the highly active anti-retroviral therapy (HAART) used to treat AIDS.¹ Prevention of the HIV-1 infection of its target cells^{1,2} is however a legitimate goal and the viral attachment process is an important target for the development of the drugs which could forestall such infection. The dynamic and supramolecular entry of human immunodeficiency virus type 1 (HIV-1) into target cells is initiated by the interaction of a viral envelope glycoprotein gp120 with the cell surface protein CD4.³ Sequential binding of CD4 and a co-receptor (CCR5 or CXCR4) to gp120 can trigger a series of conformational rearrangements of gp41, a viral transmembrane glycoprotein mediating fusion between the viral and cellular membranes.^{3–6} Control, especially of dynamic conformational changes of the envelope glycoproteins is a very attractive option.⁷ To date, several small molecules that mimic CD4 have been developed as HIV-1 entry inhibitors, which competitively block the binding of gp120 to CD4⁸ and the potential of these CD4 mimics has been explored (Fig. 1).^{9–11} Furthermore, the interaction of CD4 mimics with a highly conserved pocket on gp120, designated as the 'Phe43 cavity', induces conformational

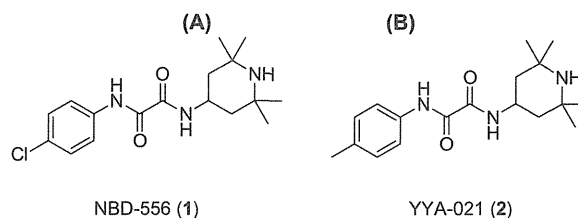


Figure 1. Structures of NBD-556 (**1**) and YYA-021 (**2**).

changes in gp120,¹² a process which occurs with unfavorable binding entropy, and leads to a favorable enthalpy change similar to that caused by binding of the soluble CD4 binding to gp120. Thus, these unique properties render CD4 mimics valuable not only as entry inhibitors but also as 'envelope protein openers' and putatively, stimulants when combined with neutralizing antibodies.¹³

Through our SAR studies a lead compound YYA-021 (**2**) with relatively high potency and low cytotoxicity has been found,¹¹ although the original compound NBD-556 (**1**) had relatively high cytotoxicity. Pharmacokinetic (PK) studies were performed to assess the potential of compound **2** for clinical application and in addition, the possibility and the effectiveness of its use in combination with neutralization antibodies are discussed.

* Corresponding author. Tel.: +81 3 5280 8036; fax: +81 3 5280 8039.

E-mail address: [tamamura.mr@tmd.ac.jp](mailto:tamura.mr@tmd.ac.jp) (H. Tamamura).