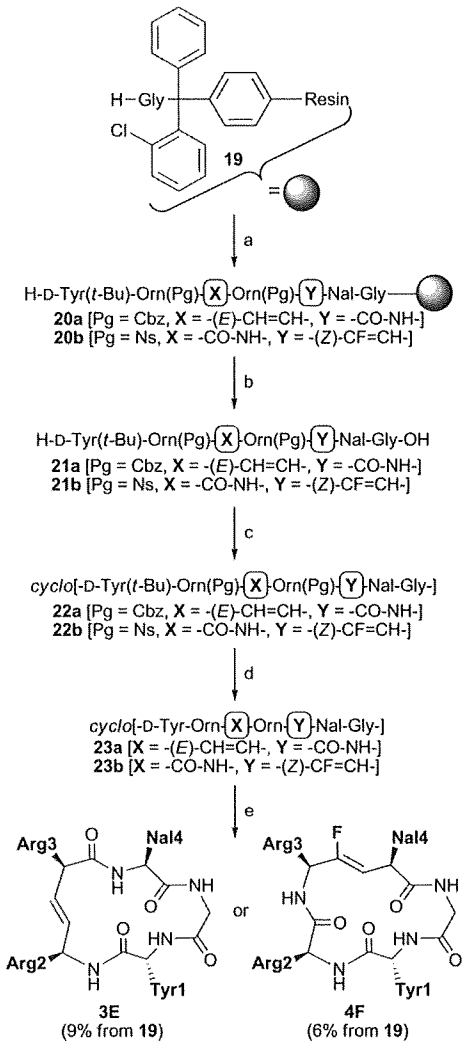

Scheme 3 Synthesis of the alkene analogues of FC131. Reagents and conditions: (a) Fmoc-based SPPS; (b) 1,1,1,3,3,3-hexafluoroisopropanol (HFIP), CH_2Cl_2; (c) diphenylphosphoryl azide (DPPA), $NaHCO_3$, DMF, –40 °C to rt; (d) **23a**: 1 M TMSBr/thioanisole in TFA, *m*-cresol, 1,2-ethandithiol, 6 h; **23b**: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

Biological evaluation of FC131 analogues with EADI and FADI

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

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

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

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

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

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

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

Table 2 Anti-HIV activities of FC131 and the derivatives

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

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

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

Conclusions

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

Experimental

Synthesis

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

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

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

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

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

Peptide synthesis

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

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

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

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

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

Determination of anti-HIV activity

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

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

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Non-Cleavage Site Gag Mutations in Amprenavir-Resistant Human Immunodeficiency Virus Type 1 (HIV-1) Predispose HIV-1 to Rapid Acquisition of Amprenavir Resistance but Delay Development of Resistance to Other Protease Inhibitors▽

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In an attempt to determine whether mutations in Gag in human immunodeficiency virus type 1 (HIV-1) variants selected with a protease inhibitor (PI) affect the development of resistance to the same or a different PI(s), we generated multiple infectious HIV-1 clones carrying mutated Gag and/or mutated protease proteins that were identified in amprenavir (APV)-selected HIV-1 variants and examined their virological characteristics. In an HIV-1 preparation selected with APV (33 passages, yielding HIV_{APVp33}), we identified six mutations in protease and six apparently critical mutations at cleavage and non-cleavage sites in Gag. An infectious recombinant clone carrying the six protease mutations but no Gag mutations failed to replicate, indicating that the Gag mutations were required for the replication of HIV_{APVp33}. An infectious recombinant clone that carried wild-type protease and a set of five Gag mutations ($rHIV_{WTpro}^{12/75/219/390/409gag}$) replicated comparably to wild-type HIV-1; however, when exposed to APV, $rHIV_{WTpro}^{12/75/219/390/409gag}$ rapidly acquired APV resistance. In contrast, the five Gag mutations significantly delayed the acquisition of HIV-1 resistance to ritonavir and nelfinavir (NFV). Recombinant HIV-1 clones containing NFV resistance-associated mutations, such as D30N and N88S, had increased susceptibilities to APV, suggesting that antiretroviral regimens including both APV and NFV may bring about favorable antiviral efficacy. The present data suggest that the preexistence of certain Gag mutations related to PI resistance can accelerate the emergence of resistance to the PI and delay the acquisition of HIV resistance to other PIs, and these findings should have clinical relevance in the therapy of HIV-1 infection with PI-including regimens.

Combination antiretroviral therapy using reverse transcriptase inhibitors and protease inhibitors (PIs) produces substantial suppression of viral replication in human immunodeficiency virus type 1 (HIV-1)-infected patients (3, 27, 28, 42). However, the emergence of drug-resistant HIV-1 variants in such patients has limited the efficacy of combination chemotherapy. HIV-1 variants resistant to all of the currently available antiretroviral therapeutics have emerged both in vitro and in vivo (6, 16, 27, 30). Of note, a number of PI resistance-associated amino acid substitutions in the active site of protease have been identified, and such substitutions have considerable impact on the catalytic activity of protease. This impact is reflected by impaired processing of Gag precursors in mutated-protease-carrying virions and by decreased catalytic efficiency of the protease toward peptides with natural cleavage sites (7, 29, 31, 43).

However, the highly PI-resistant viruses frequently have amino acid substitutions at the p7-p1 and p1-p6 cleavage sites in Gag. These mutations have been identified in PI-resistant HIV-1 variants selected in vitro (2, 5, 8, 29) and in HIV-1 isolated from patients with AIDS for whom chemotherapy including PIs was failing (26, 40, 47, 48). These mutations are known to compensate for the enzymatic impairment of protease, per se, resulting from the acquisition of PI resistance-conferring mutations within the protease-encoding region. Moreover, certain mutations at non-cleavage sites in Gag have been shown previously to be essential for the replication of HIV-1 variants in the presence of PIs (14, 15). Although a few amino acid substitutions at cleavage and non-cleavage sites in Gag have been shown to be associated with resistance to PIs, the roles and impact of amino acid substitutions in Gag for the HIV-1 acquisition of PI resistance remain to be elucidated.

In the present study, we identified novel Gag non-cleavage site mutations in addition to multiple mutations in the protease gene during in vitro selection of HIV-1 variants highly resistant to amprenavir (APV). We show that the non-cleavage site mutations were important for not only the replication of the mutated-protease-carrying HIV-1 but also the accelerated acquisition of HIV-1 resistance to APV and an unrelated PI, nelfinavir (NFV). We also show that recombinant HIV-1 clones containing NFV resistance-associated mutations, such

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as D30N and N88S, had increased susceptibility to APV, suggesting that antiretroviral regimens including both APV and NFV may bring about favorable antiviral efficacy.

MATERIALS AND METHODS

Cells and antiviral agents. MT-2 and MT-4 cells were grown in RPMI 1640-based culture medium, and 293T cells were propagated in Dulbecco's modified Eagle's medium. These media were supplemented with 10% fetal calf serum (HyClone, Logan, UT), 50 U/ml penicillin, and 50 μg/ml streptomycin. APV was kindly provided by GlaxoSmithKline, Research Triangle Park, NC. Saquinavir (SQV) and ritonavir (RTV) were provided by Roche Products Ltd. (Welwyn Garden City, United Kingdom) and Abbott Laboratories (Abbott Park, IL), respectively. NFV and indinavir (IDV) were kindly provided by Japan Energy Inc., Tokyo.

Generation of PI-resistant HIV-1 in vitro. For the generation of PI-resistant HIV-1, various PI-resistant HIV-1 strains were propagated in the presence of increasing concentrations of a drug in a cell-free fashion as described previously (44, 45). In brief, on the first passage, MT-2 or MT-4 cells (5×10^5) were exposed to 500 50% tissue culture infective doses ($TCID_{50}$) of each infectious molecular HIV-1 clone and cultured in the presence of various PIs at initial concentrations of 0.01 to 0.06 μM. On the last day of each passage (approximately day 7), 1 ml of the cell-free supernatant was harvested and transferred to a culture of fresh uninfected cells in the presence of increased concentrations of the drug for the following round of culture. In this round of culture, three drug concentrations (increased by one-, two-, and threefold compared to the previous concentration) were employed. When the replication of HIV-1 in the culture was confirmed by substantial Gag protein production (greater than 200 ng/ml), the highest drug concentration among the three concentrations was used to continue the selection (for the next round of culture). This protocol was repetitively used until the drug concentration reached the targeted concentration. Proviral DNA from the lysates of infected cells at various passages was subjected to nucleotide sequencing.

Determination of nucleotide sequences. Molecular cloning and the determination of nucleotide sequences of HIV-1 passaged in the presence of each PI were performed as described previously (44, 45). In brief, high-molecular-weight DNA was extracted from HIV-1-infected MT-2 and MT-4 cells by using the InstaGene matrix (Bio-Rad Laboratories, Hercules, CA) and was subjected to molecular cloning, followed by sequence determination. The primers used for the first-round PCR amplification of the entire Gag- and protease-encoding regions of the HIV-1 genome were LTR F1 (5′-GAT GCT ACA TAT AAG CAG CTG C-3′) and PR12 (5′-CTC GTG ACA AAT TTC TAC TAA TGC-3′). The first-round PCR mixture consisted of 5 μl of proviral DNA solution, 2.0 U of premix *Taq* (Ex *Taq* version; Takara Bio Inc., Otsu, Japan), and 12.5 pmol of each of the first-round PCR primers in a total volume of 50 μl. The PCR conditions used were an initial 2-min step at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 3 min at 72°C, with a final 8 min of extension at 72°C. The first-round PCR products (1 μl) were used directly in the second round of PCR with primers LTR F2 (5′-GAG ACT CTG GTA ACT AGA GAT C-3′) and Ksma2.1 (5′-CCA TCC CGG GCT TTA ATT TTA CTG GTA C-3′) under the same PCR conditions described above. The second-round PCR products were purified with spin columns (MicroSpin S-400 HR; Amersham Biosciences Corp., Piscataway, NJ), cloned directly, and subjected to sequencing with a model 377 automated DNA sequencer (Applied Biosystems, Foster City, CA).

Generation of recombinant HIV-1 clones. The PCR products obtained as described above were digested with two of the three enzymes BssHII, ApaI, and SmaI, and the obtained fragments were introduced into $pHIV\text{-}1_{NLSma}$, designed to have a SmaI site by changing two nucleotides (2590 and 2593) of $pHIV\text{-}1_{NL4\text{-}3}$ (15, 19). To generate HIV-1 clones carrying the mutations, site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was performed, and the mutation-containing genomic fragments were introduced into $pHIV\text{-}1_{NLSma}$. Determination of the nucleotide sequences of plasmids confirmed that each clone had the desired mutations but no unintended mutations. 293T cells were transfected with each recombinant plasmid by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), and the thus-obtained infectious virions were harvested 48 h after transfection and stored at −80°C until use.

Drug sensitivity assays. Assays for HIV-1 p24 Gag protein production were performed with MT-4 cells as described previously (1, 20, 24). In brief, MT-4 cells (10^5/ml) were exposed to 100 $TCID_{50}$ of infectious molecular HIV-1 clones in the presence or absence of various concentrations of drugs and were incubated at 37°C. On day 7 of culture, the supernatant was harvested and the amounts of p24 Gag protein were determined by using a fully automated chemiluminescent enzyme immunoassay system (Lumipulse F; Fujirebio Inc., Tokyo). The drug concentrations that suppressed the production of p24 Gag protein by 50% (50% inhibitory concentrations [IC_{50}]) were determined by comparing the levels of p24 production with that in a drug-free control cell culture. All assays were performed in triplicate.

Replication kinetic assay. MT-2 or MT-4 cells (10^5) were exposed to each infectious HIV-1 clone (5 ng of p24 Gag protein/ml) for 3 h, washed twice with phosphate-buffered saline, and cultured in 10 ml of complete medium as described previously (1, 14). Culture supernatants (50 μl) were harvested every other day, and the p24 Gag amounts were determined as described above.

CHRA. Two titrated infectious clones to be compared for their replicative capabilities or fitness in the competitive HIV-1 replication assay (CHRA) were combined and added to freshly prepared MT-4 cells (2×10^5) in the presence or absence of various concentrations of PIs as described previously (21, 36). Briefly, a fixed amount (200 $TCID_{50}$) of one infectious clone was combined with three different amounts (100, 200, and 300 $TCID_{50}$) of the other infectious clone, and the mixture was added to the culture of MT-4 cells. On the following day, one-third of infected MT-4 cells were harvested and washed twice with phosphate-buffered saline, and cellular DNA was extracted and subjected to nested PCR and sequencing as described above. The HIV-1 coculture that best approximated a 50:50 mixture on day 1 was further propagated, and the remaining cultures were discarded. Every 7 days, the cell-free supernatant of the virus coculture was transmitted to fresh uninfected MT-4 cells. The cells harvested at the end of each passage were subjected to direct DNA sequencing, and viral population changes were determined. The persistence of the original amino acid substitutions was confirmed for all infectious clones used in this assay.

Statistical analysis of selection profiles of infectious HIV-1 clones. The selection profiles of various infectious HIV-1 clones were compared as follows. The logarithms of the concentrations were modeled as normally distributed variables with possible left censoring. The mean was assumed to be a quadratic function of the passage number. The difference between two curves was assessed by combining the estimated covariance-weighted differences of the linear and quadratic coefficients and comparing the result to computer simulations for the same quantity generated under the specific null hypothesis for that difference. SAS 9.1.3 (SAS Institute, Cary, NC) was used for all the computations. All *P* values are two tailed, and for figures with more than two curves, the values were corrected by the Hochberg method for multiple pairwise comparisons.

RESULTS

Amino acid sequences of Gag and protease of HIV-1 passaged in the presence of APV. A wild-type HIV-1 strain (HIV_{WT}) was propagated in MT-2 cells in the presence of increasing concentrations of APV, and the proviral DNA sequences in those MT-2 cells were determined at passages 3, 12, and 33 (Fig. 1). By passage 3, when HIV-1 was propagating in the presence of 0.04 μM APV (yielding HIV_{APVp3}), no amino acid substitutions in protease were identified but 5 of 10 clones had acquired the substitution of arginine for leucine at position 75 (L75R) in Gag. By passage 12 (at 0.18 μM APV), two APV-related resistance mutations (L10F and M46L) in protease had emerged and one mutation (H219Q) in Gag had been added. By passage 33 (at 10 μM; yielding HIV_{APVp33}), six APV-related amino acid substitutions, one primary mutation (I84V) and five secondary mutations (L10F, V32I, M46I, I54M, and A71V), in protease had emerged (Fig. 1A). In addition, a p1-p6 cleavage site mutation in Gag (L449F) was identified in all 10 HIV-1 clones of HIV_{APVp33} examined, and five non-cleavage site mutations (E12K, L75R, H219Q, V390D, and R409K) were seen in Gag of HIV_{APVp33} (Fig. 1B). Cleavage site mutations have been known to emerge when amino acid substitutions in protease are accumulated and HIV-1 develops resistance to PIs both in vitro and in vivo (5, 8). Intriguingly, the present data suggest that certain amino acid substitutions in non-cleavage sites of Gag (i.e., L75R and

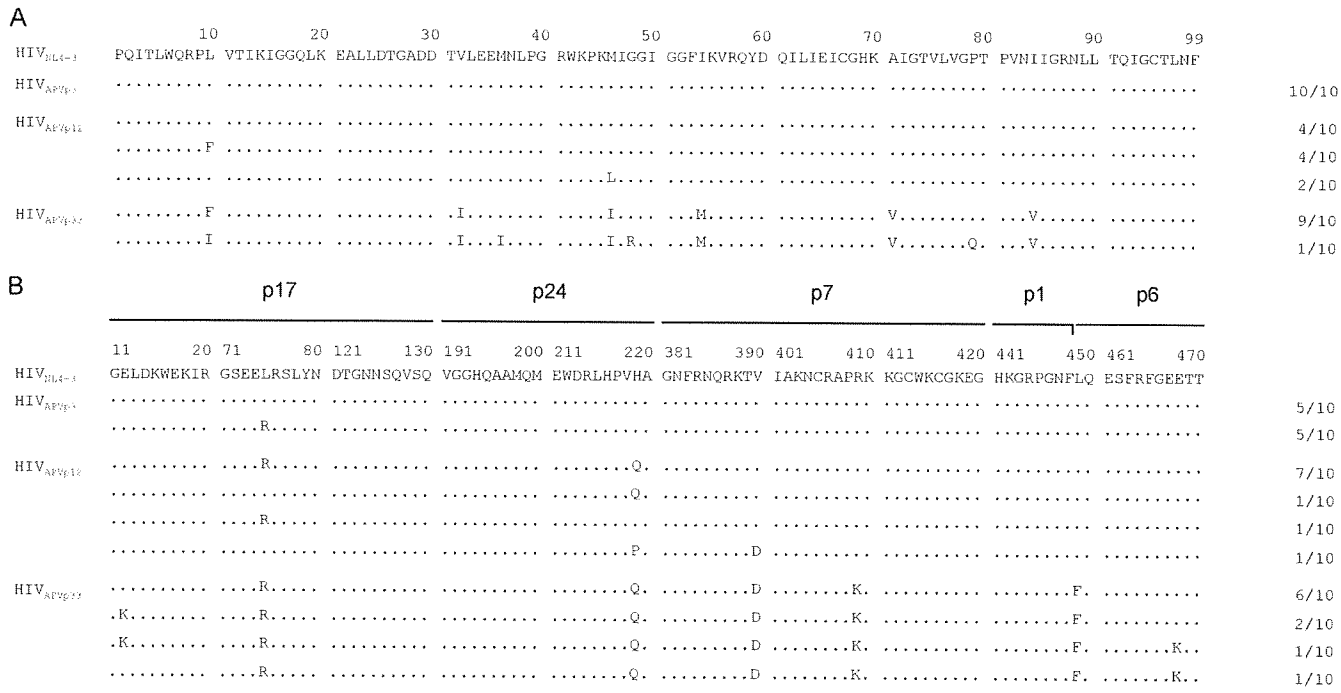

FIG. 1. Amino acid sequences deduced from the nucleotide sequences of protease (A)- and Gag (B)-encoding regions of proviral DNA isolated at the indicated passages (p3, p12, and p33) from HIV-1_{NL4-3} variants selected in the presence of APV. The amino acid sequences of the protease and Gag proteins of wild-type HIV-1_{NL4-3} are shown at the top as a reference. Identity to the sequence at individual amino acid positions is indicated by dots. The numbers of clones with the given amino acid substitutions among a total of 10 clones are listed.

H219Q) may emerge earlier and in greater numbers than amino acid substitutions in protease, at least in the case of HIV-1 selection with APV. The amino acid substitutions that emerged in the virus and the pattern and order of such substitutions were largely in agreement with the data in the previous report by Gatanaga et al. (15). The present results suggested that the non-cleavage site mutations observed may play a key role in the development of HIV-1 resistance against PIs and that especially the two Gag mutations H219Q and R409K may be required for the development of PI resistance.

Mutations in Gag are required for the replication of HIV_{APVp33}. In order to examine the effects of the mutations identified in Gag as described above on the replication profile of HIV-1, we generated infectious recombinant HIV-1 clones containing the six mutations (L10F, V32I, M46I, I54V, A71V, and I84V) in protease seen in HIV_{APVp33}. A recombinant HIV-1 clone containing the protease of HIV_{APVp33} plus a wild-type Gag ($rHIV_{APVp33pro}^{WTgag}$) or the L449F cleavage site mutation-containing Gag ($rHIV_{APVp33pro}^{449gag}$) failed to replicate in MT-2 cells over the 7-day period of culture (Fig. 2A), indicating that these Gag species do not support the growth of HIV_{APVp33}. Therefore, we next generated a recombinant HIV-1 clone containing the protease of HIV_{APVp33} and the Gag protein with the five non-cleavage site mutations (E12K, L75R, H219Q, V390D, and R409K; $rHIV_{APVp33pro}^{12/75/219/390/409gag}$), which replicated moderately under the same conditions (Fig. 2A). The addition of the cleavage site mutation L449F, generating $rHIV_{APVp33pro}^{12/75/219/390/409/449gag}$, further improved the replication of the virus. In MT-4 cells, in which HIV-1 generally replicates more quickly and efficiently than in MT-2 cells, $rHIV_{APVp33pro}^{WTgag}$ and $rHIV_{APVp33pro}^{449gag}$ replicated moderately; however, both $rHIV_{APVp33pro}^{12/75/219/390/409gag}$ and $rHIV_{APVp33pro}^{12/75/219/390/409/449gag}$ replicated comparably to HIV_{WT} (Fig. 2B), due presumably to the greater replication of HIV-1 in MT-4 cells, making the difference relatively indistinct. These data clearly indicate that both non-cleavage site and cleavage site mutations in Gag contribute to the robust fitness of HIV_{APVp33}. We also attempted to examine the effects of combined Gag mutations on the replication of HIV-1 containing wild-type protease and generated three recombinant HIV clones, $rHIV_{WTpro}^{75/219gag}$, $rHIV_{WTpro}^{219/409gag}$, and $rHIV_{WTpro}^{12/75/219/390/409gag}$. The replication rates of these three recombinant clones turned out to be comparable to that of HIV_{WT} when examined in MT-2 and MT-4 cells (Fig. 2C and D), unlike the finding by Doyon and his colleagues that the cleavage site mutation L449F compromised the replication of HIV-1 containing wild-type protease (8).

Gag mutations predispose HIV-1 to rapidly acquire APV resistance. The appearance of two non-cleavage site mutations (L75R and H219Q) in Gag prior to the emergence of mutations in protease (Fig. 1) prompted us to examine whether these two Gag mutations predisposed the virus to the acquisition of APV resistance-associated mutations in protease. We thus attempted to select APV-resistant HIV-1 by propagating HIV_{NL4-3} (HIV_{WT}) and $rHIV_{WTpro}^{75/219gag}$ in the presence of increasing concentrations of APV (Fig. 3). When we compared the selection curves of these two viruses, there was no significant difference (P, 0.53 and 0.65 for propagation in MT-2 and MT-4 cells, respectively). We then examined the effects of two mutated Gag species containing two and five mutations (H219Q and R409K and E12K, L75R, H219Q, V390D, and

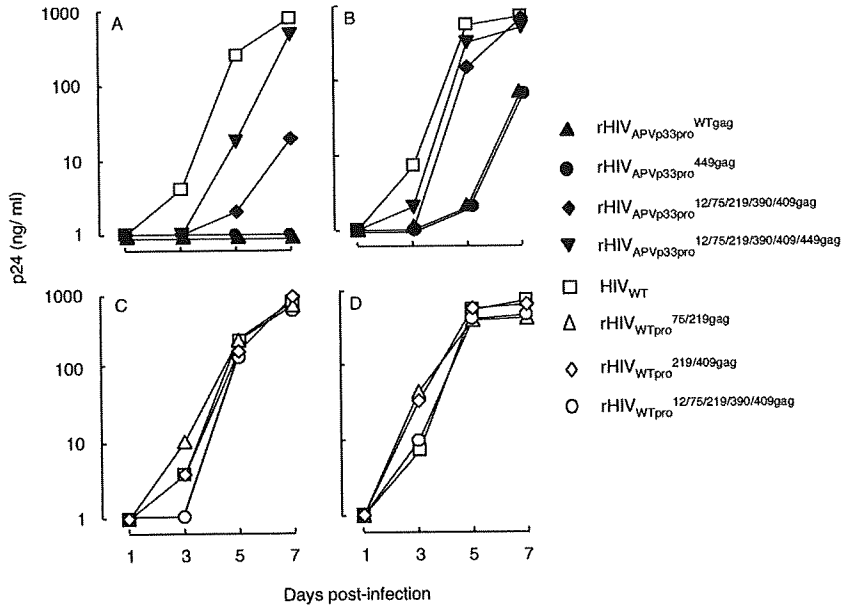

FIG. 2. Replication kinetics of Gag mutant clones with or without protease mutations. MT-2 cells (A and C) and MT-4 cells (B and D) were exposed to Gag mutant clones with (A and B) or without (C and D) protease mutations. Virus replication was monitored by the amounts of p24 Gag produced in the culture supernatants. The results shown are representative of results from three independent experiments. HIV_{APVp33} variants had six mutations (L10F, V32I, M46I, I54M, A71V, and I84V) in the viral protease.

R409K [yielding $mGag^{12/75/219/390/409gag}$], respectively) on the selection curves. The selection profile of a newly generated recombinant HIV clone ($rHIV_{WTpro}^{219/409gag}$) was not different from that of HIV_{WT} in MT-2 cells ($P = 0.22$); however, $rHIV_{WTpro}^{219/409gag}$ acquired resistance to APV much earlier than HIV_{WT} when propagated in MT-4 cells ($P < 0.0001$). The recombinant clone with five non-cleavage site mutations ($rHIV_{WTpro}^{12/75/219/390/409gag}$) started to propagate in both cell lines in the presence of APV significantly earlier than HIV_{WT}, with P values of 0.0080 and <0.0001 for MT-2 and MT-4 cells, respectively (Fig. 3).

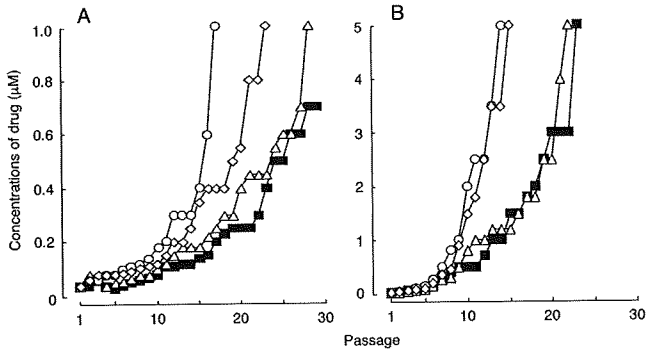

FIG. 3. In vitro selection of APV-resistant variants using HIV-1 carrying Gag mutations. HIV_{WT} (■) and three infectious HIV clones, $rHIV_{WTpro}^{75/219gag}$ (△), $rHIV_{WTpro}^{219/409gag}$ (◇), and $rHIV_{WTpro}^{12/75/219/390/409gag}$ (○), were propagated in the presence of increasing concentrations of APV (starting at 0.03 μM) in MT-2 cells (A) or MT-4 cells (B). The selection was carried out in a cell-free manner for a total of 14 to 29 passages. The results of statistical evaluation of the selection profiles are as follows: panel A, HIV_{WT} versus $rHIV_{WTpro}^{75/219gag}$, $P = 0.53$; HIV_{WT} versus $rHIV_{WTpro}^{12/75/219/390/409gag}$, $P = 0.0080$; HIV_{WT} versus $rHIV_{WTpro}^{219/409gag}$, $P = 0.22$; $rHIV_{WTpro}^{75/219gag}$ versus $rHIV_{WTpro}^{12/75/219/390/409gag}$, $P = 0.0065$; $rHIV_{WTpro}^{75/219gag}$ versus $rHIV_{WTpro}^{219/409gag}$, $P = 0.15$; and $rHIV_{WTpro}^{12/75/219/390/409gag}$ versus $rHIV_{WTpro}^{219/409gag}$, $P = 0.0018$, and panel B, HIV_{WT} versus $rHIV_{WTpro}^{75/219gag}$, $P = 0.65$; HIV_{WT} versus $rHIV_{WTpro}^{12/75/219/390/409gag}$, $P < 0.0001$; HIV_{WT} versus $rHIV_{WTpro}^{219/409gag}$, $P < 0.0001$; $rHIV_{WTpro}^{75/219gag}$ versus $rHIV_{WTpro}^{12/75/219/390/409gag}$, $P < 0.0001$; $rHIV_{WTpro}^{75/219gag}$ versus $rHIV_{WTpro}^{219/409gag}$, $P < 0.0001$; and $rHIV_{WTpro}^{12/75/219/390/409gag}$ versus $rHIV_{WTpro}^{219/409gag}$, $P = 0.088$.

We then asked whether additional amino acid substitutions occurred and accelerated the acquisition of APV resistance by the virus when the Gag mutations were present. To investigate this issue, we determined the nucleotide sequence of the protease-encoding gene of each virus. Only one protease mutation (L10F) was seen by passage 20 when HIV_{WT} and $rHIV_{WTpro}^{75/219gag}$ were propagated in MT-2 cells in the presence of APV (Fig. 4A and B). In contrast, two mutations (M46L and I84V) had been acquired by $rHIV_{WTpro}^{219/409gag}$ by passage 20. Of note, when $rHIV_{WTpro}^{12/75/219/390/409gag}$ was propagated in MT-2 cells in the presence of APV, a mutation (L10F) had occurred by an early passage (passage 5) and four mutations (L10F, V32I, M46I, and I84V) had emerged by passage 17 (Fig. 4D). When examined in MT-4 cells, HIV_{WT} and $rHIV_{WTpro}^{75/219gag}$ had acquired two mutations (L10F and I84V and M46L and I84V, respectively) by passage 10, although $rHIV_{WTpro}^{219/409gag}$ and $rHIV_{WTpro}^{12/75/219/390/409gag}$ had acquired three and four mutations (L10F, M46I, and I84V and L10F, V32I, M46I, and I84V, respectively) by the same passage (Fig. 4E to H). These data, taken together, indicate that the two sets of Gag mutations (H219Q and R409K and E12K, L75R, H219Q, V390D, and R409K) clearly predisposed the virus to rapidly acquire APV resistance-associated mutations in protease and begin to propagate in the presence of APV.

Gag mutations in HIV_{APVp33} delay viral acquisition of resistance to other PIs. We next asked whether the presence of

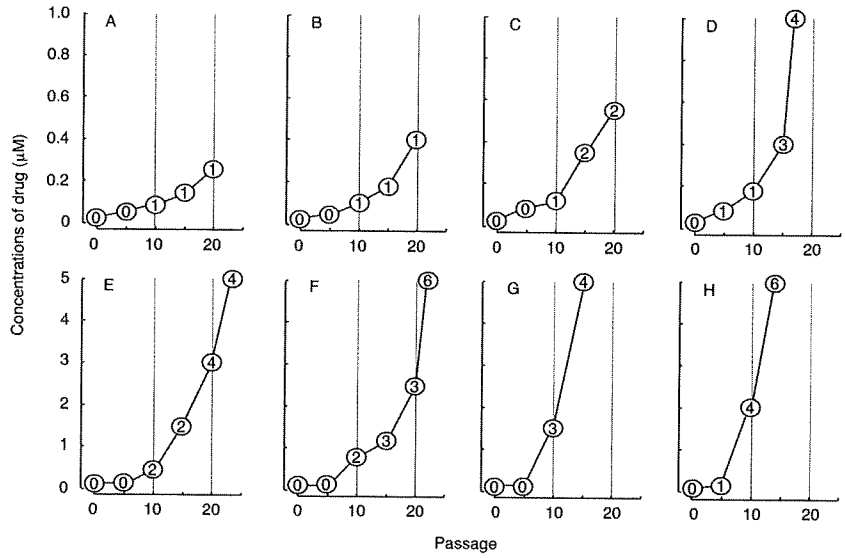

FIG. 4. Number of amino acid substitutions corresponding to the protease-encoding region of each infectious HIV-1 clone selected in the presence of APV. Nucleotide sequences of proviral DNA of HIV_{WT} (A and E) and three infectious HIV-1 clones, $rHIV_{WTpro}^{75/219gag}$ (B and F), $rHIV_{WTpro}^{219/409gag}$ (C and G), and $rHIV_{WTpro}^{12/75/219/390/409gag}$ (D and H), were determined using lysates of HIV-1-infected MT-2 cells (A to D) and MT-4 cells (E to H) at the termination of each passage and compared to the nucleotide sequence of $HIV\text{-}1_{NL4\text{-}3}$. The number within each symbol represents the number of mutations identified in the protease when each infectious HIV-1 clone was selected in the presence of APV.

the five Gag mutations (E12K, L75R, H219Q, V390D, and R409K) accelerated the viral acquisition of resistance to other currently available PIs (SQV, IDV, RTV, and NFV) (Fig. 5). To this end, we propagated two HIV-1 strains (HIV_{WT} and $rHIV_{WTpro}^{12/75/219/390/409gag}$) in MT-4 cells in the presence of increasing concentrations of each PI and compared the replication profiles. The initial drug concentrations used were 0.01 μM for SQV, 0.03 μM for IDV and NFV, and 0.06 μM for RTV, and each virus was selected by a concentration of up to 5 μM. The selection was carried out in a cell-free manner for a total of 13 to 32 passages as described previously (44, 45). There was no significant difference in the selection profiles of the two strains when they were passaged in the presence of SQV ($P = 0.8$) or IDV ($P = 0.22$) (Fig. 5A and B). However, $rHIV_{WTpro}^{12/75/219/390/409gag}$ started to replicate significantly later in the presence of RTV ($P = 0.0001$ (Fig. 5C). The selection profiles of HIV_{WT} and $rHIV_{WTpro}^{12/75/219/390/409gag}$ in the presence of NFV were examined in two independent experiments. Both curves in the first and second sets depicted in Fig. 5D showed statistically significant difference, with P values of <0.0001 and 0.0016, respectively. These data strongly suggest that the Gag mutations seen in HIV_{APVp33} predispose HIV-1 to the rapid acquisition of APV resistance; however, such Gag mutations delay the viral acquisition of resistance to other PIs.

Gag mutations seen in HIV_{APVp33} do not affect viral susceptibilities to PIs. Since the Gag mutations seen in HIV_{APVp33} were found to contribute to the rapid acquisition of viral resistance to APV but they delayed the emergence of viral resistance to other PIs, we examined whether such Gag mutations affected the susceptibilities of HIV-1 to various PIs in the HIV-1 drug susceptibility assay. As shown in Table 1, none of three sets of Gag mutations, as examined in the context of $rHIV_{WTpro}^{75/219gag}$, $rHIV_{WTpro}^{219/409gag}$, and

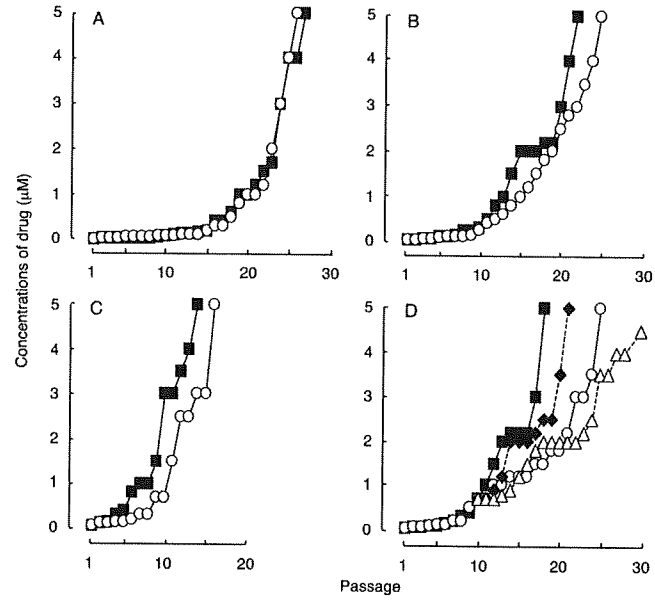

FIG. 5. In vitro selection of PI-resistant variants using HIV-1 carrying Gag mutations. HIV_{WT} (■ and ◆) and $rHIV_{WTpro}^{12/75/219/390/409gag}$ (○ and △) were propagated in MT-4 cells in the presence of increasing concentrations of SQV (A), IDV (B), RTV (C), or NFV (D). The initial drug concentrations used were 0.01 μM for SQV, 0.03 μM for IDV and NFV, and 0.06 μM for RTV, and each virus was selected by up to a 5 μM concentration of each PI. The selection was carried out in a cell-free manner for a total of 13 to 32 passages. NFV selection was performed twice. Data from the first selection are shown with a solid line; the second selection was started using the HIV-1 from passage 10 of the first selection (with NFV at 0.7 μM), and the data are shown with a dashed line. The results of statistical evaluation of the selection profiles are as follows: panel A, $P = 0.80$; panel B, $P = 0.22$; panel C, $P = 0.0001$; and panel D, first selection, $P < 0.0001$, and second selection, $P = 0.0016$.

TABLE 1. Sensitivities of infectious HIV-1 clones with Gag mutations to various PIs

Infectious HIV-1 clone	IC_{50}[a] (μM) of:				
	APV	SQV	IDV	RTV	NFV
HIV_{WT}	0.031 ± 0.0008	0.021 ± 0.002	0.032 ± 0.002	0.032 ± 0.0005	0.028 ± 0.002
$rHIV_{WTpro}^{75/219gag}$	0.031 ± 0.003	0.017 ± 0.003	0.032 ± 0.003	0.031 ± 0.0007	0.029 ± 0.003
$rHIV_{WTpro}^{219/409gag}$	0.029 ± 0.003	0.020 ± 0.01	0.032 ± 0.001	0.031 ± 0.004	0.028 ± 0.002
$rHIV_{WTpro}^{12/75/219/390/409gag}$	0.032 ± 0.0001	0.023 ± 0.005	0.032 ± 0.003	0.032 ± 0.0001	0.028 ± 0.002

[a] Data shown are mean values (with 1 standard deviation) derived from the results of three independent experiments conducted in triplicate. The IC_{50}s were determined by employing MT-4 cells exposed to each infectious HIV-1 clone (50 $TCID_{50}$) in the presence of each PI, with the inhibition of p24 Gag protein production as an end point.

$rHIV_{WTpro}^{12/75/219/390/409gag}$, affected the susceptibility of HIV-1 to any of five PIs (APV, SQV, IDV, RTV, and NFV). Indeed, the IC_{50}s for HIV_{WT} were highly comparable to those for any of the three recombinant clones carrying combined Gag mutations.

Replication rate difference is not the cause of the contrasting resistance acquisition patterns. Our observations of the contrasting resistance acquisition patterns, in which $rHIV_{WTpro}^{12/75/219/390/409gag}$ acquired resistance to APV more rapidly than HIV_{WT} when selected with APV (Fig. 3) and $rHIV_{WTpro}^{12/75/219/390/409gag}$ significantly delayed the acquisition of resistance to other PIs compared to HIV_{WT} (Fig. 5), prompted us to ask whether the replication rates of $rHIV_{WTpro}^{12/75/219/390/409gag}$ and HIV_{WT} were differentially affected by the presence of PIs. We therefore compared the replication rates of $rHIV_{WTpro}^{12/75/219/390/409gag}$ and HIV_{WT} in the presence or absence of APV, SQV, IDV, RTV, or NFV by using the CHRA (21). As shown in Fig. 6, $rHIV_{WTpro}^{12/75/219/390/409gag}$ outgrew HIV_{WT} regardless of the absence or presence of PIs. Comparing the divergence patterns of the curves for $rHIV_{WTpro}^{12/75/219/390/409gag}$ and HIV_{WT} in the absence and presence of APV (Fig. 6A and B) revealed that those for growth in the presence of APV diverged more quickly than those for growth in the absence of APV (Fig. 6B). However, similar divergence patterns were seen with SQV, IDV, RTV, and NFV (Fig. 6C, D, E, and F), suggesting that the replication advantage of $rHIV_{WTpro}^{12/75/219/390/409gag}$ seen in the CHRA was not the cause for the observed contrasting resistance acquisition patterns.

NFV resistance-conferring protease mutations increase HIV-1 susceptibility to APV. There have been reports that an

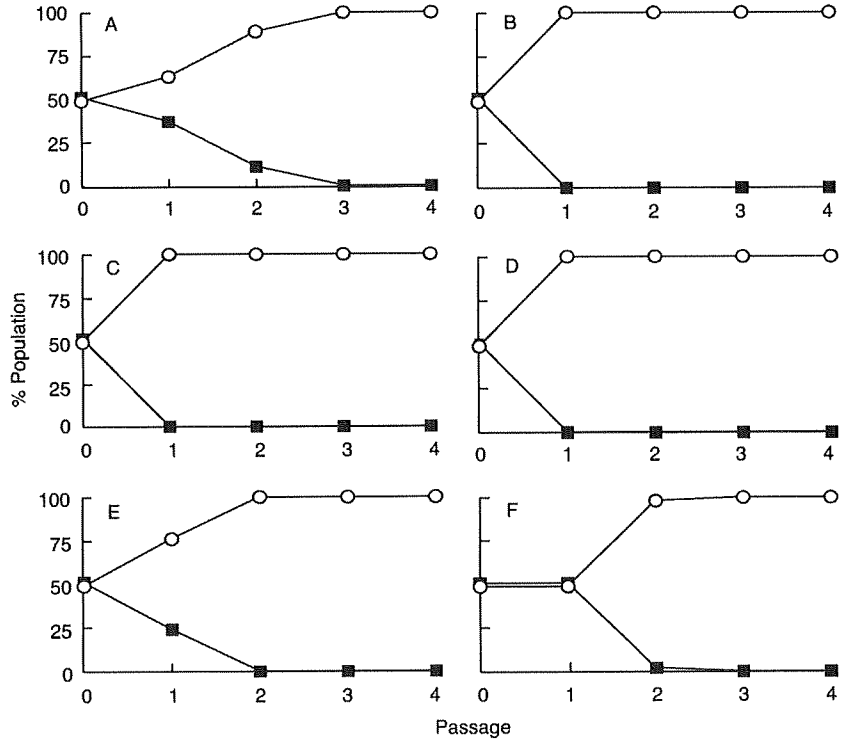

FIG. 6. Results from the CHRA for HIV_{WT} and $rHIV_{WTpro}^{12/75/219/390/409gag}$ in the absence or presence of each drug. Replication profiles of HIV_{WT} (■) and $rHIV_{WTpro}^{12/75/219/390/409gag}$ (○) in the absence (A) or presence of 0.03 μM APV (B), 0.02 μM SQV (C), 0.03 μM IDV (D), 0.03 μM RTV (E), or 0.03 μM NFV (F) were examined by the CHRA. The cell-free supernatant was transferred to fresh MT-4 cells every 7 days. High-molecular-weight DNA extracted from infected cells at the end of each passage was subjected to nucleotide sequencing, and the proportions of Arg and Lys at position 409 in Gag were determined.

TABLE 2. Phenotypic sensitivities of recombinant HIV-1 clones passaged with NFV[a]

Infectious HIV-1 clone	IC_{50} (μM) ± SD (change, *n*-fold) of:	
	APV	NFV
HIV_{WT}	0.031 ± 0.0008 (1)	0.028 ± 0.002 (1)
$rHIV_{N88Spro}{}^{WTgag}$	0.0015 ± 0.0007 (0.05)	0.028 ± 0.001 (1)
$rHIV_{D30Npro}{}^{WTgag}$	0.0031 ± 0.0001 (0.1)	0.045 ± 0.001 (1.6)
$rHIV_{10/30/45/71pro}{}^{WTgag}$	0.014 ± 0.0021 (0.45)	0.26 ± 0.03 (9)
$rHIV_{10/30/45/71pro}{}^{12/75/219/390/409gag}$	0.020 ± 0.002 (0.64)	0.32 ± 0.03 (11)
$rHIV_{30/46/77pro}{}^{WTgag}$	0.0069 ± 0.0024 (0.22)	0.25 ± 0.04 (9)
$rHIV_{30/46/77pro}{}^{12/75/219/390/409gag}$	0.0046 ± 0.0019 (0.15)	0.21 ± 0.06 (8)

[a] Recombinant HIV clones $rHIV_{10/30/45/71pro}{}^{WTgag}$ and $rHIV_{10/30/45/71pro}{}^{12/75/219/390/409gag}$ were generated to have a set of four protease mutations (L10F, D30N, K45I, and A71V) and wild-type Gag or Gag with five mutations, while other clones, $rHIV_{30/46/77pro}{}^{WTgag}$ and $rHIV_{30/46/77pro}{}^{12/75/219/390/409gag}$, were generated with three protease mutations (D30N, M46I, and V77I) and wild-type Gag or Gag with five mutations. Both sets of protease mutations were seen when HIV-1 was propagated in the presence of NFV. The IC_{50}s were determined by employing MT-4 cells exposed to each recombinant HIV-1 clone (50 $TCID_{50}$) in the presence of each PI, with the inhibition of p24 Gag protein production as an end point. All values were determined in triplicate, and the data are shown as mean values ±1 standard deviation of results from two or three independent experiments. The numbers in parentheses are changes (*n*-fold) compared to the IC_{50} of each PI for HIV_{WT}.

NFV-related resistance mutation, N88S, renders HIV-1 susceptible to APV (33, 49). Since the acquisition of viral resistance to PIs such as NFV was significantly delayed when HIV-1 had the Gag mutations seen in HIV_{APVp33}, we asked if another NFV-related resistance mutation (D30N) would render HIV-1 more susceptible to APV. We also asked whether the presence of multiple NFV resistance-associated mutations (D30N, M46I, and V77I) would make HIV-1 susceptible to APV. Moreover, we examined the effects of the Gag mutations seen in HIV_{APVp33} on HIV-1 susceptibilities to APV and NFV.

As shown in Table 2, the N88S mutant clone $rHIV_{N88Spro}{}^{WTgag}$ was more susceptible to APV than HIV_{WT} by a factor of 20, in agreement with the reports by Ziermann et al. and Resch et al. (33, 49). We found that the D30N mutation in $rHIV_{D30Npro}{}^{WTgag}$ also made HIV-1 more susceptible to APV, by a factor of 10. Interestingly, $rHIV_{10/30/45/71pro}{}^{WTgag}$, with the four mutations L10F, D30N, K45I, and A71V, was more resistant to NFV than HIV_{WT} by a factor of 9; however, the recombinant virus remained more susceptible to APV than HIV_{WT} (Table 2). The introduction of the five Gag mutations (E12K, L75R, H219Q, V390D, and R409K) into $rHIV_{10/30/45/71pro}{}^{WTgag}$, generating $rHIV_{10/30/45/71pro}{}^{12/75/219/390/409gag}$, did not change the susceptibility profile (Table 2). Another recombinant HIV-1 clone with three protease mutations (D30N, M46I, and V77I), $rHIV_{30/46/77pro}{}^{WTgag}$, was also more resistant to NFV (by a factor of 9) and more susceptible to APV than HIV_{WT}. The introduction of the five Gag mutations, generating $rHIV_{30/46/77pro}{}^{12/75/219/390/409gag}$, did not affect the susceptibility of $rHIV_{30/46/77pro}{}^{WTgag}$ to APV or NFV (Table 2).

Taken together, the data suggest that, as seen in the case of the lamivudine (3TC) resistance-associated mutation M184V that restores zidovudine (ZDV) sensitivity (37), NFV resistance-associated mutations paradoxically render HIV-1 more susceptible to APV.

DISCUSSION

Certain amino acid substitutions in Gag are known to occur in common with resistance to PIs (11, 15, 32, 36); however, no salient features such as patterns and orders of the occurrence have been identified for a number of amino acid substitutions seen in Gag in PI-resistant HIV-1 variants. The roles and impact of such amino acid substitutions in Gag for the replication of HIV-1 have not been delineated, either. These limitations have been worsened since the functions and tertiary structures of entire HIV-1 Gag proteins remain to be determined, although some structures of certain parts of Gag proteins have been lately elucidated (13, 34, 41).

In the present study, we attempted to determine the effects of non-cleavage site mutations in Gag which emerged during the in vitro selection of HIV-1 in the presence of APV on the viral acquisition of resistance to APV and other currently existing PIs. When we selected HIV-1 in vitro in the presence of increasing concentrations of APV, six amino acid substitutions apparently critical for the development of APV resistance emerged. Such substitutions included five non-cleavage site mutations (E12K, L75R, H219Q, V390D, and R409K) and one cleavage site mutation, L449F (Fig. 1B).

HIV-1 variants containing PI resistance-conferring amino acid substitutions in protease plus wild-type Gag often have highly limited replicative abilities (7, 31). Indeed, in the present study, the recombinant HIV-1 clone containing the protease of HIV_{APVp33} plus a wild-type Gag ($rHIV_{APVp33pro}{}^{WTgag}$) or the L449F cleavage site mutation-containing Gag ($rHIV_{APVp33pro}{}^{449gag}$) failed to replicate in MT-2 cells (Fig. 2A), indicating that neither of the two Gag species supported the growth of HIV_{APVp33}. However, a recombinant HIV-1 clone containing the protease of HIV_{APVp33} and the five Gag non-cleavage site mutations, $rHIV_{APVp33pro}{}^{12/75/219/390/409gag}$, replicated moderately under the same conditions (Fig. 2A), an observation in agreement with reports by others that some PI resistance-associated mutations compromise the catalytic activity of protease and/or alter polyprotein processing, often leading to slower viral replication (29, 36, 43). Since some of the five non-cleavage site mutations emerged before mutations in protease developed, we examined the effects of three sets of non-cleavage site amino acid mutations upon the emergence of APV resistance. Interestingly, HIV-1 with either of two sets of Gag mutations ($rHIV_{WTpro}{}^{219/409gag}$ and $rHIV_{WTpro}{}^{12/75/219/390/409gag}$) acquired APV resistance significantly faster than HIV_{WT} (Fig. 3), while such mutations alone did not alter the susceptibilities of HIV to the PIs examined (Table 1), a finding providing the first report that Gag mutations expedite the emergence of PI-resistant HIV-1 variants. At this time, it is apparently unknown whether certain Gag mutations associated with viral resistance to PIs persist when highly active antiretroviral therapy (HAART) regimens including a PI(s) are interrupted or changed to regimens containing no PIs. However, the non-cleavage site mutations in Gag examined in this study did not reduce the viral fitness (Fig. 2 and 6), suggesting that Gag mutations may persist longer in circulation and/or in the HIV-1 reservoir in the body than mutations in protease when antiretroviral therapy including a PI(s) is interrupted. Such persisting Gag mutations may enable HIV-1 to rapidly acquire resistance

to that very PI when treatment with the PI is resumed. It is of note that on the other hand, two sets of Gag non-cleavage site mutations seen in HIV_{APVp33} (H219Q and R409K and E12K, L75R, H219Q, V390D, and R409K) significantly delayed the emergence of resistance to other PIs such as RTV and NFV (Fig. 5). These data suggest that if a HAART regimen including APV is changed to an alternative regimen, the inclusion of a different PI in the alternative regimen is likely to delay the emergence of resistance to the different PI.

It is known that the L449F cleavage site mutation renders recombinant HIV-1 carrying a protease mutation (I50V) more resistant to APV (25). In the present study, a recombinant HIV-1 clone containing the protease of HIV_{APVp33} plus the L449F cleavage site mutation-containing Gag ($rHIV_{APVp33pro}{}^{449gag}$) failed to replicate (Fig. 2A). These data strongly suggest that the L449F mutation alone prevents HIV_{APVp33} from replicating, although HIV_{APVp33} did not contain the I50V mutation. The observation in the present study that the addition of five non-cleavage site mutations to $rHIV_{APVp33pro}{}^{449gag}$, generating $rHIV_{APVp33pro}{}^{12/75/219/390/409/449gag}$, restored the replicative ability of the virus indicates that the presence of non-cleavage site Gag mutations plays an important role in the replication of APV-resistant HIV-1 variants.

Since $rHIV_{WTpro}{}^{12/75/219/390/409gag}$ acquired resistance to APV more rapidly than HIV_{WT} (Fig. 3), while $rHIV_{WTpro}{}^{12/75/219/390/409gag}$ significantly delayed the acquisition of resistance to other PIs (Fig. 5), we examined whether the replication rates of $rHIV_{WTpro}{}^{12/75/219/390/409gag}$ and HIV_{WT} were associated with the observed contrasting resistance acquisition patterns by using the CHRA (21). We found that $rHIV_{WTpro}{}^{12/75/219/390/409gag}$ outgrew HIV_{WT} regardless of the presence or absence of PIs (Fig. 6), suggesting that the difference in the replication rates of $rHIV_{WTpro}{}^{12/75/219/390/409gag}$ and HIV_{WT} was not the cause for the contrasting resistance acquisition patterns. As for the reason why $rHIV_{WTpro}{}^{12/75/219/390/409gag}$ outgrew HIV_{WT}, it is well explained by the presence of the H219Q mutation. His-219 is located within the cyclophilin A (CypA) binding loop of p24 Gag protein. It is thought that CypA plays an essential role in the HIV-1 replication cycle (4, 35) by destabilizing the capsid (p24 Gag protein) shell during viral entry and uncoating (12) and/or by performing an additional chaperone function, thus facilitating correct capsid condensation during viral maturation (17, 39). CypA is also known to support the replication of HIV-1 by binding to the Ref-1 restriction factor and/or TRIM5α, the human cellular inhibitors that impart resistance to retroviral infection (18, 38). It has also been demonstrated previously that the effect of CypA on HIV-1 replicative ability is bimodal: both high and low CypA contents limit HIV-1 replication (14). We have demonstrated previously that certain human cells, such as MT-2 and H9 cells, contain large amounts of CypA (14). We have determined more recently that MT-2 cells contain more CypA by about fivefold and that MT-4 cells contain about three times more than peripheral blood mononuclear cells (PBMCs) (unpublished data). In fact, HIV-1 produced in MT-4 cells contains large amounts of CypA, presumably resulting in compromised replication of the HIV-1. However, the H219Q mutation apparently reduces the incorporation of CypA into the virions through significantly distorting the CypA binding loop and restores the replicative ability of virions produced in MT-4 cells (14). Therefore, H219Q should contribute at least in part to the replication advantage of $rHIV_{WTpro}{}^{12/75/219/390/409gag}$. It is noteworthy that of 156 different HIV-1 strains whose sequences were compiled in the *HIV Sequence Compendium 2008* (22), 95 and 45 strains had histidine and glutamine, respectively, at position 219. Hence, position 219 is a polymorphic amino acid site, and it is thought that this polymorphic position is associated with the acquisition of resistance to certain PIs. Indeed, we have observed that $rHIV_{WTpro}{}^{219gag}$ overgrew $rHIV_{WTpro}{}^{WTgag}$ in the CHRA using fresh phytohemagglutinin-stimulated PBMCs (14). Since H219Q confers a replication advantage on HIV-1 in PBMCs, it is likely that HIV-1 with H219Q may acquire resistance more rapidly than HIV-1 without H219Q.

Two groups, Ziermann et al. and Resch et al., have reported that an NFV-related resistance mutation, N88S, renders HIV-1 susceptible to APV (33, 49), and indeed, Zachary et al. have reported an anecdotal finding that the infection of an individual with HIV-1 containing N88S was successfully managed with an ensuing APV-based regimen (46). Therefore, we examined the effect of another NFV resistance-associated mutation, D30N, in addition to that of the N88S mutation on HIV-1 susceptibility to APV. It was found that the mutations (D30N and N88S) clearly increased the susceptibility of HIV-1 to APV by 10- and 20-fold, respectively. These data are reminiscent of the observation that the 3TC resistance-associated mutation M184V in a background of mutations conferring resistance to ZDV restores ZDV sensitivity (37) and that ZDV-3TC combination therapy has proven to be more beneficial than ZDV monotherapy in patients harboring HIV-1 with the M184V mutation (9, 23), although the structural mechanism of the restoration of ZDV sensitivity by M184V is not clear. When a set of four protease mutations (L10F, D30N, K45I, and A71V), which had emerged by passage 10 when HIV_{WT} was selected with NFV, were introduced into HIV_{WT}, generating $rHIV_{10/30/45/71pro}{}^{WTgag}$, the recombinant HIV-1 clone was more resistant to NFV than HIV_{WT} by a factor of 9 while the clone was slightly more sensitive to APV (Table 2). When we introduced $mGag^{12/75/219/390/409gag}$ into HIV-1 containing a set of three NFV resistance-associated protease mutations (D30N, M46I, and V77I), generating $rHIV_{30/46/77pro}{}^{12/75/219/390/409gag}$, the recombinant clone was more resistant to NFV by a factor of 8 but more sensitive to APV by a factor of 6.7 (Table 2).

There has been a report that dual PI therapy with APV plus NFV is generally safe and well tolerated and that the combination of APV with NFV may have the most beneficial pharmacokinetic interactions, based on the results of a phase II clinical trial of dual PI therapies, APV in combination with IDV, NFV, or SQV, although this phase II trial was handicapped by the presence of substantial PI resistance at the baseline and the small number of patients in the study, precluding conclusions about the relative activities or toxicities of the dual PI combinations (10). The hypothesis that a HAART regimen combining APV with NFV may bring about more

favorable antiviral efficacy for HIV-1-infected individuals should merit further study.

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CD4 mimics targeting the mechanism of HIV entry

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ABSTRACT

A structure–activity relationship study was conducted of several CD4 mimicking small molecules which block the interaction between HIV-1 gp120 and CD4. These CD4 mimics induce a conformational change in gp120, exposing its co-receptor-binding site. This induces a highly synergistic interaction in the use in combination with a co-receptor CXCR4 antagonist and reveals a pronounced effect on the dynamic supramolecular mechanism of HIV-1 entry.

Recently, remarkable success has attended the clinical treatment of HIV-infected and AIDS patients, with 'highly active antiretroviral therapy (HAART)'. This approach involves a combination of two or three agents from two categories: reverse transcriptase inhibitors and protease inhibitors.[1] In addition, the molecular mechanism involved in HIV-entry and -fusion into host cells has been described in detail.[2] The complex interactions of surface proteins on cellular and viral membranes, which are designated as a dynamic supramolecular mechanism of HIV entry, are reported to be crucial to the viral infection. In a first step, an HIV envelope protein, gp120 interacts with a cell surface protein, CD4, leading to a conformational change in gp120 followed by subsequent binding of gp120 to a co-receptor CCR5[3] or CXCR4.[4] CCR5 and CXCR4 are the major co-receptors for the entry of macrophage-tropic (R5-) and T cell line-tropic (X4-) HIV-1, respectively. The interaction of gp120 with CCR5 or CXCR4 triggers entry of another envelope protein, gp41 to the cell membrane and formation of a gp41 trimer-of-hairpins structure, which causes fusion of HIV/cell-membranes and completes the infection.

Informed by this mechanism, a fusion inhibitor, enfuvirtide (fuzeon, Trimers & Roche)[5] and a CCR5 antagonist, maraviroc (Pfizer)[6] in addition to an integrase inhibitor, raltegravir (Merck)[7] have been used clinically. However, serious problems with chemotherapy still persist, including the emergence of viral strains with multi-drug resistance (MDR), considerable adverse effects and high costs. Consequently, development of novel drugs possessing mechanisms of action different from those of the above inhibitors is currently required. We have previously developed selective CXCR4 antagonists[8] and fusion inhibitors.[9] Furthermore, *N*-(4-Bromophenyl)-*N'*-(2,2,6,6-tetramethylpiperidin-4-yl)-oxalamide (**1**) and *N*-(4-chlorophenyl)-*N'*-(2,2,6,6-tetramethylpiperidin-4-yl)-oxalamide (**2**) were previously found using chemical library screening to inhibit syncytium formation by other researchers.[10] **1** and **2** bind to gp120 with binding affinities of K_d = 2.2 μM and 3.7 μM, respectively, blocking the interaction of gp120 with CD4 in the first step of an HIV-1 entry. Thus, in the present study we focus on the development of CD4 mimics that can block the interaction between gp120 and CD4. We have investigated the effect of CD4 mimics on conformational changes of gp120 and on their use in combination use with a CXCR4 antagonist.

Initially, molecular modeling of compound **2** docked into gp120 was carried out using docking simulations performed by the FlexSIS module of SYBYL 7.1 (Tripos, St. Louis) (Fig. 1).[11] The atomic coordinates of the crystal structure of gp120 with soluble CD4 (sCD4) were retrieved from Protein Data Bank (PDB) (entry 1RZJ) (Fig. 1a) and it was observed that Phe^{43} and Arg^{59} of the CD4 have multiple contacts with Asp^{368}, Glu^{370} and Trp^{427} of gp120, which are all conserved residues. An inspection of the environment of compound **2** docked in gp120 revealed the presence of a large cavity around the *p*-position of the phenyl ring of compound **2**, which could interact with the viral surface protein gp120 (Fig. 1b and c). Several analogs of **2** with substituents on the phenyl ring were therefore synthesized.

All compounds except **12** were synthesized by previously published methods (Scheme 1).[10b,12,13] Aniline derivatives (**3**) were coupled with ethyl oxalyl chloride to yield the corresponding ethyl oxalamates **4**. Saponification of the above oxalamates to the corresponding free acids and the subsequent coupling with 4-ami-

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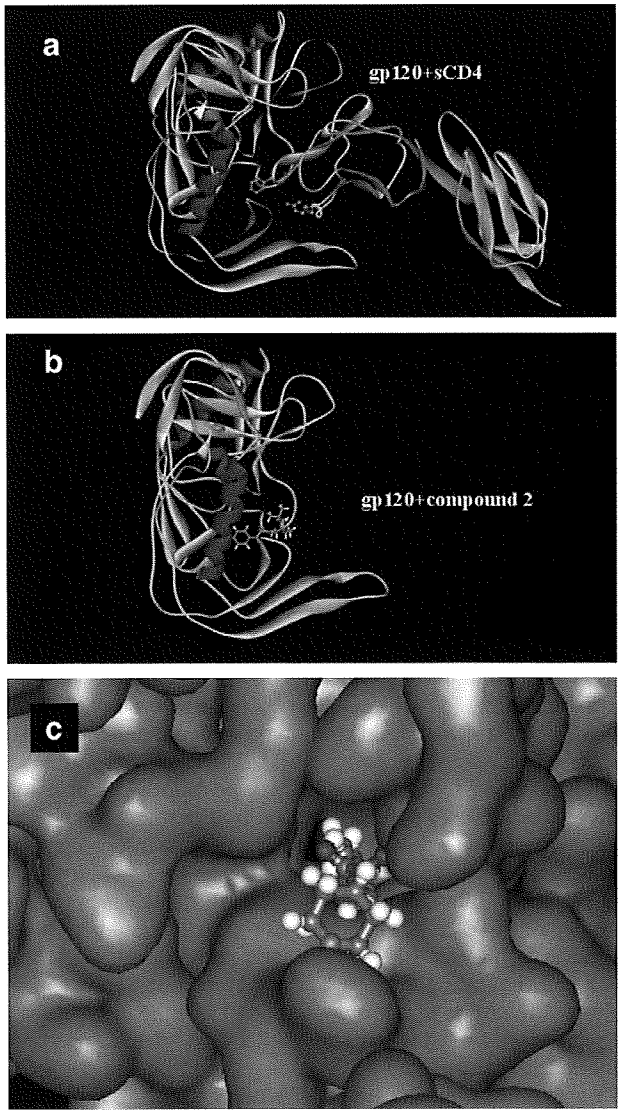
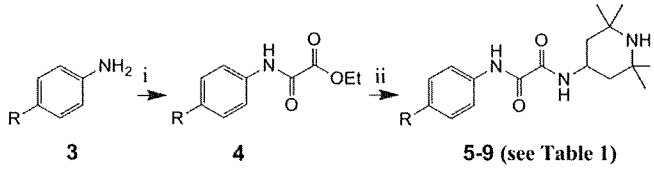
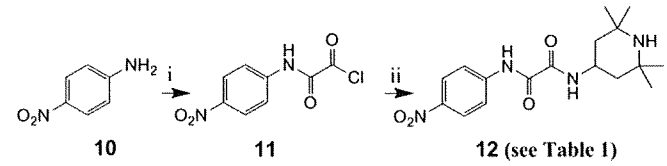


Figure 1. (a) The crystal structure of gp120 with soluble CD4 (sCD4) retrieved from the PDB (entry 1RZJ); (b) docking structure of compound **2** and gp120; (c) a focused figure of (b) shown by space-filling model.



Scheme 1. Reagents and conditions: (i) ethyl oxalyl chloride, Et_3N; (ii) 1 M NaOH; 4-amino-2,2,6,6-tetramethylpiperidine, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, 1-hydroxybenzotriazole, Et_3N.

no-2,2,6,6-tetramethylpiperidine using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt) yielded compounds **5–9**. In the case of compound **12**, whose amide bond is not stable during the reaction of the saponification of the corresponding oxalamates, an alternative synthetic scheme was used (Scheme 2).[14] The reaction of *p*-nitroaniline (**10**) with oxalyl chloride gave the corresponding oxoacetamide **11**, which was subsequently coupled with 4-amino-2,2,6,6-tetramethylpiperidine to yield the desired compound **12**.



Scheme 2. Reagents and conditions: (i) oxalyl chloride, Et_3N; (ii) 4-amino-2,2,6,6-tetramethylpiperidine, Et_3N.

The anti-HIV activity of the synthetic compounds was evaluated against various viral strains including both laboratory and primary isolates (Table 1). IC_{50} values were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method[15] as the concentrations of the compounds which conferred 50% protection against HIV-1-induced cytopathogenicity in PM1/CCR5 cells. Cytotoxicity of the compounds based on the viability of mock-infected PM1/CCR5 cells was also evaluated using the MTT method. CC_{50} values were determined as the concentrations achieving 50% reduction of the viability of mock-infected cells. Compounds **1** and **2** showed potent anti-HIV activity against laboratory isolates, IIIB (X4, Sub B) and 89.6 (dual, Sub B) strains, and compound **2** also possessed potent activity against a primary isolate, an fTOI strain (R5, Sub B). All of the IC_{50} values were between 4 μM and 10 μM. Compound **1** was not tested against primary isolates. The potencies of compounds **1** and **2** are comparable to the reported binding affinities for gp120 (K_d = 2.2 and 3.7 μM, respectively).[10] Several of the new analogs of compounds **1** and **2** showed significant anti-HIV activity. Compound **5**, which has a phenyl group in place of the *p*-chlorophenyl group of compound **2**, did not show significant anti-HIV activity at concentrations below 100 μM against all strains tested except for an fTOI strain (R5, Sub B). This result suggests that a substituent at the *p*-position of the phenyl ring is critical for potent activity. Compound **6**, which has a fluorine atom at the *p*-position of the phenyl ring, showed moderate anti-HIV activity against laboratory isolates, IIIB (X4, Sub B) and 89.6 (dual, Sub B) strains (IC_{50} = 61 and 81 μM, respectively), but, at concentrations below 100 μM, failed to show significant anti-HIV activity against a primary isolate, a KYAG strain (R5, Sub B). Among halogen atoms, fluorine is less suitable than bromine or chlorine as a substituent at the *p*-position of the phenyl ring, as evidenced by compound **6**, which is 8–15-fold less potent than compounds **1** and **2** against IIIB (X4, Sub B) and 89.6 (dual, Sub B) strains. Compound **7**, which has a methyl group at the *p*-position of the phenyl ring, showed relatively more potent activity against IIIB (X4, Sub B) and 89.6 (dual, Sub B) strains (IC_{50} = 23 and 41 μM, respectively) than compound **6**. Compound **7** also showed significant anti-HIV activity against primary isolates, fTOI (R5, Sub B) and KYAG (R5, Sub B) strains (IC_{50} = 16 and 51 μM, respectively). Compound **8**, with a methoxy group at the *p*-position of the phenyl ring, did not show significant anti-HIV activity against all strains tested until a concentration of 100 μM was reached. In the biological assays, derivatives having electron-withdrawing substituents such as bromine, chlorine and fluorine at the *p*-position of the phenyl ring are relatively potent, whereas derivatives having electron-donating groups such as methoxy at this position are not potent. Furthermore, the steric effect of a substituent at the *p*-position of the phenyl ring appears to be critical to anti-HIV activity. The sum of Hammett constants (σ) of benzoic acid substituents[16] shown in Table 1 can be used to evaluate the electron-withdrawing or -donating effect of the substituents on the aromatic ring. The Taft E_s values[16a,17] were used as steric parameters for substituents at the *p*-position of the phenyl ring. The order of potency found for the halogen-containing derivatives in anti-HIV activity against laboratory isolates, IIIB (X4, Sub B) and 89.6 (dual, Sub B), is: compound **1** (R = Br) (σ = 0.23, E_s = −1.16), **2**

Table 1
Hammett constants (σ) and steric effects (E_s) of substituted aromatic rings and anti-HIV activity and cytotoxicity of synthetic compounds

Compd	R[a]	σ[b]	E_s[c]	IC_{50}[e] (μM)				CC_{50}[e] (μM)
				Lab. isolates		Primary isolates		
				IIIB (X4)	89.6 (dual)	fTOI (R5)	KYAG (R5)	
1	Br	0.23	−1.16	4	9	ND	ND	150
2	Cl	0.23	−0.97	8	10	5	>30	170
5	H	0	0	>100	>100	81	>100	350
6	F	0.06	−0.46	61	81	ND	>100	320
7	CH_3	−0.17	−1.24	23	41	16	51	210
8	OCH_3	−0.27	−0.55	>100	>100	ND	>100	340
9	CF_3	0.54	−2.40	ND	27	ND	ND	72
12	NO_2	0.78	−1.77[d]	ND	42	ND	ND	230
sCD4				0.010	0.021	0.0044	ND	ND

[a] See Schemes 1 and 2.
[b] σ = Hammett constant of a substitutent on a benzoic acid derivative.[16]
[c] E_s = steric effect of a substituent at the *para* position on the aromatic ring.[16a,17]
[d] The average value of −1.01 and −2.52, which are E_s values of the NO_2 group, −1.77, was used.
[e] Values are means of at least three experiments (ND = not determined).

(R = Cl) (σ = 0.23, E_s = −0.97), **6** (R = F) (σ = 0.06, E_s = −0.46) and **5** (R = H) (σ = 0, E_s = 0). This is the order of substituents' electron-withdrawing ability and also of their size. Methyl (σ = −0.17, E_s = −1.24) is an electron-donating group, but is almost as bulky as a bromine atom. Thus, the *p*-methyl derivative **7** has relatively potent anti-HIV activity against laboratory isolates, IIIB (X4, Sub B) and 89.6 (dual, Sub B), higher than that of compound **6** (R = F) but lower than that of compound **1** (R = Br) or **2** (R = Cl). The electron-donating ability of a methoxy group is stronger (σ = −0.27), but the bulk size is smaller (E_s = −0.55), than that of a methyl group. Thus, the *p*-methoxy derivative **8** has no significant anti-HIV activity against all strains tested at concentrations below 100 μM. Two derivatives containing bulkier and more potent electron-withdrawing substituents such as trifluoromethyl (R = CF_3) (σ = 0.54, E_s = −2.40) and nitro (R = NO_2) (σ = 0.78, E_s = −1.77) at the *p*-position of the phenyl ring were evaluated. Compounds **9** (R = CF_3) and **12** (R = NO_2) showed significant anti-HIV activity against an 89.6 (dual, Sub B) strain. These are less potent than compounds **1** and **2** and this is perhaps due to the excessive size of the substituents at the *p*-position. This suggests that a certain level of the bulk size and a potent electron-withdrawing ability of the substituents are preferable for anti-HIV activity. It is estimated that a cavity around the *p*-position of the phenyl ring of CD4 mimicking compounds would be optimally filled by bromine (E_s = −1.16) or a methyl group (E_s = −1.24) at *p*-position, and that an electron-deficient aromatic ring might interact tightly with a negatively charged group such as carboxy of Glu^{370}. In isothermal titration calorimetry (ITC) experiments reported elsewhere,[10c] compound **5** (R = H) does not have significant affinity for gp120, and compound **6** (R = F) has less potent affinity for gp120 than compound **2**, consistent with the present data. In all but one of the compounds, no significant cytotoxicity was detected (CC_{50} >150 μM, Table 1), the exception being compound **9** (R = CF_3) (CC_{50} = 72 μM). Compounds **7** and **12** have relatively low cytotoxicities, compared to compounds **1** and **2**.

Fluorescence activated cell sorting (FACS) analysis was performed[15] to investigate whether these synthetic compounds interact with gp120 inducing the conformational change necessary for the approach of an anti-envelope antibody or a co-receptor to the gp120. The profile of binding of an anti-envelope CD4-induced monoclonal antibody, 4C11, to the Env-expressing cell surface (an R5-HIV-1 strain, JR-FL,-infected PM1 cells) pretreated with the above CD4 mimic analogs was examined. Comparison of the binding of 4C11 to the cell surface was measured in terms of the mean fluorescence intensity (MFI), and is shown in Figure 2. Pretreatment of the Env-expressing cells with compound **2** (MFI = 38.42) produced a remarkable increase in binding affinity for 4C11, similar to that observed in pretreatment with sCD4 (MFI = 37.90). This is consistent with the results in the previous paper[10] where it was reported that compound **2** enhances the binding of gp120 to the 17b monoclonal antibody which recognizes the co-receptor binding site of gp120. Env-expressing cells, which were not pretreated with sCD4 or a CD4 mimic compound, did not show significant binding affinity for 4C11 (Fig. 2, blank). The increase in binding affinity for monoclonal antibodies may be due to conformational changes in gp120, which were caused by the interaction of sCD4 or a CD4 mimic with gp120. It is hypothesized that such conformational changes involve the exposure of the co-receptor binding site of gp120 (the V3 loop), which is hidden internally, since the binding of gp120 to 17b is enhanced. Compound **5**, which failed to show significant anti-HIV activity, and compounds **7**, **9** and **12**, which had significant anti-HIV activity, were assessed in the FACS analysis. The profile of the binding of 4C11 to the Env-expressing cell surface pretreated with compound **5** (MFI = 14.34) was similar to that of the blank (MFI = 11.24), suggesting that compound **5** offers no significant enhancement of binding affinity for 4C11. This result is compatible with the anti-HIV activity of compound **5**. The profile of the binding of 4C11 to the Env-expressing cell surface pretreated with compound **7** (MFI = 38.33) was entirely similar to that of compound **2** used as a pretreatment. Pretreatment of the cell surface with compounds **9** and **12** (MFI = 29.09 and 30.01, respectively) produced a slightly lower enhancement of binding affinity for 4C11, compared to those of compounds **2** and **7** as pretreatments. However, in the ITC experiments reported elsewhere,[10c] compound **9** (R = CF_3) has a high affinity for gp120, comparable to that of compound **2**, but compound **12** (R = NO_2) does not have significant affinity for gp120, indicating that these are not consistent with the current FACS studies, possibly due to the difference in the assay systems. Although the anti-HIV activity of **7** is weaker than that of compound **2**, the level of compound **7** inducing an enhancement of binding affinity of gp120 for 4C11 is comparable to that of compound **2**. The concentration of compounds used in the FACS analysis was 100 μM, much beyond the IC_{50} values of compounds **2** and **7**. A concentration of 100 μM would be also sufficient for the expression of anti-HIV activity caused by compounds **2** and **7**.

An effect on the use of compound **2** combined with another entry inhibitor was investigated. Analysis of the synergistic effects of anti-HIV agents was performed according to the median effect principle using the CalcuSyn version 2 computer program[18] to estimate IC_{50} values of compounds in different combinations. Combination indices (CI) were estimated from the data evaluated using the MTT assay

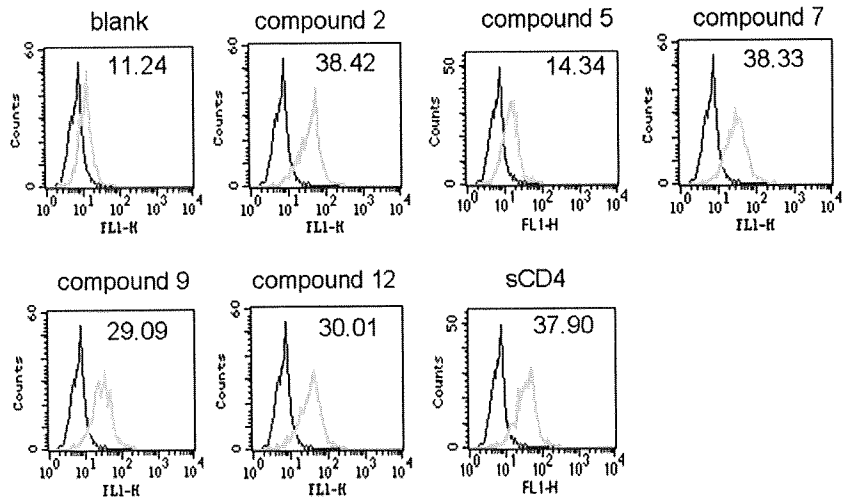


Figure 2. JR-FL (R5, Sub B) chronically infected PM1 cells were preincubated with 100 μM of a CD4 mimic or sCD4 (11 nM) for 15 min, and then incubated with an anti-HIV-1 mAb, 4C11, at 4 °C for 15 min. The cells were washed with PBS, and fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG antibody was used for antibody-staining. Flow cytometry data for the binding of 4C11 (green lines) to the Env-expressing cell surface in the presence of sCD4 or 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 top of each graph shows the mean fluorescence intensity (MFI) of the antibody 4C11.

Table 2
Combination indices (CI) for compound **2** or sCD4 and a CXCR4 antagonist, T140, against an HIV IIIB strain

Combination	HIV strain	CI values at different IC[a]		
		IC_{50}	IC_{75}	IC_{90}
2 + T140	IIIB	0.786	0.713	0.655
sCD4 + T140	IIIB	0.705	0.528	0.400

[a] The multiple-drug effect analysis reported by Chou et al. was used to analyze the effects of combinational uses of compounds.[18] CI <0.9: synergy, 0.9 < CI < 1.1: additivity, CI >1.1: antagonism.

(Table 2).[15] Compound **2** showed a highly remarkable synergistic anti-HIV activity with a co-receptor CXCR4 antagonist, T140,[8a] against an X4-HIV-1 strain, IIIB at various IC values (IC_{50}, IC_{75} and IC_{90}). However, sCD4 exhibited a higher synergistic effect (lower CI values) with T140 (Table 2). The interaction of sCD4 or a CD4 mimic with gp120 would expose the co-receptor-binding site of gp120, and the co-receptor CXCR4 could then easily approach gp120. Thus, an inhibitory effect of a CXCR4 antagonist would be meaningful, and a significant synergistic effect might also be brought about by a combination of sCD4 or a CD4 mimic and T140.

In summary, a series of CD4 mimic compounds were synthesized and evaluated for their anti-HIV activity. Several compounds showed significant anti-HIV activity with relatively low cytotoxicity. SAR studies showed that a certain level of size and electron-withdrawing ability of the substituents at the *p*-position of the phenyl ring are suitable for potent anti-HIV activity. In addition, the treatment of Env-expressing cells with several CD4 mimicking compounds causes a conformational change, exposing the co-receptor-binding site of gp120 externally. Thus, a CD4 mimic exhibited a remarkable synergistic effect with a co-receptor antagonist. These compounds are essential probes directed to the dynamic supramolecular mechanism of HIV entry, and important leads for the cocktail therapy of AIDS.

Acknowledgments

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11. The structure of compound **2** was built in Sybyl and minimized with the MMFF94 force field and partial charges. (see: Halgren, T. A. *J. Comput. Chem.* **1996**, *17*, 490.) Docking was then performed using FlexSIS through its SYBYL

module, into the crystal structure of gp120 (PDB, entry 1RZJ). The binding site was defined as residues Val^{255}, Asp^{368}, Glu^{370}, Ser^{375}, Ile^{424}, Trp^{427}, Val^{430} and Val^{475}, and included residues located within a radius 4.4 Å. The ligand was considered to be flexible, and all other options were set to their default values. Figures were generated with ViewerLite version 5.0 (Accelrys Inc., San Diego, CA).

12. *For example, the synthesis of compound* **7**: To a solution of ethyl oxalyl chloride (0.400 mL, 3.48 mmol) in THF (20 mL) were added triethylamine (Et_3N) (0.480 mL, 3.48 mmol) and *p*-toluidine (373 mg, 3.48 mmol) with stirring at 0 °C. The reaction mixture was allowed to warm to room temperature, and then stirred for 6 h. After removal by filtration of the resulting salts, the filtrate was concentrated under reduced pressure. The residue was extracted with EtOAc (50 mL), and the extract was washed successively with brine (20 mL), 1 M HCl (20 mL × 2), brine (20 mL), saturated $NaHCO_3$ (20 mL × 2) and brine (20 mL × 3), then dried over $MgSO_4$. Concentration under reduced pressure gave the crude ethyl oxalamate, which was used without further purification. To a solution of the crude ethyl oxalamate (640 mg, 3.09 mmol) in THF (30 mL) were added aqueous 1 M NaOH (3.40 mL, 3.40 mmol), water (50 mL) and MeOH (20 mL) with stirring at 0 °C. The reaction mixture was allowed to warm to room temperature, and then stirred for 20 h. After the addition of aqueous 1 M HCl (5 mL), MeOH and THF were evaporated under reduced pressure. The residue was acidified to pH 2 with 1 M HCl, and extracted with EtOAc (50 mL × 2). The combined organic layer was washed with brine (20 mL × 3), and dried over $MgSO_4$. Concentration under reduced pressure gave the crude acid, which was used for the next reaction without further purification. To a solution of the above crude acid (514 mg, 2.87 mmol) in THF (10 mL) were added 1-hydoxybenzotriazole (484 mg, 3.16 mmol), 4-amino-2,2,6,6-tetramethylpiperidine (446 μL, 2.58 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (606 mg, 3.16 mmol) and Et_3N (0.439 mL, 3.16 mmol) with stirring at 0 °C. The reaction mixture was allowed to warm to room temperature, and then stirred for 20 h. After evaporation of THF, the residue was dissolved in $CHCl_3$ (50 mL). The mixture was washed with saturated $NaHCO_3$ (20 mL × 2) and brine (20 mL × 3), and dried over $MgSO_4$. Concentration under reduced pressure gave the crude crystalline mass. The usual work-up followed by recrystallization from EtOAc–*n*-hexane gave the title compound **7** (363 mg, 1.14 mmol, 39.8%) as colorless crystals, mp = 176 °C; δ_H (400 MHz; $CDCl_3$) 1.07 (1H, m, NH), 1.16 (6H, s, CH_3), 1.29 (6H, s, CH_3), 1.44 (2H, m, CH_2), 1.91 (1H, d, *J* 3.7, C*H*H), 1.94 (1H, d, *J* 3.7, CH*H*), 2.34 (3H, s, CH_3), 4.25 (1H, m, CH), 7.17 (2H, d, *J* 8.3, ArH), 7.33 (1H, m, NH), 7.50 (2H, d, *J* 8.4, ArH), 9.18 (1H, s, NH); HRMS (FAB), *m*/*z* calcd for $C_{18}H_{28}N_3O_2$ $(MH)^+$ 318.2182, found 318.2173.
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14. *The synthesis of compound* **12**: To a solution of Et_3N (417 μL, 3.00 mmol) and 4-nitroaniline (138 mg, 1.00 mmol) in THF (1.3 mL) was added oxalyl dichloride (85.8 μL, 1.00 mmol) with stirring at 0 °C. After being stirred for 30 min at 0 °C, Et_3N (167 μL, 1.20 mmol) and 4-amino-2,2,6,6-tetramethylpiperidine (156 μL, 0.90 mmol) were added. The reaction mixture was stirred for 6 h at 0 °C. After removal by filtration of the resulting salts, the filtrate was concentrated under reduced pressure. The residue was dissolved in $CHCl_3$ (20 mL), and the mixture was washed successively with brine (10 mL), saturated $NaHCO_3$ (10 mL × 2) and brine (10 mL × 3), and dried over $MgSO_4$. Concentration under reduced pressure followed by flash chromatography over silica gel with $CHCl_3$–MeOH (9:1) gave 42.4 mg (0.122 mmol, 13.5%) of the title compound **12** as colorless crystals, mp = 190 °C; δ_H (400 MHz; $CDCl_3$) 1.09 (1H, m, NH), 1.17 (6H, s, CH_3), 1.29 (6H, s, CH_3), 1.43 (2H, m, CH_2), 1.92 (1H, d, *J* 3.8, C*H*H), 1.95 (1H, d, *J* 3.8, CH*H*), 4.28 (1H, m, CH), 7.29 (1H, m, NH), 7.82 (2H, d, *J* 9.1, ArH), 8.28 (2H, d, *J* 9.1, ArH), 9.55 (1H, s, NH); HRMS (FAB), *m*/*z* calcd for $C_{17}H_{25}N_4O_4$ $(MH)^+$ 349.1876, found 349.1871.
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Gram-positive bacteria enhance HIV-1 susceptibility in Langerhans cells, but not in dendritic cells, via Toll-like receptor activation

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Although numerous studies have shown a higher risk of acquiring HIV infection in the presence of other sexually transmitted diseases, the biologic mechanisms responsible for enhanced HIV acquisition are unclear. Because Langerhans cells (LCs) are suspected to be the initial HIV targets after sexual exposure, we studied whether microbial components augment HIV infection in LCs by activating Toll-like receptor (TLR) and nucleotide-binding oligomerization domain (NOD) pattern recognition receptors. We found that TLR1/2 and TLR2/6 agonists dramatically enhanced both HIV susceptibility and replication in immature monocyte-derived LCs, whereas TLR3-5, TLR7-9, and NOD1,2 agonists did not significantly affect HIV infection. The same infection-enhancing effects were observed when LCs were incubated with other related bacterial components as well as with whole Gram$^+$ bacteria. In resident LCs in human skin, TLR2 agonists also significantly increased HIV susceptibility. By contrast, TLR2 agonists and related bacterial components decreased HIV susceptibility in monocyte-derived dendritic cells (DCs). We found that TLR2 activation of LCs, but not DCs, resulted in a significant down-regulation of APOBEC3G, which is a cellular restriction factor for HIV. Given these data, we hypothesize that ligation of TLR2 by Gram$^+$ bacterial products may underlie enhanced sexual transmission of HIV that occurs with concomitant bacterial sexually transmitted disease infections. (Blood. 2009;113:5157-5166)

Introduction

Epidemiologic studies have suggested a strong association between the acquisition of HIV and other sexually transmitted diseases (STDs).[1] The risk ratio for HIV acquisition for a person with genital ulcer disease ranges from 2.2 to 11.3, whereas for nonulcerative STDs, risk ratios range from 3 to 4.[2] Additional studies have reported that bacterial vaginosis (BV) is associated with an increase in HIV acquisition.[3,4] Genital ulcer disease, such as syphilis or herpes simplex infections, are thought to enhance HIV transmission, because genital ulcerations associated with these infections reduce epithelial barriers to HIV.[1] Two nonulcerative diseases, *Chlamydia* and gonorrhea, are responsible for up to 80% of the cases of all notifiable diseases in the United States,[5] whereas nonulcerative STDs, such as BV, *Candida*, and *Trichomonas*, are common in Africa.[6] Mechanisms responsible for the enhanced HIV transmission by nonulcerative STDs are as of yet unknown, but they may be related to breaching of physical barriers to infection, disturbances in normal vaginal flora, or biologic effects on potential target cells for HIV infection.[1]

During sexual transmission of HIV, virus crosses mucosal epithelium and is eventually disseminated to proximally located lymphoid organs, where it establishes a permanent infection. Most evidence has indicated that Langerhans cells (LCs) are the initial cellular targets for HIV, and that these cells play a crucial role in disseminating HIV.[7-10] LCs are abundantly present within genital mucosal epithelium, and after contact with pathogens, they readily emigrate from tissue to draining lymph nodes. Immature resident LCs express surface CD4 and C-C chemokine receptor (CCR) 5, but not surface CXC chemokine receptor 4. These LCs are readily infected ex vivo with R5 HIV, but not with X4 HIV.[11-14] This is consistent with previous epidemiologic observation, which have found that the majority of HIV strains isolated from patients are R5 HIV after initial infection.[15] It has been reported that persons with CCR5 homozygous defects are largely protected from sexually acquiring HIV.[16] In rhesus macaques, within an hour after intravaginal inoculation of simian immunodeficiency virus (SIV), up to 90% of the SIV-infected cells were LCs, which also supports the role of LCs as initial target cells.[17]

Studies on dendritic cell–specific intercellular adhesion molecule-3–grabbing non-integrin (DC-SIGN), a C-type lectin receptor (CLR) that is expressed on dermal macrophages and monocyte-derived dendritic cells (mDCs),[18,19] have shown that it can bind to HIV glycoprotein (gp) 120 and facilitate HIV infection of T cells in trans.[18] Although results from other studies indicate a minor contribution by DC-SIGN in the transmission of HIV from mDCs to T cells,[19,20] DC-SIGN may be involved in viral dissemination. In addition, langerin, a LC-specific CLR, has been shown to bind HIV gp120, suggesting that it also participates in viral dissemination.[21] However, a recent study revealed that langerin impairs both infection of LCs by HIV and its subsequent viral dissemination.[22] This study also showed that langerin was involved in capture of HIV and subsequent internalization within Birbeck granules, where it was degraded. Nevertheless, when LCs were exposed to high viral concentrations of HIV, there was significant infection of LCs by R5 virus, followed by viral transmission to

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