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Enhancement of the T140-based pharmacophores leads to the development of more potent and bio-stable CXCR4 antagonists †

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Received 10th June 2003, Accepted 2nd September 2003
First published as an Advance Article on the web 1st October 2003

A CXCR4 antagonistic peptide, T140, and its bio-stable analogs, such as Ac-TE14011, were previously developed. These peptides inhibit the entry of T cell line-tropic strains of HIV-1 (X4-HIV-1) into T cells. Herein, a series of TE14011 analogs having modifications in the *N*-terminal region were synthesized to develop effective compounds with increased biostability. Among these analogs, 4F-benzoyl-TE14011 (TF14013) showed the strongest anti-HIV activity derived from CXCR4-antagonism, suggesting that a 4-fluorobenzoyl moiety at the *N*-terminus of T140 analogs constitutes a novel T140-based pharmacophore for CXCR4 antagonists. Structure–activity relationship (SAR) studies on TE14011 analogs with N^{α}-acylation by several benzoic acid derivatives have disclosed a significant relationship between the anti-HIV activity and the Hammett constant (σ) of substituted benzoic acids. TF14013 was found to be stable in mouse serum, but not completely stable in rat liver homogenate due to deletion of the *C*-terminal Arg^{14}-NH_2 from the parent peptide. This biodegradation was completely suppressed by *N*-alkyl-amidation at the *C*-terminus. Taken together, the enhancement of the T140-based pharmacophores led to development of a novel CXCR4 antagonist, 4F-benzoyl-TE14011-Me (TF14013-Me), which has very high anti-HIV activity and increased biostability.

Introduction

Several diseases relevant to the interaction axis between a chemokine receptor, CXCR4, and its natural ligand, stromal cell-derived factor-1α (SDF-1α), have been reported in the past few years (described in the preceding paper[1]). A 14-residue peptide, T140, is a specific CXCR4 antagonist that suppresses X4-HIV-1 entry through this coreceptor.[2] Pharmacophore identification of T140[3] and Glu and/or L-citrulline (Cit)-scanning study[4] led to development of TE14011 ([Cit^6, D-Glu^8]-T140 with the *C*-terminal amide), which possesses strong anti-HIV activity and low cytotoxicity.[1] TE14011 is completely stable in mouse serum whereas it is unstable in rat liver homogenate, mainly due to the cleavage of Arg^1-Arg^2-L-3-(2-naphthyl)alanine (Nal)[3] from the *N*-terminus of the parent peptide. *N*-Terminal acetylation, which mostly suppressed the biodegradation, led to the development of a new lead compound, Ac-TE14011, however, this was not sufficient for complete stability in liver homogenate. In this study, a series of TE14011 derivatives, with modifications in the *N*- and *C*-terminal regions, were synthesized to increase potency and biostability. In addition, we attempted to refine the T140-based pharmacophore to develop new CXCR4 antagonists.

Biological results and discussion

Structures of *N*-terminal-modified T140 analogs, which were designed for the purpose of suppression of biodegradation from the *N*-terminus, are shown in Figs. 1 and 2. Anti-HIV activity was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Table 1).[5] EC_{50} values show the concentrations for 50% inhibition of X4-HIV-1 ($HIV\text{-}1_{IIIB}$)-induced cytopathogenicity in MT-4 cells. EC_{50} ratios (Q) were adopted as activity indicators between assays performed with different screens, since EC_{50} values showed a marked tendency to flexibly depend on the conditions of cells and viruses. N^{α}-Acetylated TE14011 (Ac-TE14011, TF14001) showed almost the same anti-HIV activity as that of TE14011, as previously reported.[1] Guanylation of the N^{α}-amino group of TE14011 (TF14002) caused a significant increase in anti-HIV activity (7-fold), whereas N^{α}-tetramethylguanylation (TF14003) brought a decrease in activity (0.3-fold), suggesting that an N^{α}-guanidino group is more suitable than an amino group and an N^{α}-tetramethylguanidino group. TE14011 analogs lacking the N^{α}-amino group (TF14004–TF14006) showed almost the same or higher anti-HIV activity, compared with TE14011. An N^{α}-amino group is not necessary for anti-HIV activity (see TF14005), and an N^{δ}-guanidino group of Arg^1 can be replaced by an amino group and a tetramethylguanidino group (see TF14004 and TF14006). However,

† Electronic supplementary information (ESI) available: Fig. S1: behaviors of TF14013, TF14016 and TF14013-Me in mouse serum; Fig. S2: behaviors of TF14002 (a), TF14005 (b), TF14006 (c), TF14013 (d), TF14016 (e) and TF14013 analogs (f) in rat liver homogenate; Table S1: characterization data of novel synthetic peptides; and HPLC charts for synthetic compounds of 4F-benzoyl-TE14011 (TF14013), 4F-benzoyl-TN14003 (TF14016), nicotinoyl-TE14011 (TF14031) and 4F-benzoyl-TE14011-Me (TF14013-Me), and for a degraded sample of 4F-benzoyl-TE14011 (TF14013) in rat liver homogenate and its co-injection with an authentic compound des-[Arg^{14}-NH_2]-4F-benzoyl-TE14011. See http://www.rsc.org/suppdata/ob/b3/b306613b/

DOI: 10.1039/b306613b

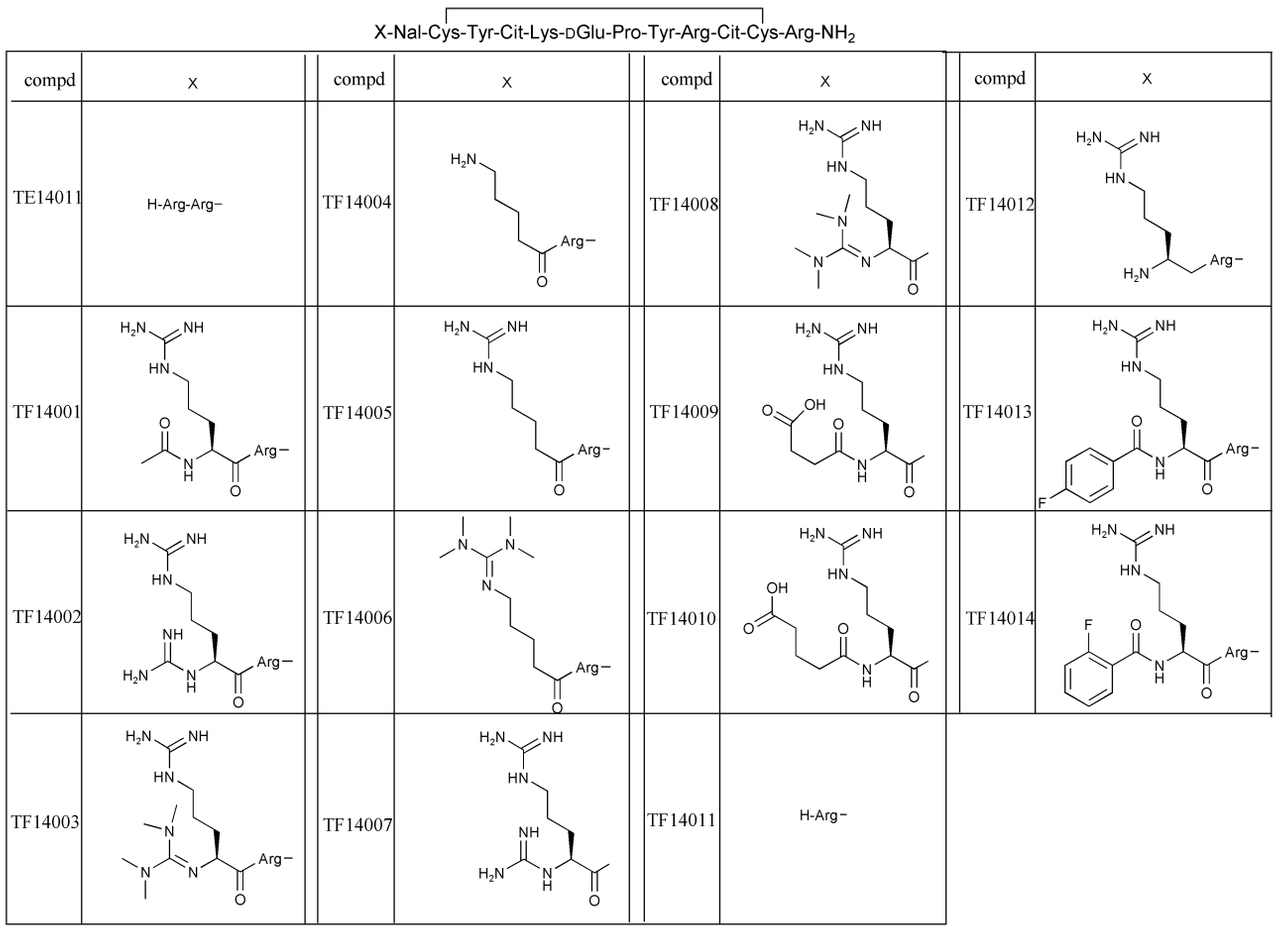


Fig. 1 Structures of TE14011 analogs (TF14001–TF14014). Each peptide has a disulfide linkage, which is shown by a solid line.

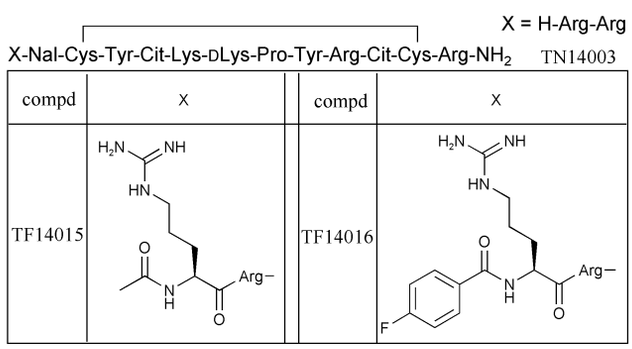


Fig. 2 Structures of TN14003 analogs (TF14015 and TF14016).

the deletion of the Arg[1] residue in the *N*-terminal region caused one or two orders of magnitude decrease in anti-HIV activity in spite of the *N*-terminal modification by a guanidino group, a tetramethylguanidino group, a hemisuccinyl group or a hemiglutaryl group (see TF14007–TF14011). In addition, a TE14011 analog, TF14012, with replacement by a reduced amide bond in Arg[1]-Arg[2], exhibited lower anti-HIV activity than TE14011, suggesting that the carbonyl group of Arg[1] is important. It is noteworthy that N^{α}-benzoyl derivatives, TF14013 (4F-benzoyl-TE14011) and TF14014 (2F-benzoyl-TE14011), were 200-fold and 50-fold more potent than TE14011, respectively. To examine the generality of the above results, *N*-terminal-modified derivatives of another active compound, TN14003,[4] were evaluated for anti-HIV activity. N^{α}-Acetylation of TN14003 (TF14015) brought a slight increase in anti-HIV activity,[1] while N^{α}-4-fluorobenzoylation (TF14016) caused a remarkable increase in potency (35-fold).

Since potent enhancement of anti-HIV activity by a 4-fluorobenzoyl moiety suggested the beneficial extension of the T140-based pharmacophore at the *N*-terminus, several derivatives containing other substituted benzoyl moieties were examined for anti-HIV activity (Fig. 3 and Table 2). Since the EC_{50} values of TF14013 obtained in experiments No. 1 and No. 2 in the MTT assay were different, EC_{50} ratio values (Q) of compounds

Table 1 Anti-HIV activity and cytotoxicity of T140 analogs (TF14011–TF14016)

Compound	Q^a	EC_{50}/nM[b]	CC_{50}/μM[c]	SI[d]
TE14011	200	40	>200	>5000
TF14001	140	28	>200	>7100
TF14002	27	5.3	130	25000
TF14003	650	130	>200	>1500
TF14004	27	5.3	>200	>38000
TF14005	140	28	>200	>7100
TF14006	34	6.7	>200	>30000
TF14007	3100	620	>200	>320
TF14008	800	160	>200	>1300
TF14009	18000	3600	>200	>55
TF14010	3900	780	>200	>260
TF14011	4400	870	>200	>230
TF14012	3900	780	140	180
TF14013	1	0.20	140	700000
TF14014	4.1	0.81	160	200000
TN14003	700	140	90	640
TF14015	150	29	110	3800
TF14016	20	4.0	56	14000
T140	600	120	>10	>83
AZT	90	18	140	7800
ddC	17000	3400	2200	650

[a] $Q = EC_{50}$ of each compound/EC_{50} of TF14013. [b] All data are the mean values for at least three experiments. [c] All data are the mean values for at least three experiments. The estimation of T140 at higher concentrations was omitted in this study. [d] $SI = CC_{50}/EC_{50}$.

Table 2 Hammett constant (σ) and steric effect (E_S) of substituted benzoic acids and anti-HIV activity and cytotoxicity of TE14011 analogs (TF14013, TF14014 and TF14017–TF14032)

Compound	σ [a]	E_S [b]	Q [c]	EC_{50}/nM [d]		CC_{50}/μM [e]	SI	
				No. 1	No. 2		No. 1	No. 2
TF14013	0.06	−0.46	1	2.4	0.28	>100	>42000	>360000
TF14014	0.06	0	4.6	11		>100	>9100	
TF14017	0.23	−0.97	29	69		>100	>1400	
TF14018	0.54	−2.4	6.1	15		>100	>6700	
TF14019	0.12	−0.46	5.7		1.6	>100		>63000
TF14020	0.18	−0.46	1.6		0.45	>100		>220000
TF14021	0.78	−1.77 [f]	6.0	14		>100	>7100	
TF14022	0	0	3.0	7.1		>100	>14000	
TF14023	−0.11		3.3	7.9		>100	>13000	
TF14024	−0.66	−0.61	35	83		>100	>1200	
TF14025	−0.37	−0.55	34	81		>100	>1200	
TF14026	−0.17	−1.24	31		8.9	>100		>11000
TF14027	−0.2	−2.78	290		83	47		570
TF14028	−0.27	−0.55	33		9.4	>100		>11000
TF14029	−0.51	−1.24	650		180	53		290
TF14030			1.1	2.7		>100	>37000	
TF14031			0.83	2.0		>100	>50000	
TF14032			3.2	7.6		>100	>13000	
AZT			5.4	13		210	16000	
ddC			480	1100		>5000	>4400	

[a] σ = Hammett constant of a substituted benzoic acid condensed at the *N*-terminus.[6–8] [b] E_S = steric effect of a substituent at the *para* position on the aromatic ring.[8,11] [c] $Q = EC_{50}$ of each compound/EC_{50} of TF14013. [d] All data are the mean values for at least three experiments. [e] All data are the mean values for at least three experiments. [f] The average value of −1.01 and −2.52, which are E_S values of the NO_2 group, −1.77, was used.

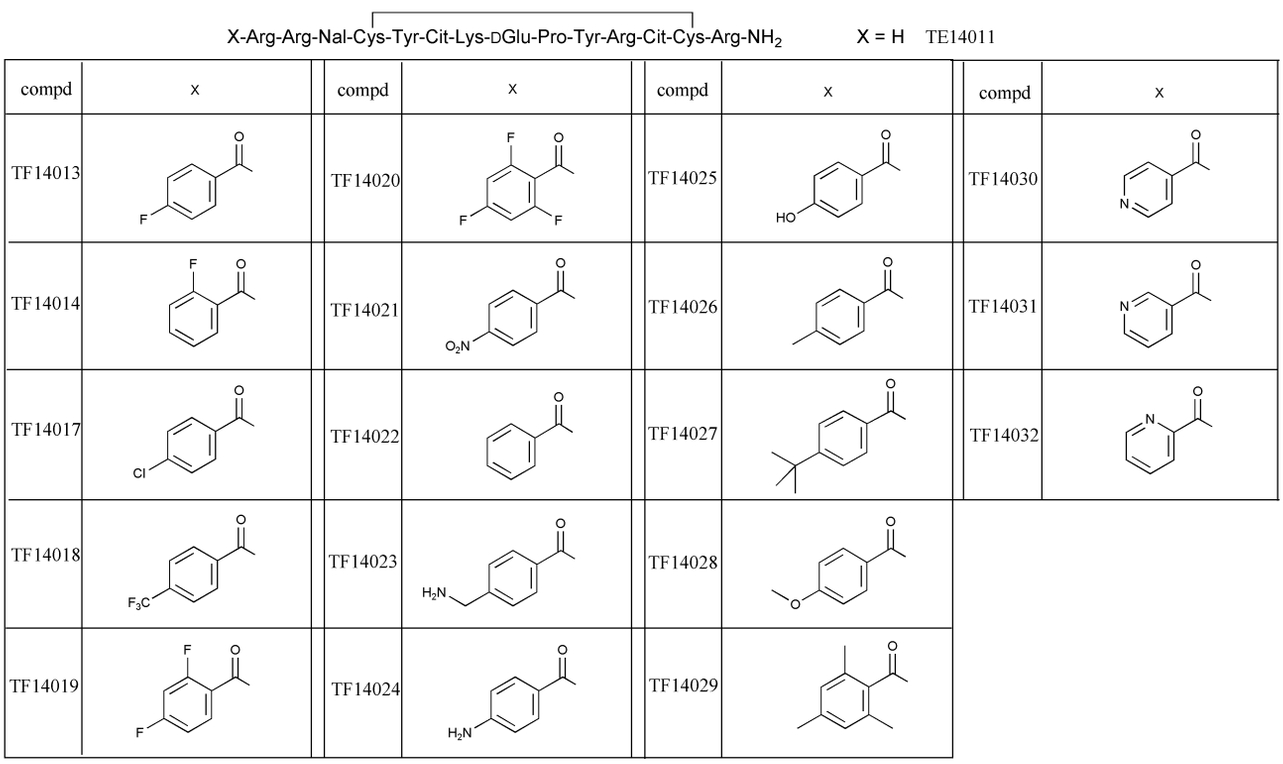


Fig. 3 Structures of TE14011 analogs (TF14017–TF14032).

were used for the SAR study as activity indicators. The sum of Hammett constants (σ) of substituents on benzoic acids[6–8] attached at the *N*-terminus is shown in Table 2 to evaluate the electron-withdrawing or -donating effect of the substituents on the aromatic ring. In general, analogs possessing electron-withdrawing substituents on the aromatic ring (TF14013, TF14014 and TF14018–TF14021) showed higher anti-HIV activity than analogs with electron-donating substituents (TF14024–TF14029), while the analog containing an unmodified normal benzoyl group (TF14022) exhibited relatively high potency (3-fold less potent than TF14013). However, several exceptions were observed. In TF14017, 4-chloro is an electron-withdrawing group (σ = 0.23), whereas anti-HIV activity of TF14017 is lower than that of TF14022, possibly due to a steric effect of the chlorine atom. A 4-fluorobenzoyl moiety (TF14013) was preferable to a 2-fluorobenzoyl moiety (TF14014) in terms of anti-HIV activity in spite of the same values of σ. An analog having a 2,4-difluorobenzoyl moiety (TF14019) showed comparable potency to the analog with a 2-fluorobenzoyl moiety (TF14014), while an analog having a 2,4,6-trifluorobenzoyl moiety (TF14020) exhibited strong anti-HIV activity equivalent to that of TF14013, having a 4-fluorobenzoyl moiety. Analogs possessing a 4-trifluoromethylbenzoyl moiety (TF14018) and a 4-nitrobenzoyl moiety

(TF14021) showed relatively low anti-HIV activity in spite of the high σ values of their substituents (0.54 and 0.78, respectively). A possible rationalization would be the steric effect of a bulky group, such as CF_3 or NO_2. To investigate the quantitative relationships between anti-HIV activity and the electronic and steric effects of substituents on the phenyl ring, the log(1/EC_{50} ratio) values were analyzed by the classical QSAR procedure.[9,10] The Taft E_S was used as a steric parameter.[8,11] The following eqns. (1) and (2) were formulated for the activity of 4-monosubstituted benzoyl derivatives and the whole compound set, respectively. TF14023 was excluded from the analyses because of the lack of its E_S value.

$$\log(1/EC_{50}\ \text{ratio}) = 1.28\ (\pm 0.90)\ \sigma + 0.57\ (\pm 0.43)\ E_S - 0.55\ (\pm 0.59) \quad n = 10,\ s = 0.45,\ r = 0.83 \quad (1)$$

$$\log(1/EC_{50}\ \text{ratio}) = 1.56\ (\pm 0.73)\ \Sigma\sigma + 0.63\ (\pm 0.34)\ E_S - 0.55\ (\pm 0.42) \quad n = 14,\ s = 0.45,\ r = 0.86 \quad (2)$$

In eqns. (1) and (2), n is the number of compounds, s is the standard deviation, r is the correlation coefficient, and the figures in parentheses are the 95% confidence intervals. The positive slope of σ and $\Sigma\sigma$ in eqns. (1) and (2) showed that the stronger the electron-withdrawing effect of substituents on the aromatic ring, the higher the anti-HIV activity of the compound. However, other effects on anti-HIV activity, such as steric effects, are important. Since the bulkier substituent has the smaller E_S value, the equations represent the bulky substituent at the 4-position of the phenyl ring as being unfavorable for the activity. The plot between observed and calculated values from eqn. (2) is shown in Fig. 4. Although the effect of the 2-substituent on the phenyl ring was introduced in eqn. (2) because of the small number of the 2-substituted benzoyl derivatives, the proximity effect owing to a 2-substituent may unfavorably affect the activity. Since aromatic rings possessing electron-withdrawing substituents are electron-deficient, TF14013 analogs having electron-deficient pyridine rings were also examined for anti-HIV activity. Compounds containing isonicotinoyl and nicotinoyl groups (Fig. 3, TF14030 and TF14031, respectively) showed almost the same anti-HIV activity as that of TF14013, whereas a compound with a picolinoyl group (TF14032) exhibited slightly lower potency than TF14013. It was confirmed that an electron-deficient aromatic ring at the *N*-terminus of TE14011 analogs is favorable for anti-HIV activity, suggesting the potential for a π interaction of the *N*-terminal aromatic ring of TE14011 analogs with an aromatic ring of a CXCR4 amino acid residue. In all the compounds, significant cytotoxicity was not detected (CC_{50} > 45 μM, Tables 1 and 2).

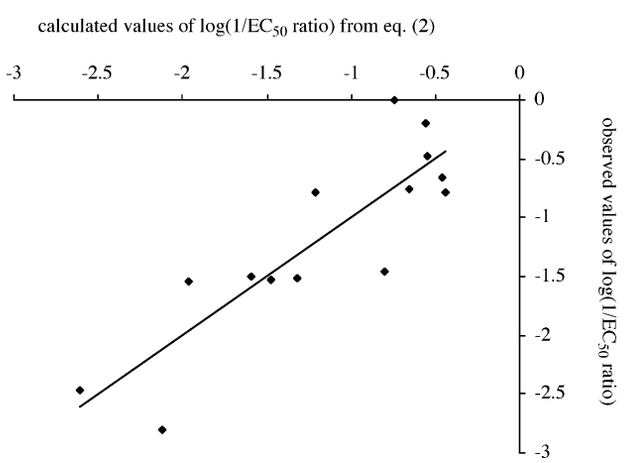
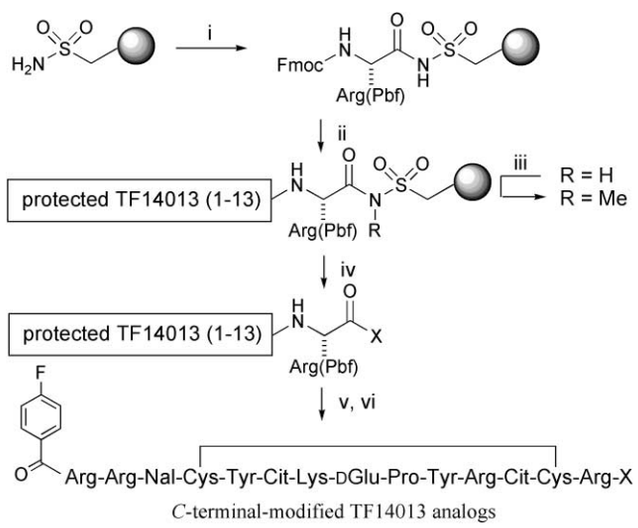


Fig. 4 Plots of observed values of log(1/EC_{50} ratio) *versus* calculated values of log(1/EC_{50} ratio) from eqn. (2).

The biostability of these *N*-terminal-modified analogs was investigated. Two compounds, TF14013 and TF14016, were proven to be stable in mouse serum (Electronic Supplementary Information (ESI), Fig. S1), comparable to TE14011 and Ac-TE14011.[1] Since all these compounds possessing the *C*-terminal carboxy-amide forms are thought to be stable in serum, other analogs were not tested.[4] However, TF14002 (N^{α}-guanyl), TF14005 (des-N^{α}-amino), TF14006 (des-N^{α}-amino, N^{δ}-tetramethylguanyl) and TF14013 (N^{α}-4-fluorobenzoyl) were found to be unstable by incubation with rat liver homogenate[12] at 37 °C (ESI, Fig. S2 a–d), whereas Ac-TE14011 (N^{α}-acetyl, TF14001) was highly resistant against biodegradation (71% recovery at 24 h).[1] In biodegradation of all these peptides, a *C*-terminal-deleted derivative, des-[Arg^{14}-NH_2]-derivative, was identified by ion-spray MS analysis and HPLC analysis by co-injection with an authentic sample as seen in biodegradation of Ac-TE14011. The order of the rate of degradation of these parent peptides and appearance of des-[Arg^{14}-NH_2]-derivative is shown as follows: TF14006 (fast), TF14002, TF14005, TF14013 and Ac-TE14011[1] (slow). All the *N*-terminal modifications efficiently suppressed the cleavage of the parent peptide from the *N*-terminus. However, the rate of the deletion of Arg^{14}-NH_2 from the *C*-terminus depended on the species of *N*-terminal modifications, suggesting the influence of the *N*-terminal region on an enzymatic cleavage in the *C*-terminal region, possibly due to close proximity in space. The N^{α}-4-fluorobenzoyl derivative of TN14003 (TF14016) was also found to be unstable in rat liver homogenate, due to the cleavage of Arg^{14}-NH_2 (ESI, Fig. S2 e) as seen in the biodegradation of TF14013.

In order to suppress the biodegradation of TF14013 at the *C*-terminus, a series of derivatives with *C*-terminal *N*-alkylamides were prepared to evaluate anti-HIV activity and stability in liver homogenate (Scheme 1, Table 3), since *C*-terminal Arg^{14} is a necessary moiety in the anti-HIV pharmacophore. A *C*-terminal *N*-methylamide derivative, TF14013-Me, showed almost the same potency as that of TF14013. It was found that the larger the *N*-alkyl group of the *C*-terminal amide, the weaker the anti-HIV activity. TF14013-tyramine, with replacement of the NH_2 moiety of the *C*-terminal amide in TF14013 by tyramine, exhibited the lowest potency among these derivatives. In all these compounds, significant cytotoxicity was not detected (CC_{50} > 100 μM). Furthermore, these *C*-terminal modifications completely suppressed biodegradation of TF14013 derivatives with rat liver homogenate (ESI, Fig. S2 f). Appearance of des-[Arg^{14}-NHR]-derivatives was not observed in any modifications. Treatment of TE14011, TF14001 and



Scheme 1 *Reagents*: (i) Fmoc–Arg(Pbf)–OH, DIPEA, PyBOP; (ii) solid-phase peptide synthesis; (iii) TMS–CHN_2; (iv) substituted amine (HX); (v) TFA, thioanisole; (vi) $AcONH_4$ buffer pH 7.5; X is shown in Table 3.

Table 3 Anti-HIV activity and cytotoxicity of *C*-terminal-modified TF14013 analogs

Compound	X[a]	*Q*[b]	EC_{50}/nM[c]	CC_{50}/μM[d]	SI
TF14013	NH_2	1	2.4	>100	>42000
TF14013-Me	NHMe	1.1	2.7	>100	>37000
TF14013-Et	NHEt	2.5	6.1	>100	>16000
TF14013-Pr^i	$NHPr^i$	5.7	14	>100	>7400
TF14013-tyramine	tyramine	7.3	17	>100	>5700
AZT		5.4	13	210	16000
ddC		480	1100	5000	>4400

[a] The whole structure is shown in Scheme 1. [b] $Q = EC_{50}$ of each compound/EC_{50} of TF14013. [c] All data are the mean values for at least three experiments. [d] All data are the mean values for at least three experiments.

Table 4 Inhibitory activities of TE14011 analogs against SDF-1α-induced Ca^{2+} mobilization and against binding of ^{125}I-SDF-1α to CXCR4

Compound	Ca^{2+} mobilization IC_{50}/nM[a]	Binding of SDF IC_{50}/nM[b]
T140	3.6	0.93
TF14013	3.7	1.5
TF14016	4.5	0.91
TF14030	4.3	1.6
TF14031	3.3	1.3
TF14013-Me	4.9	1.9
TF14013-Et	9.9	3.0
TF14013-Pr^i	6.4	3.3
TF14013-tyramine	5.2	2.7

[a] IC_{50} values are the concentrations for 50% inhibition of Ca^{2+} mobilization induced by SDF-1α-stimulation through CXCR4. [b] IC_{50} values are the concentrations for 50% inhibition of binding of ^{125}I-SDF-1α to CXCR4. All data are the mean values for at least two experiments.

TF14013 with carboxy peptidase B (from bovine pancreas, Worthington Biochemical Corp., NJ, USA, 2 units/100 nmol peptide) caused a partial cleavage of Arg-NH_2 from the parent peptides (14–20%, at 37 °C, 24 h) whereas TF14013-Me showed complete resistance against this enzyme (data not shown), suggesting that biodegradation of these TE14011 analogs from the *C*-terminus in liver homogenate is performed by a carboxypeptidase-like enzyme. Thus, TF14013-Me possesses high anti-HIV activity as well as highly increased biostability. It is a matter of course that TF14013-Me was found to be completely stable in mouse serum (ESI, Fig. S1).

To investigate whether the present compounds interact with CXCR4, inhibitory activity of several representative compounds against CXCR4 was evaluated by the following assays: inhibition against Ca^{2+} mobilization induced by SDF-1α-stimulation through CXCR4[13] and binding of SDF-1α to CXCR4.[14] 4-Fluorobenzoyl derivatives, TF14013 and TF14016, and TE14011 derivatives containing pyridine rings at the *N*-terminus, TF14030 and TF14031, and a *C*-terminal *N*-methylamide TF14013 derivative, TF14013-Me, showed almost the same levels of inhibitory activity against Ca^{2+} mobilization and SDF binding as those of T140 (Table 4). Inhibitory activity of these strong CXCR4 antagonists against Ca^{2+} mobilization and binding of SDF reached the maximum peaks of IC_{50} at 3–5 nM and 1–2 nM, respectively, thus the difference of inhibitory potencies among these antagonists was not clearly observed.[1] Other *C*-terminal *N*-alkylamide TF14013 derivatives, TF14013-Et, TF14013-Pr^i and TF14013-tyramine, have relatively low anti-HIV activity, and also showed slightly lower inhibitory activity against Ca^{2+} mobilization and SDF binding, compared with T140 and TF14013. Therefore, TF14013 and TF14013-Me were proven to be strong CXCR4 inhibitors.

Finally, the solution structures of novel compounds, TF14013, TF14016, TF14031 and TF14013-Me, were analyzed by CD. T140 and TE14011 form β-sheet structures as shown by NMR and CD analyses.[1,15] All of the CD spectra for TF14013, TF14016, TF14031 and TF14013-Me showed the characteristic pattern of β-sheet structures as seen in the spectra of T140 and TE14011: a strong negative band near 210 nm and a strong positive band near 197 nm (Fig. 5).[2] Thus, these compounds have no significant changes in the secondary structure, suggesting that the spacial disposition of pharmacophores of T140, Arg^2, Nal^3, Tyr^5 and Arg^{14}, is maintained.[3] Therefore, the novel extension of the pharmacophore of a 4-fluorobenzoyl or nicotinoyl moiety is thought to be located in the flexible region at the *N*-terminus.

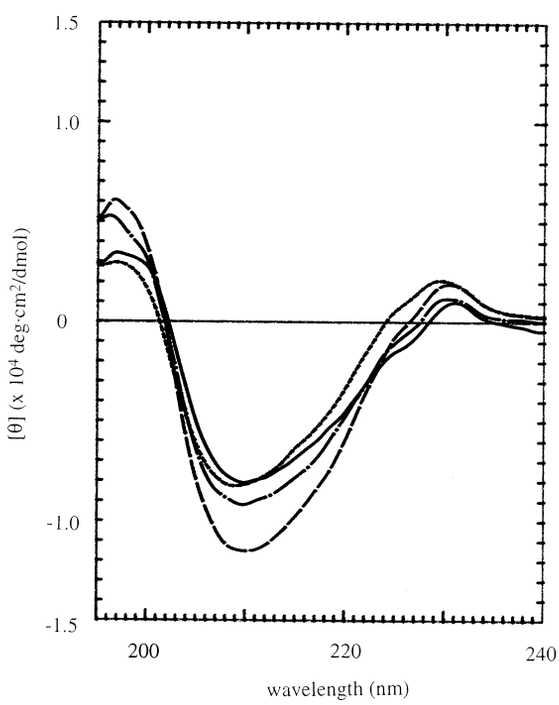


Fig. 5 CD spectra of 4F-benzoyl-TE14011 (TF14013) (dashed line), 4F-benzoyl-TN14003 (TF14016) (center-dotted line), nicotinoyl-TE14011 (TF14031) (solid line) and 4F-benzoyl-TE14011-Me (TF14013-Me) (dotted line).

Chemistry

The synthesis of TE14011 and TF14001 (Ac-TE14011) was reported in the previous paper.[1] The protected TF14002–TF14014 resins were constructed using the Fmoc-based solid-phase synthesis followed by *N*-terminal modifications. Cleavage of the peptides from the resins and deprotection were performed by the TFA–thioanisole system, and the resulting SH-peptides were air-oxidized to obtain corresponding peptides, which were purified by HPLC and gel-filtration. Each *N*-terminal modification was performed after the Fmoc-based solid-phase synthesis as follows. In the synthesis of TF14002 and TF14003, the N^{α}-amino group of Arg(Pbf)[1] [Pbf = 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl] was guan-

ylated and tetramethylguanylated by treatment of 1*H*-pyrazole-1-carboxamidine–DIPEA and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU),[16] respectively. In the synthesis of TF14004, N^{δ}-Fmoc-5-aminopentanoic acid was condensed to the N^{α}-amino group of Arg(Pbf)^2 instead of Fmoc-Arg(Pbf)-OH using 1,3-diisopropylcarbodiimide (DIPCDI) and *N*-hydroxybenzotriazole (HOBt) followed by deprotection of the Fmoc group. In the synthesis of TF14005 and TF14006, the amino group of the aminopentanoyl moiety on the above protected TF14004 resin was guanylated and tetramethylguanylated by treatment of 1*H*-pyrazole-1-carboxamidine–DIPEA and TBTU, respectively. In the synthesis of TF14007, TF14008, TF14009 and TF14010, after condensation of Fmoc-Arg(Pbf)-OH at position 2 and the subsequent deprotection of the Fmoc group, the N^{α}-amino group of Arg(Pbf)^2 was guanylated, tetramethylguanylated, hemisuccinylated and hemiglutarylated, respectively. In the synthesis of TF14012, an argininal derivative, Boc-Arg(Mts)-H, was coupled to the N^{α}-amino group of Arg(Pbf)^2 instead of Fmoc-Arg(Pbf)-OH by reductive amination with $NaB(CN)H_3$. In the synthesis of TF14013 and TF14014, 4-fluorobenzoic acid and 2-fluorobenzoic acid were condensed to the N^{α}-amino group of Arg(Pbf)^1, respectively. The synthesis of TF14015 (Ac-TN14003) was reported in the preceding paper.[1] TF14016 was synthesized based on the TN14003 sequence in the same way as in the synthesis of TF14013.

TE14011 analogs, TF14017–TF14032, were synthesized in the same manner as in the synthesis of TF14013, except for using several benzoic acid derivatives instead of 4-fluorobenzoic acid for the *N*-terminal modifications.

In the synthesis of TF14013 analogs having *C*-terminal *N*-alkylamide, the first amino acid, Fmoc-Arg(Pbf)-OH, was loaded on a sulfonamide resin, 4-sulfamylbutyryl AM NovaGel-resin,[17–19] using benzotriazol-1-yloxytris(pyrrolidino)-phosphonium hexafluorophosphate (PyBop)[20] and DIPEA, followed by construction of the protected peptide resin using the standard Fmoc-based solid-phase synthesis (Scheme 1). After *N*-methylation of sulfonamide with the trimethylsilyldiazomethane (TMS-CHN_2) activation, *N*-alkylamide derivatives of protected TF14013 were cleaved from the resin by treatment with several substituted amines. Removal of all protecting groups with the TFA–thioanisole system and air oxidation yielded the corresponding crude *C*-terminal-modified derivatives, which were purified by HPLC and gel-filtration.

Conclusion

CXCR4 represents an important target for the development of new drugs, since the interaction between CXCR4 and SDF-1α critically links several diseases. In this study, an SAR study of TE14011 analogs containing several benzoyl derivatives at the *N*-terminus has shown that there is a significant relationship between anti-HIV activity of TE14011 analogs and the Hammett constant (σ) of substituted benzoic acids, and that an electron-deficient aromatic ring at the *N*-terminus of TE14011 analogs is preferable for strong anti-HIV activity. As a result, TE14011 analogs, with a novel pharmacophore at the *N*-terminus, such as a 4-fluorobenzoyl moiety, suggest the presence of the π interaction with an aromatic ring of a CXCR4 amino acid residue. Furthermore, *N*-alkylamidation at the *C*-terminus of a TE14011 analog increased biostability in rat liver homogenate, and 4F-benzoyl-TE14011-Me (TF140013-Me) possesses very high anti-HIV activity and biostability. Since T140 is an inverse agonist for a constitutively active mutant of CXCR4 and wild type CXCR4 and T140 lacks partial agonistic activity that might induce toxicities,[21] these CXCR4 inhibitors are also thought to be useful compounds for chemotherapy of AIDS, cancer and rheumatoid arthritis.

Experimental

General

HPLC solvents were H_2O and CH_3CN, both containing 0.1% (v/v) TFA. For analytical HPLC, a Cosmosil 5C18-AR column (4.6 × 250 mm, Nacalai Tesque Inc., Kyoto, Japan) was eluted with a linear gradient of CH_3CN at a flow rate of 1 mL min^{-1} on a Waters™ 717 plus autosampler (Nihon Millipore, Ltd., Tokyo, Japan) equipped with a Hitachi D-2500 chromato-integrator (Tokyo, Japan). Preparative HPLC was performed on a Waters Delta Prep 4000 equipped with a Cosmosil 5C18-AR column (20 × 250 mm, Nacalai Tesque Inc.) using a linear gradient of CH_3CN at a flow rate of 15 mL min^{-1}. For gel-filtration, the solution was applied to a column of Sephadex G-15 (2.1 × 30 cm), which was eluted with 1 M AcOH. Ion-spray (IS)-mass spectra were obtained with a Sciex API*III*E triple quadrupole mass spectrometer (Toronto, Canada). Optical rotation of a peptide in aqueous solution was measured with a JASCO DIP-360 digital polarimeter (Tokyo, Japan) or a Horiba high-sensitive polarimeter SEPA-200 (Kyoto, Japan). Fmoc-protected amino acids, a 4-(2′,4′-dimethoxyphenylaminomethyl)phenoxy (SAL) resin and a 4-sulfamylbutyryl AM NovaGel™ resin were purchased from Watanabe Chemical Industries, Ltd. (Hiroshima, Japan) or Calbiochem-Novabiochem Japan, Ltd. (Tokyo, Japan). All the other chemicals were purchased from either Nacalai Tesque Inc. or Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The procedures of biological assays and CD spectroscopy are described in the previous paper.[1]

Synthesis of TF14002–TF14032

TF14002–TF14032 were synthesized using Fmoc-based solid-phase synthesis followed by *N*-terminal modifications in a similar manner to the synthesis of TF14001 (Ac-TF14011) as reported in the previous paper.[1] Procedures of *N*-terminal modifications in the synthesis of TF14002–TF14032 are given below.

TF14002. After deprotection of the Fmoc group of Arg(Pbf)^1, the protected resin (0.05 mmol) was reacted with 1*H*-pyrazole-1-carboxamidine (37 mg, 0.25 mmol) and DIPEA (87 μL, 0.5 mmol) in DMF for 2 h.

TF14003. After deprotection of the Fmoc group of Arg(Pbf)^1, the protected resin (0.05 mmol) was reacted with TBTU (80 mg, 0.25 mmol) in DMF for 2 h.

TF14004. After deprotection of the Fmoc group of Arg(Pbf)^2, the protected resin (0.05 mmol) was reacted with N^{δ}-Fmoc-5-aminopentanoic acid (41 mg, 0.125 mmol), DIPCDI (2.5 equiv.) and HOBt (2.5 equiv.) in DMF for 2 h instead of condensation with Fmoc-Arg(Pbf)-OH, and then treated with 20% (v/v) piperidine–DMF for 1 and 15 min.

TF14005. The above protected TF14004 resin (0.05 mmol) was reacted with 1*H*-pyrazole-1-carboxamidine (37 mg, 0.25 mmol) and DIPEA (87 μL, 0.5 mmol) in DMF for 2 h.

TF14006. The above protected TF14004 resin (0.05 mmol) was reacted with TBTU (80 mg, 0.25 mmol) in DMF for 2 h.

TF14007 and TF14008. The N^{α}-amino group of Arg(Pbf)^2 was guanylated or tetramethylguanylated in the same manner as in the synthesis of TF14002 or TF14003, respectively.

TF14009 and TF14010. After deprotection of the Fmoc group of Arg(Pbf)^2, the protected resin (0.05 mmol) was reacted with succinic anhydride (25 mg, 0.25 mmol) or glutaric anhydride (29 mg, 0.25 mmol) in pyridine for 2 h instead of condensation with Fmoc-Arg(Pbf)-OH.

TF14011. After deprotection of the Fmoc group of Arg(Pbf)[2], no amino acid was condensed at the position 1.

TF14012. After deprotection of the Fmoc group of Arg(Pbf)[2], the protected resin (0.05 mmol) was reacted with Boc-Arg(Mts)-H (0.05 mmol), $NaB(CN)H_3$ (9 mg, 0.15 mmol) and AcOH (3 μL, 0.05 mmol) in DMF for 15 h. Boc-Arg(Mts)-H was prepared by treatment of Boc-Arg(Mts)-NMe(OMe) (25 mg, 0.05 mmol) with DIBAL–toluene (1.0 M, 100 μL) in CH_2Cl_2 (100 μL) at −78 °C for 3 h, followed by quenching with saturated aq. citric acid (1 mL) at −78 °C. The mixture was extracted with EtOAc (3 mL) and the extract was then washed with saturated aq. NaCl (1 mL), and dried over $MgSO_4$, followed by concentration to give crude Boc-Arg(Mts)-H, which was used for the above reductive amination. After the reaction of the reductive amination, the protected resin (0.05 mmol) was treated with 1 M TMSBr-thioanisole–TFA (5 mL), instead of 1 M thioanisole–TFA, in the presence of *m*-cresol (250 μL, 55 equiv.) and 1,2-ethanedithiol (100 μL, 33 equiv.) at room temp. for 15 h.

TF14013, TF14014 and TF14016. After deprotection of the Fmoc group of Arg(Pbf)[1], the protected resin (0.05 mmol) was reacted with 4-fluorobenzoic acid or 2-fluorobenzoic acid (18 mg, 0.125 mmol), DIPCDI (2.5 equiv.) and HOBt (2.5 equiv.) in DMF for 2 h.

TF14017–TF14032. After deprotection of the Fmoc group of Arg(Pbf)[1], the protected resin (0.05 mmol) was reacted with each benzoic acid derivative (5 equiv.), DIPCDI (5 equiv.) and HOBt (5 equiv.) in DMF for 2 h.

Synthesis of TF14013 analogs with *C*-terminal *N*-alkylamide. TF14013-Me

On a 4-sulfamylbutyryl AM NovaGel-resin (0.60 meq/g, 0.1 mmol scale), Fmoc-Arg(Pbf)-OH (4.0 equiv.) was loaded using DIPEA (6.0 equiv.) and PyBOP (3.0 equiv.) in $CHCl_3$ at −20 °C for 8 h. After this condensation reaction was repeated twice, the protected TF14013 resin was constructed using standard Fmoc-based solid-phase synthesis. After the resin was swollen with THF (2 mL), 2 M TMS-CHN_2–hexane (2 mL, 40 equiv.) was added, and the mixture was then stirred at room temp. for 4 h. After washing the activated resin with DMF, THF and $CHCl_3$ (10 mL × 3 each), 2 M methylamine–THF (5 mL, 100 equiv.) and DMF (5 mL) were added, and the mixture was then stirred at room temp. for 24 h. After removal of the resin by filtration, the filtrate was concentrated *in vacuo* to give the protected TF14013 with *C*-terminal *N*-methylamide. Removal of all protecting groups and air oxidation were performed in the same manner as in the synthesis of TF14013 to yield TF14013-Me.

TF14013-Et, TF14013-Pr^i and TF14013-tyramine

TF14013-Et, TF14013-Pr^i and TF14013-tyramine were synthesized in the same manner as in the synthesis of TF14013-Me, except for treatment with 2 M ethylamine–THF (5 mL, 100 equiv.), isopropylamine (0.85 mL, 100 equiv.) and tyramine (1.37 g, 100 equiv., stirring at 60 °C) instead of 2 M methylamine–THF, respectively.

Characterized data of all the synthetic peptides can be found in ESI, Table S1.

Acknowledgements

The authors wish to thank Dr Fumiyoshi Yamashita, Ms Yuko Kawaguchi and Mr Shintaro Fumoto from the Graduate School of Pharmaceutical Sciences, Kyoto University for the preparation of rat liver homogenates, and Drs Hiromu Habashita and Nobuyuki Hamanaka from the Minase Research Institute, Ono Pharmaceutical Co., Ltd. for technical assistance. This work was supported in part by a 21st Century COE program 'Knowledge Information Infrastructure for Genome Science a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan and the Japan Health Science Foundation.

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