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Synthesis of potent CXCR4 inhibitors possessing low cytotoxicity and improved biostability based on T140 derivatives†

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A peptidic CXCR4 antagonist T140 efficiently blocks the entry of T cell line-tropic strains of HIV-1 (X4-HIV-1) into target cells. In this study, a series of T140 derivatives, replacing the basic amino acid residues with Glu (D-Glu) and/or L-citrulline (Cit), were synthesized in order to reduce non-specific binding and cytotoxicity. Among them, TE14011 ([Cit^6, D-Glu^8]-T140 with the *C*-terminal amide) exhibited strong anti-HIV activity and low cytotoxicity. TE14011 was found to be stable in mouse serum, but unstable in rat liver homogenate due to the deletion of the *N*-terminal Arg^1-Arg^2-L-3-(2-naphthyl)alanine (Nal)^3 residues from the parent peptide. *N*-Terminal acetylation of TE14011 led to the development of a novel lead compound, Ac-TE14011, which possesses a high selectivity index as well as increased stability in serum and liver homogenate.

Introduction

Recently, the multiple drug-combination chemotherapy, "highly active anti-retroviral therapy (HAART)", which involves reverse transcriptase/protease inhibitors, has dramatically improved the clinical treatment of individuals with HIV-infection or AIDS.[1] However, there are concerns with HAART, as there still remain several serious problems, including the emergence of viral strains with multi-drug resistance, significant adverse effects and high cost. Discovery and development of a new generation of agents, such as entry/fusion inhibitors, are required for the multiple drug-combination chemotherapy. The recent identification of chemokine receptors, CCR5 and CXCR4, as coreceptors for macrophage-tropic HIV-1 (R5-HIV-1)[2–6] and T cell line-tropic HIV-1 (X4-HIV-1),[7] respectively, provided us with an ideal therapeutic approach to discovery of new entry/fusion inhibitors. We and others have reported several specific antagonists for CCR5 or CXCR4.[8–18] According to very recent papers, several diseases, besides AIDS, are linked to CXCR4. Müller[19] and we[20] reported that the interaction between CXCR4 and its natural ligand, stromal cell-derived factor-1α (SDF-1α),[21–24] is primarily involved in cancer metastasis and progression. In addition, the CXCR4–SDF-1α system is involved in rheumatoid arthritis.[25,26] We developed a 14-residue peptide, T140, as a specific CXCR4 antagonist that prevents X4-HIV-1 entry mediated by this coreceptor (anti-HIV activity: 50% effective concentration (EC_{50}) = 3.5 nM, antagonism of entry by X4-HIV-1: EC_{50} = 0.43 nM).[27] T140 possesses the highest level of anti-HIV activity by antagonising the entry of X4-HIV-1 among all the CXCR4 antagonists that have been reported up to 1998. In addition, T140 was proven to be an inverse agonist for a constitutively active mutant of CXCR4:[28] T140 lacks partial agonistic activity that might induce toxicities. Subsequently, the anti-HIV activity pharmacophore of T140 was identified through a conventional Ala-scanning study to define the critical residues: Arg^2, L-3-(2-naphthyl)alanine (Nal)^3, Tyr^5 and Arg^{14}.[29] However, T140 was found to be unstable in feline and mouse sera due to cleavage of the *C*-terminal Arg^{14} residue, whereas a *C*-terminal-amidated analog of T140 was completely stable, suggesting that T140 analogs require *C*-terminal arginine protection *via* amidation to retain *in vivo* activity.[30] Furthermore, T140 has relatively strong cytotoxicity (CC_{50} = 45 μM). T140 is a positively charged peptide containing basic amino acid residues (Arg^1, Arg^2, Arg^6, Lys^7, D-Lys^8, Arg^{11} and Arg^{14}). The electrostatic interaction of such peptides with cell-membranes might be related to its cytotoxicity[31,32] and might also have non-specific binding to several proteins, lipids and mucosugars. An apparent correlation was found between the number of net-positive charges and anti-HIV activity and cytotoxicity of T140 analogs. Moderate reduction of total net-positive charges resulted in less cytotoxicity, while extreme reduction caused a significant decrease in anti-HIV activity. Reduction of the number of the total positive charges by the substitution of the basic residues with a non-basic polar amino acid, L-citrulline (Cit), was useful for developing effective analogs with high anti-HIV activity and low cytotoxicity.[29] Cit is an analog with an Arg-like isosteric structure without positive charge. A Cit-scanning study, based on the *C*-terminally amidated form, led to the development of novel effective CXCR4 inhibitors, TN14003 ([Cit^6]-T140 with the *C*-terminal amide) and TC14012 ([Cit^6, D-Cit^8]-T140 with the *C*-terminal amide), which possess high selectivity indexes (SIs, SI = CC_{50}/EC_{50}) and complete stability in serum.[30] An exploratory search for potent CXCR4 inhibitors, based on anti-HIV activity as an index parameter, will lead to the development of compounds that

† Electronic supplementary information (ESI) available: Fig. S1: behaviors of TE14005 (a), TE14011 and Ac-TE14011 (b) in mouse serum; Fig. S2: behaviors of TE14011 (a), Ac-TE14011 (b), TN14003 (c), Ac-TN14003 (d), TC14012 (e) and Ac-TC14012 (f) in rat liver homogenate; Table S1: characterization data of novel synthetic peptides; HPLC charts for synthetic compounds of TE14005, TE14011 and Ac-TE14011, and for a degraded sample of TE14011 in rat liver homogenate and its co-injection with an authentic compound des-[Arg^1, Arg^2, Nal^3]-TE14011. See http://www.rsc.org/suppdata/ob/b3/b306473p/

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Table 1 Anti-HIV activity and cytotoxicity of TE14001–TE14007

Compound	Net-positive charges	EC_{50}/nM [a]	EC_{50} ratio [b]	CC_{50}/μM [c]	SI [d]
T140	7	1.6	1	45	28000
TE14001	5	52	32	>100	>1900
TE14002	5	3900	2400	>100	>26
TE14003	5	8.7	5.4	>100	>11000
TE14004	5	54	34	>100	>1900
TE14005	5	0.4	0.25	>100	>250000
TE14006	5	65	41	>100	>1500
TE14007	5	85	53	>100	>1200
AZT		7.9	4.9	12	1500
ddC		88	55	14	160

[a] EC_{50} values are based on the inhibition of HIV-induced cytopathogenicity in MT-4 cells. [b] EC_{50} ratio is EC_{50} of each compound/EC_{50} of T140. [c] CC_{50} values are based on the reduction of the viability of mock-infected MT-4 cells. All data are the mean values for at least three experiments. [d] Selectivity index (SI) is shown as CC_{50}/EC_{50}.

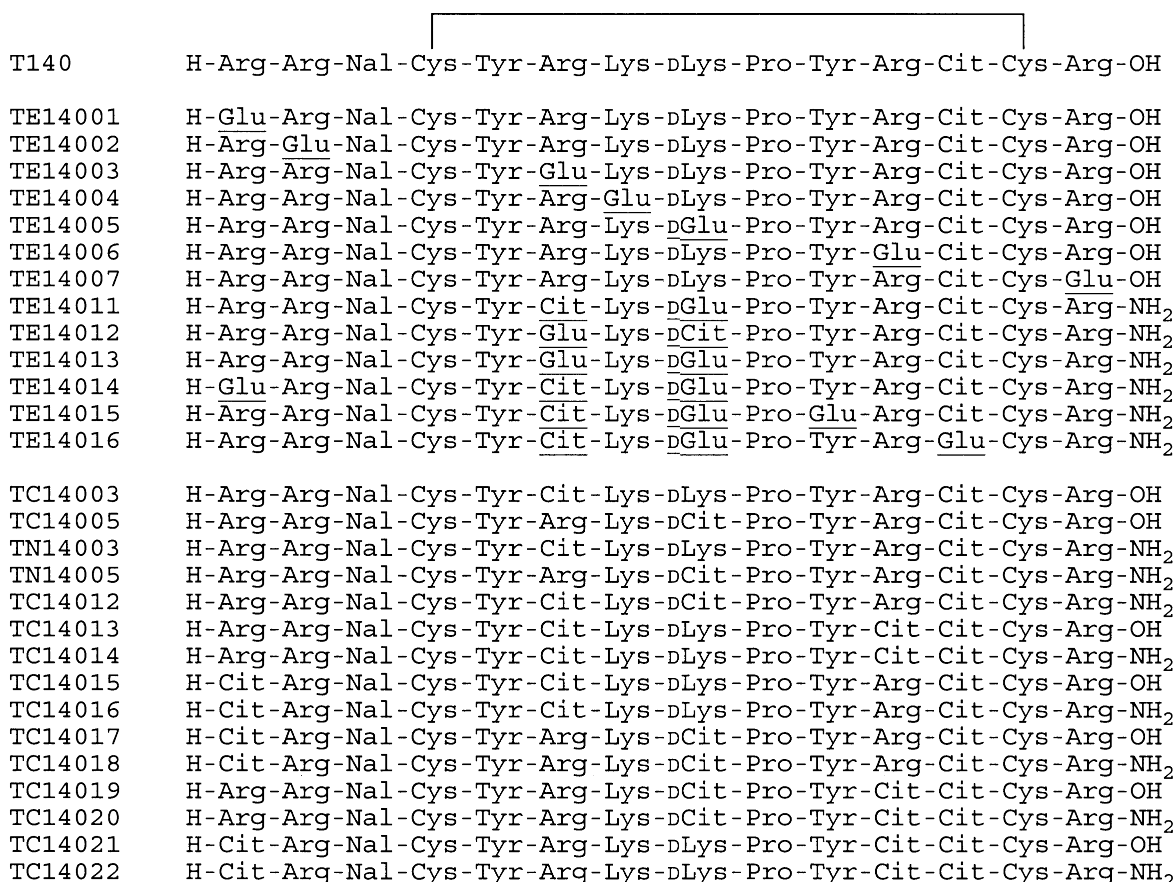

Fig. 1 Amino acid sequences of T140 analogs. Each peptide has a disulfide linkage between Cys^4 and Cys^{13}, which is shown by a solid line.

may be candidates for several diseases, such as cancer and rheumatoid arthritis. In this study, we initially tried to reduce the total net-positive charge of T140 by the replacement of basic residues with Glu (or D-Glu) to find a novel lead compound. Glu-substitution for a basic residue reduces the total charge by −2 while Cit-substitution causes a reduction of −1. Subsequently, Glu- and Cit-substitutions and *N*- and *C*-terminal modifications of T140 analogs were conducted to develop effective CXCR4 inhibitors with increased biostability as described in consecutive papers ‡.

Biological results and discussion

Glu-substitution scanning was conducted for all Arg and Lys residues in T140 (Fig. 1, TE14001–TE14007). Anti-HIV activity was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method (Table 1).[33] EC_{50} values are based on the inhibition of X4-HIV-1 (HIV-1_{IIIB})-induced cytopathogenicity in MT-4 cells. Since EC_{50} values showed a marked tendency to flexibly depend on the conditions of cells and viruses, EC_{50} ratios were adopted as activity indicators between assays performed with different screens. Glu-substitution for Arg^2 and Arg^{14} caused a remarkable decrease in anti-HIV activity (see TE14002 and TE14007). This result is compatible with the previous identification of Arg^2 and Arg^{14} as critical residues.[29] Arg^6 and D-Lys^8 could be replaced by Glu and D-Glu, respectively, without a significant decrease in anti-HIV activity (see TE14003 and TE14005). This is also compatible with our previous findings on TC14003 ([Cit^6]-T140) and TC14005 ([D-Cit^8]-T140).[29] These analogs, TE14001–TE14007, did not show significant cytotoxicity (CC_{50} > 100 μM), underlining that these analogs are less cytotoxic than T140. Therefore, TE14005 may be a useful lead. However, TE14005 was not

‡ This is the first of a pair of papers, preceding *Org. Biomol. Chem.*, 2003, DOI 10.1039/b306613h.

Table 2 Anti-HIV activity and cytotoxicity of TE14011–TE14013

Compound	Net-positive charges	EC_{50}/nM [a]	EC_{50} ratio	CC_{50}/μM [b]	SI
T140	7	6.6	1	>10	>1500
TE14005	5	5.3	0.80	>100	>19000
TE14011	5	1.6	0.24	>100	>63000
TE14012	5	17	2.6	>100	>6000
TE14013	4	25	3.8	>100	>4100
des-[Arg[1], Arg[2], Nal[3]]-TE14011	3	>20000		>20	
AZT		2.0	0.30	36	18000
ddC		160	24	95	580

[a] All data are the mean values for at least three experiments. [b] All data are the mean values for at least three experiments. The estimation of T140 at higher concentrations was omitted in this study.

Table 3 Anti-HIV activity and cytotoxicity of TE14014–TE14016 and *N*-terminal-acetylated derivatives of Glu- or Cit-substituted T140 analogs

Compound	Net-positive charges	EC_{50}/nM [a]	EC_{50} ratio	CC_{50}/μM [b]	SI
T140	7	120	1	>10	>81
TE14011	5	29	0.24	>200	>6800
Ac-TE14011	4	17	0.14	>200	>14000
TE14014	3	340	2.8	>200	>600
Ac-TE14014	2	17000	140	>200	>13
TE14015	4	140	1.2	>200	>1500
Ac-TE14015	3	150	1.3	>200	>1400
TE14016	4	810	6.8	>200	>260
Ac-TE14016	3	600	5.0	>200	>350
TC14003	6	33	0.28	120	3600
Ac-TC14003	5	29	0.24	>200	>6700
TC14005	6	24	0.20	180	7300
Ac-TC14005	5	32	0.27	>200	>6000
TN14003	7	140	1.2	90	650
Ac-TN14003	6	29	0.24	110	4100
TN14005	7	36	0.30	90	2600
Ac-TN14005	6	32	0.27	100	3100
TC14012	6	37	0.31	180	4800
Ac-TC14012	5	29	0.24	>200	>6800
TC14013	5	37	0.31	>200	>5800
Ac-TC14013	4	750	6.3	>200	280
TC14014	6	130	1.1	>200	>1700
Ac-TC14014	5	730	6.1	>200	>290
TC14015	5	38	0.32	>200	>5300
Ac-TC14015	4	700	5.8	>200	>300
TC14016	6	170	1.4	>200	>1200
Ac-TC14016	5	170	1.4	>200	>1200
TC14017	5	140	1.2	>200	>1400
Ac-TC14017	4	170	1.4	>200	>1200
TC14018	6	110	0.92	>200	>1900
Ac-TC14018	5	120	1.0	150	1300
TC14019	5	33	0.28	>200	>6300
Ac-TC14019	4	680	5.7	>200	>310
TC14020	6	130	1.1	>200	>1600
Ac-TC14020	5	150	1.3	>200	>1400
TC14021	5	430	3.6	>200	>490
Ac-TC14021	4	5700	48	>200	>37
TC14022	6	170	1.4	>200	>1200
Ac-TC14022	5	1000	8.3	>200	>200
AZT		70	0.58	150	2100
ddC		3700	31	2300	630

[a] All data are the mean values for at least three experiments. [b] All data are the mean values for at least three experiments. The estimation of T140 at higher concentrations was omitted in this study.

stable in mouse serum due to the cleavage of the *C*-terminal Arg[14] residue (TE14005 has a *C*-terminal carboxy-free form as in the case of T140) (Electronic Supplementary Information (ESI), Fig. S1 a).[30] Therefore, Glu-substituted T140 analogs, such as TE14005, also require *C*-terminal amidation. *C*-Terminal amidation of TE14005 analogs causes a charge increase of +1 (+5 to +6). Thus, in order to decrease the net-positive charge, additional Glu- or Cit-substitution was conducted in combination with the *C*-terminal amidation. In accordance with the above results, both Arg[6] and D-Lys[8] were replaced by Glu (D-Glu) and Cit (D-Cit) to synthesize TE14011–TE14013. TE14011 ([Cit[6], D-Glu[8]]-T140 with the *C*-terminal amide) showed the strongest anti-HIV activity among analogs substituted at Arg[6] and D-Lys[8], even compared with TE14005 and T140 (Table 2). Next, for the purpose of further reduction of net-positive charge, Arg[1], Tyr[10] and Cit[12] in TE14011 were chosen as additional candidates for substitution by Glu, since Ala-substitution of Arg[1] did not cause a significant decrease in anti-HIV activity (see TE14001, Table 1) and, Tyr[10] and Cit[12] are the non-critical residues of T140.[29] TE14014 ([Glu[1]]-TE14011), TE14015 ([Glu[10]]-TE14011) and TE14016 ([Glu[12]]-TE14011) (Fig. 1) showed lower anti-HIV activity than TE14011, whereas TE14015 exhibited almost the same potency as that of T140 (Table 3). Taken together, TE14011 is the most effective compound among T140 analogs with Glu- and Cit-substitution. In addition, TE14011 was found to be completely

stable in mouse serum (ESI, Fig. S1 b). TE14011–TE14016 did not show significant cytotoxicity.

The metabolic stability of TE14011 was studied in a biological medium, rat liver homogenate, which is known to have broad enzymatic activity.[34] The behavior of TE14011 in incubation with rat liver homogenate at 37 °C was investigated by HPLC and mass analysis (ESI, Fig. S2 a). It was found that 70% of this peptide was degraded in a 4 h incubation with liver homogenate due to the cleavage of Arg^1-Arg^2-Nal^3 and Arg^{14}-NH_2 from the *N*- and *C*-terminal ends, respectively, identified by ion-spray MS (IS-MS) analysis and the HPLC analysis by co-injection with the authentic sample. The synthetic deletion peptide des-[Arg^1, Arg^2, Nal^3]-TE14011 showed no significant anti-HIV activity (Table 2). Nal was detected by the HPLC and IS-MS analysis while Arg could not be detected due to its very high hydrophilicity. An *N*- and *C*-terminal-degraded derivative of TE14011, des-[Arg^1, Arg^2, Nal^3, Arg^{14}-NH_2]-TE14011, could not be detected. No peptide bond in the disulfide loop (4–13) was cleaved, suggesting that this disulfide bridge contributes to the biostability of the cyclic decapeptide substructure. An *N*-terminal-acetylated derivative of TE14011, Ac-TE14011, was proven to be highly resistant against biodegradation with rat liver homogenate, whereas a small amount of the *C*-terminal-degraded derivative, des-[Arg^{14}-NH_2]-Ac-TE14011, was still detected (ESI, Fig. S2 b). Since N^α-acetylation suppressed the cleavage of the parent peptide from the *N*-terminus, TE14011 is thought to be cleaved stepwise from the *N*-terminus by an exopeptidase, such as aminopeptidase. Treatment of TE14011 with aminopeptidase M (porcine kidney, Calbiochem, CA, USA, 2 units/100 nmol peptide) caused complete degradation of the parent peptide (at 37 °C, 1 h) whereas Ac-TE14011 showed complete resistance against this enzyme (data not shown). Ac-TE14011 was also stable in mouse serum (ESI, Fig. S1 b). The previous compounds, TN14003 and TC14012, have the same instability in rat liver homogenate (ESI, Fig. S2 c, e) and can be similarly protected with *N*-terminal acetylation (ESI, Fig. S2 d, f).

We subsequently synthesized *N*-terminal-acetylated compounds of Glu-substituted TE14011 analogs (TE14014, TE14015 and TE14016) and used Cit-substituted T140 analogs synthesized previously (TC14003, TC14005, TN14003, TN14005 and TC14012–TC14022, Fig. 1) to evaluate anti-HIV activity (Table 3). Ac-TE14011 showed slightly higher potency, compared with TE14011. Acetylation of TE14014–TE14016 analogs did not bring a noticeable change in anti-HIV activity, except for TE14014. *N*-Terminal acetylation of TE14014 caused a remarkable reduction in potency, possibly due to a decrease in net-positive charge (+3 to +2), suggesting that these compounds require at least +3 charge for the expression of strong anti-HIV activity. Our previous lead compounds, TC14003, TC14005, TN14003, TN14005 and TC14012, did not show a significant change of anti-HIV activity upon acetylation. Acetylated analogs of TC14012–TC14022 showed somewhat lower or almost the same potency, compared with the corresponding parent compounds. Taken together, since Ac-TC14003 and Ac-TC14005 are thought to be unstable in serum due to the *C*-terminal carboxy-free form as in the case of T140, Ac-TE14011, Ac-TN14003, Ac-TN14005 and Ac-TC14012 might become useful lead compounds. Ac-TE14011, Ac-TN14003, and Ac-TC14012 were found to be highly resistant against biodegradation with mouse serum (data of Ac-TE14011 are shown in ESI, Fig. S1 b, data for others are not shown) and rat liver homogenate, although the biostability of Ac-TN14005 was not investigated. Ac-TE14011 and Ac-TC14012 did not show cytotoxicity until a concentration of 200 μM, while CC_{50} values of Ac-TN14003 and Ac-TN14005 are 110 and 100 μM, respectively.

We investigated whether these novel compounds, which possess strong anti-HIV activity, are CXCR4 inhibitors. Inhibitory activity of TE14005, TE14011 and Ac-TE14011 against CXCR4 was evaluated by two assays: inhibition against Ca^{2+} mobilization induced by SDF-1α-stimulation through CXCR4[35] and against binding of SDF-1α to CXCR4.[36] TE14005, TE14011 and Ac-TE14011 showed almost the same inhibitory activity against Ca^{2+} mobilization and against binding of SDF as that of T140 (Table 4). These inhibition assays are based on competition with SDF for binding to CXCR4, and the binding affinity of SDF for CXCR4 is less than that of the molecules tested. Therefore, inhibitory activities of strong CXCR4 antagonists against Ca^{2+} mobilization and against binding of SDF reached the maximum peaks of IC_{50} at 2–5 nM and 0.5–1 nM, respectively, and thus the difference of inhibitory potency of strong antagonists could not be clearly observed. However, it was confirmed that TE14005, TE14011 and Ac-TE14011 express anti-HIV activity mediated by their interaction with CXCR4.

Table 4 Inhibitory activities of T140 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	2.2	0.90
TE14005	3.5	0.49
TE14011	2.9	0.46
Ac-TE14011	5.6	1.4

[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.

We further investigated whether the lead compounds obtained in this study (TE14005, TE14011 and Ac-TE14011) maintain the solution structure of T140. T140 forms an antiparallel β-sheet structure with a type II′ β-turn formed by Lys^7 (i), D-Lys^8 (i + 1), Pro^9 (i + 2) and Tyr^{10} (i + 3), which was determined by NMR and molecular dynamic calculations.[37] The CD spectrum of T140 showed characteristic patterns derived from β-sheet structures: a strong negative band near 210 nm and a strong positive band near 197 nm.[27] CD spectroscopic analysis of TE14005, TE14011 and Ac-TE14011 revealed that these peptides form β-sheet structures similar to that of T140 (Fig. 2). Thus, these compounds have no significant changes in the secondary structure of the parent compound, T140. Hydrogen bonds formed by the β-sheet/β-turn structure might contribute to the biostability of these compounds, since the peptide bond-cleavage with liver homogenate was not detected in the disulfide loop (4–13) forming the β-sheet/β-turn structure.

Conclusion

A CXCR4 antagonist, T140, is a positively charged peptide possessing many basic amino acid residues in the molecule. Its basicity might cause relatively strong cytotoxicity and non-specific binding to several other proteins, lipids and mucosugars. The replacement of basic amino acid residues in T140 with Glu (or D-Glu) led to the development of an effective compound, TE14005, which possesses a high selectivity index. Since Glu-substitution for a basic residue causes a charge decrease of −2, Glu-substitution is perhaps more efficient than Cit-substitution that causes a charge decrease of −1. However, TE14005 was proven to be unstable in mouse serum. *N*-Terminal acetylation and *C*-terminal amidation in combination with additional Glu (D-Glu)- and/or Cit (D-Cit)-substitution led to development of a compound, Ac-TE14011, which has a high therapeutic index as well as increased biostability in mouse serum and rat liver homogenate. Ac-TE14011 has a net charge of +4, compared to that of +7 for T140, +6 for Ac-TN14003, and +5 for Ac-TC14012. Ac-TE14011 did

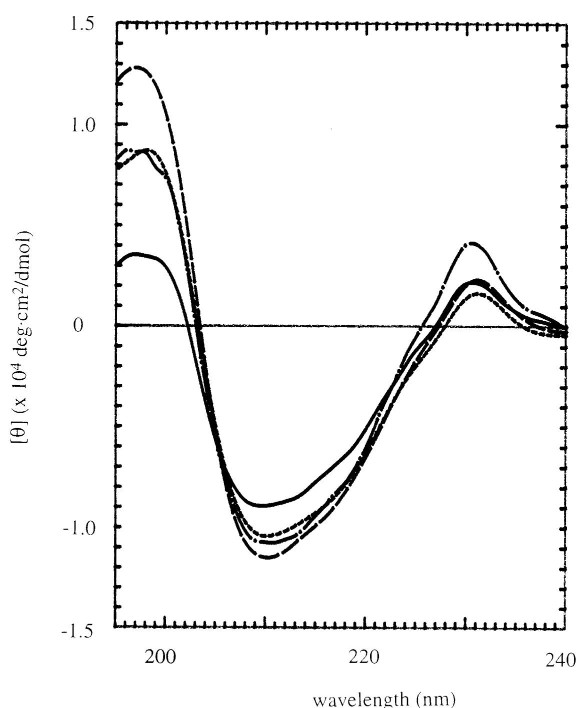

Fig. 2 CD spectra of T140 (dashed line), TE14005 (dotted line), TE14011 (center-dotted line) and Ac-TE14011 (solid line).

not show significant cytotoxicity until 200 μM, suggesting that the possibility of its non-specific binding might be also reduced. Since the CXCR4–SDF-1α system participates in a number of pathogenetical processes,[19,20,25,26] the present results will aid in the rational design and synthesis of CXCR4 inhibitors for the 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, *p*-benzyloxybenzyl alcohol (Alko)-resins, and 4-(2′,4′-dimethoxyphenylamino-methyl)phenoxy (SAL) 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).

Peptide synthesis

The protected peptidyl resin was manually constructed using Fmoc-based solid-phase synthesis on an Fmoc-Arg(Pbf)-Wang resin (0.64 meq/g, 0.1 mmol scale, Pbf = 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl, Wang resin = *p*-benzyloxybenzyl alcohol resin) for the synthesis of TE14001–TE14006, on an Fmoc-Glu(OBu^t)-Wang PEG-resin (0.21 meq/g, 0.1 mmol scale) for the synthesis of TE14007, or on a 4-(2′,4′-dimethoxy-phenylaminomethyl)phenoxy resin (0.33 meq/g, 0.2 mmol scale) for the synthesis of TE14011–TE14016. Fmoc-protected amino acid derivatives (2.5 equiv.) were successively condensed using 1,3-diisopropylcarbodiimide (DIPCDI) (2.5 equiv.) in the presence of *N*-hydroxybenzotriazole (HOBt) (2.5 equiv.) in DMF. The following side-chain protecting groups were used: Pbf for Arg, Trt for Cys, Bu^t for Tyr, Glu and D-Glu and Boc for Lys and D-Lys. The Fmoc-group was deprotected by treatment of the resin with 20% (v/v) piperidine–DMF for 1 and 15 min. In the synthesis of *N*-terminal acetylated peptides, after final deprotection of the Fmoc-group following the condensation of 14 residues, an N^{α}-amino group was acetylated by treatment of the resin with Ac_2O (200 equiv.) and pyridine (200 equiv.) in $CHCl_3$. The resulting protected peptidyl resin (50 μmol) was treated with 1 M thioanisole–TFA (5 mL) in the presence of *m*-cresol (250 μL, 55 equiv.) and 1,2-ethanedithiol (100 μL, 33 equiv.) at 4 °C for 2 h. After removal of the resin by filtration, the filtrate was concentrated *in vacuo*. Ice-cold dry diethyl ether (30 mL) was added to the residue. The resulting powder was collected by centrifugation and then washed three times with ice-cold dry diethyl ether (20 mL × 3). The crude reduced peptide was dissolved in 50% (v/v) AcOH (2 mL). Subsequently, the solution was diluted to total volume 400 mL with H_2O, and then pH was adjusted to 7.8 with concentrated NH_4OH. After air-oxidation for 1 d, the pH of the solution was adjusted to 5 with AcOH. The crude product in the solution was purified by preparative HPLC and gel-filtration to afford a fluffy white powder of the desired peptide. Characterized data of all the synthetic peptides are listed in ESI, Table S1.

Behavior of T140 analogs in mouse serum

Test compounds (100 nmol) were dissolved in mouse serum (100 μL)–H_2O (100 μL), and incubated at 37 °C. At intervals, an aliquot was sampled and examined by analytical HPLC with a linear gradient of CH_3CN (10–40%, 30 min). HPLC peaks of the starting compound and the generated products were identified by IS-MS analysis. The amounts of the starting compound and the generated products were quantitated from the corresponding peak areas.

Behavior of T140 analogs in rat liver homogenate [34]

Rat liver (21.4 g) was suspended in ice-cold PBS (85 mL) and then homogenized, followed by centrifugation at 3000 rpm for 10 min. The obtained supernatant was diluted to 40% (v/v) solution with PBS. Test compounds (100 nmol) were dissolved in PBS (100 μL), which contained 0.1% (v/v) *m*-cresol as an internal standard. After addition of 40% (v/v) rat liver homogenate solution (100 μL), the mixture was incubated at 37 °C. At intervals (0, 1, 2, 4, 6, 10 and 24 h), a 10 μL aliquot was sampled. After quenching enzymatic activities by addition of 0.1 M aq. HCl (190 μL), 6 M guanidine·HCl–1 M Tris buffer (pH 7.5, 300 μL) was added and the mixture was then stirred for 12 h. 100 μL of this solution was analyzed by analytical HPLC with a linear gradient of CH_3CN (10–40%, 30 min). HPLC peaks of the starting compound and the generated compounds were identified by IS-MS analysis. Their amounts were quantitated from the corresponding peak areas, which were corrected by the internal standard *m*-cresol.

Cell culture

Human T-cell lines, MT-4 and MOLT-4 cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, 100 IU mL^{-1} penicillin and 100 μg mL^{-1} streptomycin.

Virus

A strain of X4-HIV-1, HIV-1_{IIIB}, was used for the anti-HIV assay. This virus was obtained from the culture supernatant of HIV-1 persistently infected MOLT-4/HIV-1_{IIIB} cells, and stored at −80 °C until used.

Anti-HIV-1 assay

Anti-HIV-1 activity was determined based on the protection against HIV-1-induced cytopathogenicity in MT-4 cells. Various concentrations of test compounds were added to HIV-1-infected MT-4 cells at a multiplicity of infection (MOI) of 0.01, and placed in wells of a flat-bottomed microtiter tray (1.5×10^4 cells/well). After 5 days' incubation at 37 °C in a CO_2 incubator, the number of viable cells was determined using the MTT method (EC_{50}).[33] Cytotoxicity of compounds was determined based on the viability of mock-infected cells using the MTT method (CC_{50}). 3′-Azido-3′-dideoxythymidine (AZT)[38] and 2′,3′-dideoxycytidine (ddC)[39] were tested as controls.

Calcium fluorimetry[35]

The stable CXCR4-transfected Chinese hamster ovary (CHO) cell lines (3×10^4 cells/100 μL/well) were placed in wells of a flat-bottomed microtiter tray. After 1 day's incubation at 37 °C in a CO_2 incubator, the cells were loaded with 5 μM of Fura2-AM (Dojin, Kumamoto, Japan), 2.5 mM probenecid (Sigma) and 20 mM HEPES (pH 7.4) in Ham's F-12 buffer (80 μL/well) for 1 h at 37 °C, and then twice washed with Hank's balanced salt solution (100 μL × 2), and inserted into a spectrofluorometer (96 well Fluorescence Drug Screening System, Hamamatsu Photonix, Japan). 30 s after start of measurement, the cells were incubated with various concentrations of test compounds in Hank's balanced salt solution (10 μL/well), and after 3 min, recombinant SDF-1α (PreproTech, 30 nM/40 μL/well) was added. Real time recording of $[Ca^{2+}]_i$ changes in the stable CXCR4-transfected CHO cell lines loaded with Fura2-AM was performed by a modified procedure of the Fura-2 method.[35] Inhibitory activity of T140 analogs was determined based on the inhibition of Ca^{2+} mobilization induced by SDF-1α-stimulation through CXCR4 (IC_{50}).

CXCR4 receptor binding (oil cushion method)[36]

The CXCR4-transfected CHO cell lines suspended in Ham's F-12 buffer (0.5% bovine serum albumin, 20 mM HEPES buffer) (5×10^5 cells/120 μL/well) were placed in silicone-coated tubes. Cold SDF-1α (final concentration 0.1 μM, 15 μL/well) and various concentrations of test compounds (15 μL/well) were added to the above tubes followed by addition of ^{125}I-SDF-1α (Perkin-Elmer, final concentration 0.1 nM, 15 μL/well). After 1 h's incubation at 0 °C, oil (dibutyl phthalate : olive oil = 4 : 1 (v/v), 500 μL/well) was added followed by centrifugation at 14,000 rpm for 2 min. After removal of aqueous and organic layers and cutting the bottoms from the tubes, the bottoms were placed in RIA-tubes and the CPM was then counted by γ-counter. Inhibition percentage of T140 analogs against the binding of ^{125}I-SDF-1α was calculated by the following equation. IC_{50} was the concentration of a test compound when it showed 50% inhibition.

Inhibition (%) = (Et − Ea)/(Et − Ec) × 100

Et: the quantity of radioactivity in the absence of a test compound

Ec: the quantity of radioactivity in the presence of cold SDF-1α as a test compound

Ea: the quantity of radioactivity in the presence of a test compound

CD spectroscopy

Peptides were dissolved in H_2O at concentration of 10 μM. CD spectra were recorded on a JASCO J-720 spectropolarimeter (Tokyo, Japan) at 25 °C using 1 cm cells at 1 nm intervals, with five scans averaged for each.

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