Development of a linear type of low molecular weight CXCR4 antagonists based on T140 analogs†

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A linear type of several low molecular weight CXCR4 antagonists were developed based on T140 analogs, which were previously found to be strong CXCR4 antagonists that block X4-HIV-1 entry and have inhibitory activities against cancer metastasis/progression and rheumatoid arthritis.

Introduction

A system of a chemokine receptor, CXCR4, and its endogenous ligand, stromal cell-derived factor-1 (SDF-1/CXCL12), has multiple important functions in normal physiology involving the migration of progenitors during embryologic development of the cardiovascular, hemopoietic and central nervous systems.[1] The CXCL12/CXCR4 system has been also recognized to be involved in several pathologic conditions, such as HIV infection,[2] cancer metastasis/progression[3] and rheumatoid arthritis (RA).[4] First, CXCR4 was identified as a co-receptor that is used in the entry of T cell line-tropic (X4-) HIV-1 into T cells.[2] Second, it is found that the CXCL12/CXCR4 system is involved in the metastasis of several types of cancers, including breast cancer, pancreatic cancer, melanoma, prostate cancer, kidney cancer, neuroblastoma, non-Hodgkin's lymphoma, lung cancer, ovarian cancer, multiple myeloma, chronic lymphocytic leukemia, acute lymphoblastic leukemia and malignant brain tumor,[3] and that this system might determine the metastatic destination of tumor cells. For instance, Müller *et al.* reported that CXCR4 is highly expressed in human breast cancer cells, while CXCL12 is highly expressed in lymph nodes, bone marrow, lung and liver, which represent the primary metastatic destinations of breast cancer, and that breast cancer metastasis can be significantly inhibited by neutralization using anti-CXCR4 antibodies in mice.[3a] Third, Nanki *et al.* reported that the memory T cells highly express CXCR4 and the concentration of CXCL12 is extremely high in the synovium of RA patients, and that CXCL12 stimulates migration of the memory T cells and inhibits T cell apoptosis followed by T cell accumulation in the RA synovium.[4a] Taken together, CXCR4 is thought to represent an important therapeutic target.[5] Thus, several antagonists directed against CXCR4 have been developed. We previously found a 14-mer peptide, T140, which specifically antagonizes CXCR4,[6] and that Arg[2], L-3-(2-naphthyl)alanine (Nal)[3], Tyr[5] and Arg[14] constitute the biologically critical residues of T140 (Fig. 1).[7] Recently, its potent analogs, 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011, possessing increased stability in serum and liver homogenate, were developed by introduction of a *p*-fluorobenzoyl group, which was defined as a new pharmacophore, into the *N*-terminus.[8] 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011 showed strong anti-HIV activity *in vitro*, anti-metastatic activity against breast cancer[3b] and melanoma[3g] and anti-RA activity in experimental model mice.[4b] Furthermore, T140-related analogs exhibited significant inhibition against CXCL12-induced migration/activation/invasion of small-cell lung cancer cells,[3h] acute lymphoblastic leukemia cells[3e] and pancreatic cancer cells[3c,f] *in vitro*. Molecular-size reduction of T140 based on the above four critical residues (Arg × 2, Nal and Tyr) led to discovery of a low molecular weight CXCR4 antagonist with a cyclic pentapeptide template, FC131.[9] In this paper, identification of the enhanced pharmacophore involving an electron-deficient aromatic ring at the *N*-terminus of 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011, such as a *p*-fluorobenzoyl or *p*-trifluoromethylbenzoyl moiety, prompted us to develop novel linear-type low molecular weight CXCR4 antagonists. By combining substructure units of

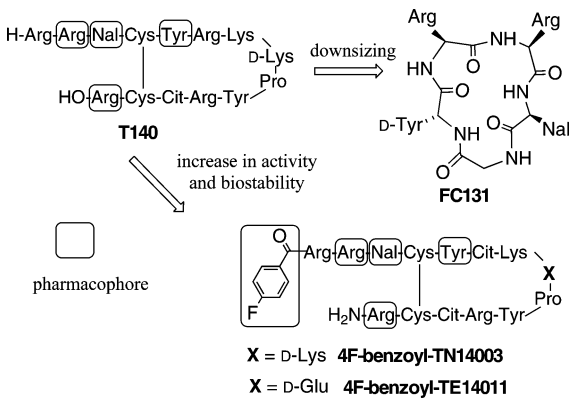

Fig. 1 Development of bio-stable CXCR4 antagonists, 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011, and a downsized antagonist, FC131. Nal = L-3-(2-naphthyl)alanine, Cit = L-citrulline.

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the above four critical residues (Arg × 2, Nal and Tyr) that were used in the development of FC131, in addition to the above electron-deficient aromatic ring, several compounds were designed and synthesized.

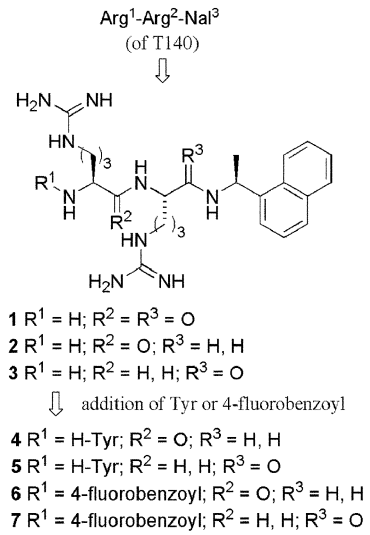


Fig. 2 Development of tri- and tetrapeptide mimetics with CXCR4-antagonistic activity.

Biological results and discussion

Biological activities of the present synthetic compounds were evaluated by two assays: the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay based on the inhibition of X4-HIV-1 (HIV-1_{IIIB})-induced cytopathogenicity in MT-4 cells by test compounds (anti-HIV activity)[10] and a blocking assay based on displacement of CXCL12 binding to CXCR4 by test compounds (binding affinity for CXCR4).[11] Initially, three tripeptide mimetics containing amide bonds and/or reduced amide bonds, **1**–**3** were designed based on the sequence of Arg^1–Arg^2–Nal^3 in the *N*-terminal region of T140 (Fig. 1 and 2) and synthesized using solution-phase techniques involving amide bond-forming condensation and reductive amination reactions. In this study, (*S*)-(−)-1-(1-naphthyl)ethylamide, which was used in another CXCR4 antagonist KRH-1636,[12] was introduced with the view to enhancement of biostability. Compounds **2** and **3** showed significant anti-HIV activity, while compound **1** did not exhibit activity until the 100 μM concentration, suggesting that a reduced amide bond possessing the conformational flexibility might be more suitable for the interaction of CXCR4 (Table 1).

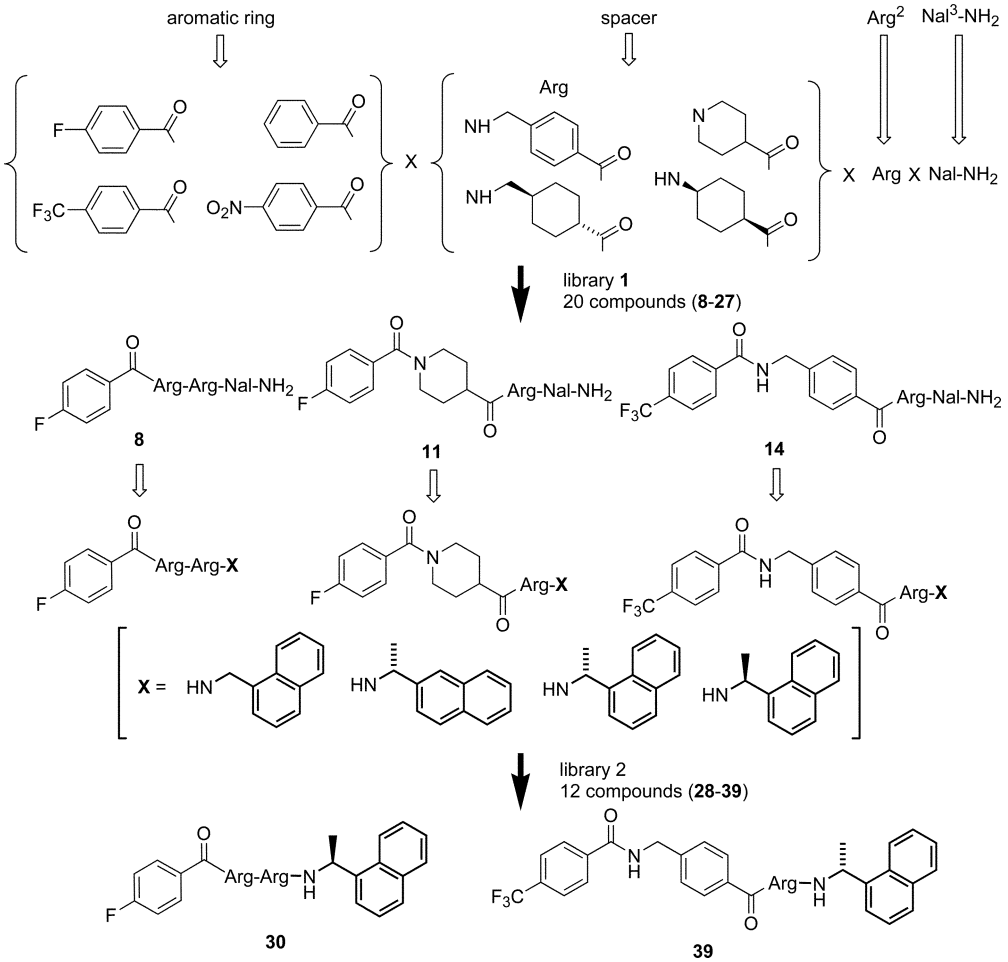


Fig. 3 Design of tripeptide library containing three pharmacophores of the *N*-terminal region of 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011 (aromatic ring, Arg^2 and Nal^3) and the development of new leads.

Table 1 Cytotoxicity, anti-HIV activity and inhibitory activity against CXCL12 binding to CXCR4 of the synthetic compounds

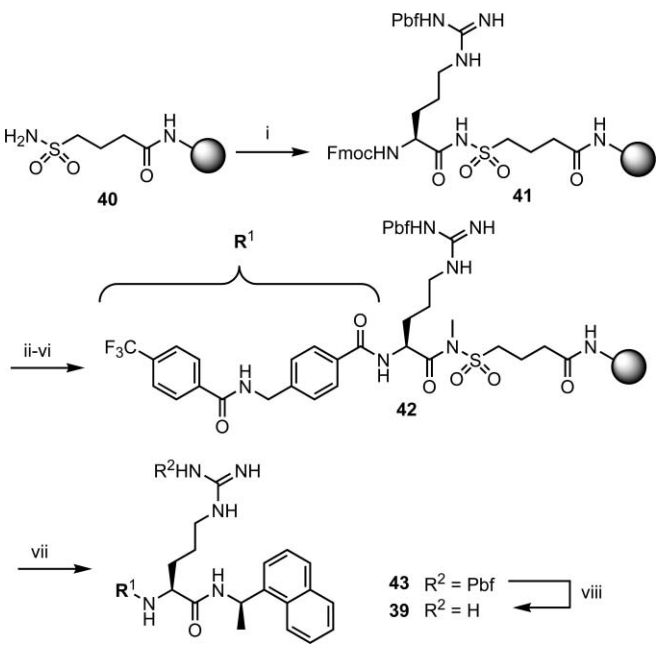
Compound	CC_{50}/μM[a]	EC_{50}/μM[b]	IC_{50}/μM[c]
1	>100	>100	0.32–1
2	>100	52	0.32–1
3	>100	46	0.32–1
4	>100	22	0.090
5	>100	26	0.30
6	>100	11	0.32–1
7	>100	1.7	>1
8	>100	45	0.30
11	>100	7.7	>1
14	>100	6.0	>1
30	>100	61	>1
39	66	7.4	>1
FC131	>100	0.073	0.0032
T140	>10	0.026	0.0045
AZT	>100	0.014	

[a] CC_{50} values are based on the reduction of the viability of mock-infected MT-4 cells. Since the cytotoxicity of T140 was previously evaluated as CC50 > 40 μM, further estimation at high concentrations was omitted in this study. [b] EC_{50} values are based on the inhibition of HIV-induced cytopathogenicity in MT-4 cells. [c] IC_{50} values are based on the inhibition of [^{125}I]-CXCL12 binding to CXCR4 transfectants of CHO cells. All data are the mean values for at least three independent experiments.

Thus, we synthesized two tetrapeptide mimetics, **4** and **5**, where a Tyr residue was added in the *N*-terminus of compounds **2** and **3**, respectively, based on the sequence of the FC131 sequence. Compounds **4** and **5** showed approximately twice stronger anti-HIV activity than compounds **2** and **3**, indicating that an *N*-terminal addition of a Tyr residue is effective for an increase in anti-HIV activity. Furthermore, compounds **4** and **5** exhibited stronger binding affinity for CXCR4, compared to compounds **1**–**3**. Next, we synthesized *p*-fluorobenzoylated tripeptide mimetics, **6** and **7**, based on the *N*-terminal sequence of 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011. As a result, *p*-fluorobenzoylation caused an increase in anti-HIV activity. Compound **7** showed strong anti-HIV activity, suggesting that introduction of a reduced amide bond between two Arg residues is more suitable than that between Arg and naphthalenylethylamine. However, binding affinity of compound **7** for CXCR4 could not be exhibited until the 1 μM concentration, and compound **6** is weaker than compounds **4** and **5** in terms of binding affinity for CXCR4, although anti-HIV activity of compounds **6** and **7** is stronger than that of compounds **4** and **5**. This discrepancy might be caused by the difference between the interactive site of HIV and the binding site of CXCL12 on CXCR4.[13]

Since hit compounds with significant anti-HIV activity were found among several compounds that were synthesized using solution-phase techniques, we attempted to prepare more compounds by solid-phase synthesis: A tripeptide library containing three pharmacophores of the *N*-terminal region of 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011 (aromatic ring, Arg^2 and Nal^3) and the *C*-terminal carboxy amide was designed (Fig. 3). Since Arg^1 is not an indispensable residue for high activity, it was replaced by several spacers involving conformationally constrained units, such as 4-piperidinecarboxylic acid and 4-(aminomethyl)benzoic acid. Use of this library involving 20 synthetic compounds, which was constructed by solid-phase peptide synthesis (Fig. 3, library 1), led to the discovery of lead compounds for anti-HIV agents, **11** and **14**, although these compounds did not show significant binding affinity for CXCR4 until the 1 μM concentration. Compound **8**, which contains Arg^1 based on the original 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011 sequence, also exhibited moderate anti-HIV activity and significant CXCR4-binding affinity. These results suggest that Arg^1 can be replaced by conformationally restricted units in terms of anti-HIV activity. The other compounds that were contained in library 1 did not show significant anti-HIV activity until the 100 μM concentration.

Next, in due consideration of an increase in biostability, focused library of compounds with the *C*-terminal substituted amide was constructed based on the structures of compounds **8**, **11** and **14** by solid-phase techniques using Kenner's sulfonamide safety-catch linker[14] (Fig. 3, library 2): *C*-terminal Nal-amide of compounds **8**, **11** and **14** was replaced by several amides possessing various naphthalene units. The synthetic scheme for compound **39** is shown as a representative in Scheme 1. Compounds **30** and **39** showed moderate and strong anti-HIV activity, respectively, although each compound did not show significant CXCR4-binding affinity until the 1 μM concentration. Anti-HIV potency of compounds is not always in proportion to binding affinity for CXCR4, especially in case of these small compounds, since there is a significant difference between the interactive site of HIV and the binding site of CXCL12 on CXCR4. There is a great interest in this result: compound **39**, possessing (*R*)-(+)-1-(1-naphthyl)ethylamide in the *C*-terminus, is stronger than compound **38**, possessing (*S*)-(−)-1-(1-naphthyl)ethylamide in the



Scheme 1 Reagents: (i) Fmoc-Arg(Pbf)–OH, DIPEA, PyBOP, $CHCl_3$; (ii) 20% (v/v) piperidine–DMF; (iii) Fmoc-(4-aminomethyl)benzoic acid, DIPCDI, HOBt, DMF; (iv) 20% (v/v) piperidine–DMF; (v) 4-trifluoromethylbenzoic acid, DIPCDI, HOBt, DMF; (vi) $TMSCHN_2$, hexane, THF; (vii) (*R*)-(+)-1-(1-naphthyl)ethylamine, DMF, reflux; (viii) thioanisole, TFA; Pbf = 2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl, DIPEA = *N*,*N*-diisopropylethylamine, PyBOP = benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate, DIPCDI = *N*,*N*-diisopropylcarbodiimide, HOBt = *N*-hydroxybenzotriazole.

C-terminus, which is a common structure unit with KRH-1636. Compound **39** is thought to be a useful lead possessing chemically modified *N*- and *C*-terminal ends. The other 10 compounds that were contained in library 2 did not show significant anti-HIV activity until the 100 μM concentration.

In summary, several compounds that were synthesized based on pharmacophores of T140 analogs showed significant anti-HIV activity and binding affinity for CXCR4. According to these results, two types of libraries based on the *N*-terminal region of 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011 were constructed to find effective lead compounds. Linear-type low molecular weight compounds obtained in this study are thought to be useful leads for chemotherapy of AIDS, cancer and RA.

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