Structure-activity relationship study on artificial CXCR4 ligands possessing the cyclic pentapeptide scaffold: the exploration of amino acid residues of pentapeptides by substitutions of several aromatic amino acids†

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Previously, downsizing of a 14-residue peptidic CXCR4 antagonist **1** has led to the development of a highly potent CXCR4 antagonist **2** [*cyclo*(-D-Tyr[1]-Arg[2]-Arg[3]-Nal[4]-Gly[5]-)]. In the present study, cyclic pentapeptide libraries that were designed by substitutions of several amino acids for D-Tyr[1] and Arg[2] in peptide **2** were prepared and screened to evaluate binding activity for CXCR4. The above structure-activity relationship study led to the finding of several potent CXCR4 ligands.

Introduction

The chemokine receptor CXCR4, which has an endogenous ligand, stromal-cell derived factor-1α (SDF-1α)/CXCL12,[1,2] belongs to the G-protein coupled receptor (GPCR) family.[3,4] The CXCR4-CXCL12 axis plays an important role in various physiological functions: chemotaxis,[5] angiogenesis[6,7] and neurogenesis[8,9] in embryonic stage. However, CXCR4 is also relevant to multiple intractable diseases: AIDS,[10,11] cancer metastasis,[12] progress of leukemia,[13] and rheumatoid arthritis[14] in adulthood. Thus, CXCR4 is thought to be an attractive drug target against these diseases, and CXCR4 antagonists would be useful for the development of potent therapeutic agents.[15–17] Various CXCR4 antagonists such as AMD3100[18,19] and KRH-1636[20] have been reported to date. A 14-residue cyclic peptide CXCR4 antagonist **1** was previously found by structure optimization of an 18-residue bicyclic peptide polyphemusin analogue (Fig. 1).[21,22] Furthermore, the downsizing of **1** using its pharmacophore residues [Arg × 2, L-3-(2-naphthyl)alanine (Nal), Tyr] brought the development of cyclic pentapeptide **2** as a CXCR4 antagonist.[23]

In addition, a biologically stable analogue **3** was derived from **1** with the addition of a 4-fluorobenzoyl group as a new pharmacophore moiety at the N-terminus.[24] We have studied structure-activity-relationship (SAR) of **2** through various modifications such as changes of the ring size and amino acid substitutions.[25–27]

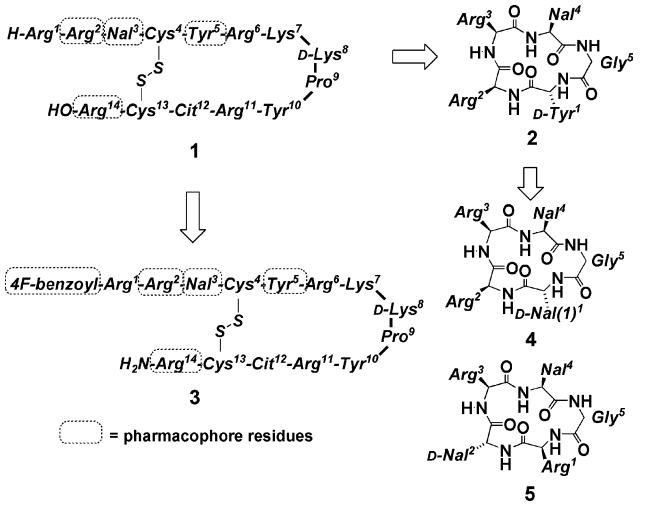

Fig. 1 Development of a cyclic pentapeptide **2** based on the pharmacophore of a CXCR4 antagonistic peptide **1**. Further conversion from **1** into a biostable derivative **3** and from **2** into new cyclic pentapeptide leads **4** and **5**. Cit = L-citrulline, Nal = L-3-(2-naphthyl)alanine, Nal(1) = L-3-(1-naphthyl)alanine.

Potent CXCR4 ligands contain aromatic and cationic groups,[28] suggesting that these groups are involved in binding to CXCR4 mediated by hydrophobic and electrostatic interactions. In a previous study, D-Tyr[1] and Arg[2] in peptide **2** were replaced by a bicyclic aromatic amino acid and a cationic amino acid to identify novel pharmacophores and to find new lead compounds. Compounds **4**, with replacement of D-Tyr[1] by D-3-(1-naphthyl)alanine (D-Nal(1)), and **5** with the sequence of Arg[1]-D-Nal[2] based on shuffling cationic and aromatic amino acids at positions 1 and 2 of compound **2** showed high CXCR4 binding activity.[29] Thus, in this study, the design of a cyclic pentapeptide library based on substitutions of several aromatic amino acids at positions 1 and 2 led to the development of novel analogues of **2** to explore new pharmacophore moieties.

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Table 1 Inhibitory activity of the synthetic compounds **6–10** against binding of [^{125}I]-SDF-1α to CXCR4

Compd	*cyclo*(-**Xaa^1**-**Xaa^2**-Arg^3-Nal^4-Gly^5-). **Xaa^1-Xaa^2**	IC_{50}/μM[a]
2	D-Tyr^1-Arg^2	0.0079
6	D-Phe(4-F)^1-Arg^2	0.22
7	D-Phe(4-F)^1-D-Arg^2	0.31
8	Phe(4-F)^1-Arg^2	0.22
9	Phe(4-F)^1-D-Arg^2	2.2
10	*cyclo*(-D-Tyr^1-Arg^2-Arg^3-Nal^4- Phe(4-F)^5-)	4.4

[a] IC_{50} values are the concentrations for 50% inhibition of the [^{125}I]-SDF-1α binding to CXCR4 transfectants of CHO cells. All data are the mean values for at least three experiments.

Biological results and discussion

SAR of analogues with L/D-Phe(4-F)^1-L/D-Arg^2

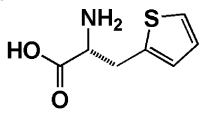
Since compound **6**, where D-Tyr^1 of **2** was replaced by 4-fluoro-D-phenylalanine [D-Phe(4-F)] relevant to the new pharmacophore 4-fluorobenzoyl group, showed relatively potent CXCR4-binding activity as reported previously,[26] initially, **6** and its three analogues **7–9**, [L/D-Phe(4-F)^1, L/D-Arg^2]-**2**, were synthesized to evaluate the configuration effects of amino acids at positions 1 and 2 (Table 1). These analogues except for **9** showed high CXCR4 binding activity (IC_{50} = 0.2–0.4 μM, Table 1), although the potencies were much lower than that of **2**. Compound **9** showed moderate potency (IC_{50} = 2.2 μM), suggesting that the combination of Phe(4-F)^1 and D-Arg^2 is not suitable. In addition, compound **10** was synthesized, where Gly^5 of **2** was replaced by Phe(4-F) with maintenance of D-Tyr^1, since both Phe(4-F) and D-Tyr are thought to be important pharmacophore residues. However, compound **10** did not show high potency (IC_{50} = 4.4 μM), probably due to a conformational change.

SAR of analogues with replacement of D-Phe(4-F)^1 of 6 by an aromatic D-amino acid

Based on the configuration of [D-Phe(4-F)^1, L-Arg^2] of **6**, a series of analogues with replacement of D-Phe(4-F)^1 by several aromatic amino acids were synthesized. The order of preference of halogen atoms as a substituent of position 4 on D-Phe^1 is fluorine, chlorine and bromine as shown in activity of **6**, **11** and **12** (IC_{50} = 0.22, 1.2 and 2.3 μM, respectively, Tables 1 & 2). It suggests that a small or electron-withdrawing group is favorable for a substituent of position 4 on D-Phe^1. Next, preference of positions of fluorine on the phenyl ring of D-Phe^1 was investigated. As a result, the order of preference is *ortho*, *meta* and *para*-positions, as shown in activity of **13**, **14** and **6** (IC_{50} = 0.059, 0.088 and 0.22 μM, respectively). In the previous paper, a D-Nal(1)^1-substituted analogue **4** (IC_{50} = 0.043 μM) showed much higher CXCR4 binding activity than a D-Nal^1-substituted analogue, [D-Nal^1]-**2** (IC_{50} > 2.0 μM).[29] Taken together, a *para*-substituent on the phenyl ring of the D-amino acid residue at position 1 is not appropriate for high potency, possibly due to the steric hindrance between the para-substituent on the phenyl ring and CXCR4. In addition, two other analogues were prepared. Compound **15**, [L/D-Phg^1]-**2** (racemic), did not show high CXCR4 binding activity. Compound **16**, [β-(2-thienyl)-D-alanine (D-Thi)^1]-**2**, showed very potent CXCR4 binding activity, suggesting a thienyl group is relatively suitable for the side-chain of the amino acid at position 1. New leads, **13** and **16**, having D-Phe(2-F)^1 and D-Thi^1, respectively, were found although the potencies were approximately one-eighth of that of **2**.

Table 2 Inhibitory activity of the synthetic compounds **11–16** against binding of [^{125}I]-SDF-1α to CXCR4

Compd	*cyclo*(-**Xaa^1**-Arg^2-Arg^3-Nal^4-Gly^5-). **Xaa^1**	IC_{50}/μM[a]
2	D-Tyr^1	0.0079
11	D-Phe(4-Cl)^1	1.2
12	D-Phe(4-Br)^1	2.3
13	D-Phe(2-F)^1	0.059
14	D-Phe(3-F)^1	0.088
15	L/D-Phg^1	1.1
16	D-Thi^1	0.056



β-(2-thienyl)-D-alanine (D-Thi)

[a] IC_{50} values are the concentrations for 50% inhibition of the [^{125}I]-SDF-1α binding to CXCR4 transfectants of CHO cells. All data are the mean values for at least three experiments.

SAR of analogues with Arg^1-aromatic D-amino acid^2

Since **5**, an analogue with the sequence of Arg^1-D-Nal^2 based on shuffling cationic and aromatic amino acids at positions 1 and 2 of compound **2**, showed high CXCR4 binding activity,[29] a series of analogues with the sequence of Arg^1-aromatic D-amino acid^2 (substitution for D-Nal^2) were synthesized. Among halogen substituents at position 4 on the phenyl ring of D-Phe^2, fluorine is the most suitable, whereas chlorine or bromine is not preferable as shown in activity of **17**,[26] **18** and **19** (IC_{50} = 0.035, 0.79 and 0.57 μM, respectively, Table 3). In addition, a 4-nitro group is not suitable although this group is an electron-withdrawing group, possibly due to steric hindrance (**20**, IC_{50} = 0.94 μM). A 4-hydroxy group with electron-donating action and a 4-amino group with strong electron-donating action is not favorable (**21**, IC_{50} = 0.97 μM, **22**, IC_{50} = 15 μM). As a result, fluorine is the most suitable substituent at position 4 on D-Phe^2 among these atoms and groups. In the investigation of the preference of positions of fluorine on the phenyl ring of D-Phe^2, *para*-position is superior to

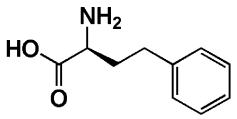
Table 3 Inhibitory activity of the synthetic compounds **17–24** against binding of [^{125}I]-SDF-1α to CXCR4

Compd	*cyclo*(-**Xaa^1**- **Xaa^2**-Arg^3-Nal^4-Gly^5-). **Xaa^1-Xaa^2**	IC_{50}/μM[a]
2	D-Tyr^1-Arg^2	0.0079
17	Arg^1-D-Phe(4-F)^2	0.035
18	Arg^1-D-Phe(4-Cl)^2	0.79
19	Arg^1-D-Phe(4-Br)^2	0.57
20	Arg^1-D-Phe(4-NO_2)^2	0.94
21	Arg^1-D-Tyr^2	0.97
22	Arg^1-D-Phe(4-NH_2)^2	15
23	Arg^1-D-Phe(2-F)^2	7.1
24	Arg^1-D-Phe(3-F)^2	6.1

[a] IC_{50} values are the concentrations for 50% inhibition of the [^{125}I]-SDF-1α binding to CXCR4 transfectants of CHO cells. All data are the mean values for at least three experiments.

Table 4 Inhibitory activity of the synthetic compounds **25–31** against binding of [^{125}I]-SDF-1α to CXCR4

Compd	*cyclo*(-D-Tyr ^{1}-**Xaa^{2}-Xaa^{3}**-Nal^{4}-Gly^{5}-). **Xaa^{2}-Xaa^{3}**	IC_{50}/μM[a]
2	Arg^{2}-Arg^{3}	0.0079
25	Hph^{2}-Arg^{3}	0.075
26	D-Phg^{2}-Arg^{3}	6.0
27	Phg^{2}-Arg^{3}	0.17
28	His^{2}-Arg^{3}	0.037
29	D-His^{2}-Arg^{3}	0.035
30	Arg^{2}-His^{3}	5.0
31	His^{2}-His^{2}	12

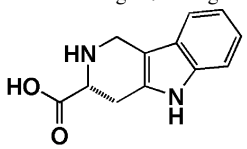


L-homophenylalanine (Hph)

[a] IC_{50} values are the concentrations for 50% inhibition of the [^{125}I]-SDF-1α binding to CXCR4 transfectants of CHO cells. All data are the mean values for at least three experiments.

Table 5 Inhibitory activity of the synthetic compounds **32–37** against binding of [^{125}I]-SDF-1α to CXCR4

Compd	*cyclo*(-**Xaa^{1}**- **Xaa^{2}**-Arg^{3}-Nal^{4}-Gly^{5}-). **Xaa^{1}-Xaa^{2}**	IC_{50}/μM[a]
2	D-Tyr^{1}-Arg^{2}	0.0079
32	Arg^{1}-His^{2}	0.40
33	Arg^{1}-D-His^{2}	0.96
34	Arg^{1}-D-Thi^{2}	1.7
35	Arg^{1}-D-Tpi^{2}	8.1
36	Arg^{1}-D-Hph^{2}	5.0
37	Arg^{1}-L/D-Phg^{2}	5.9



(3*R*)-2,3,4,9-tetrahydro-1*H*-β-carboline -3-carboxylic acid (D-Tpi)

[a] IC_{50} values are the concentrations for 50% inhibition of the [^{125}I]-SDF-1α binding to CXCR4 transfectants of CHO cells. All data are the mean values for at least three experiments.

ortho or *meta* as shown in the activity of **17**, **23** and **24** (IC_{50} = 0.035, 7.1 and 6.1 μM, respectively). In the previous paper, a D-Nal^{2}-substituted analogue **5** (IC_{50} = 0.045 μM) showed much higher CXCR4 binding activity than a D-Nal(1)^{2}-substituted analogue, [Arg^{1}-D-Nal(1)^{2}]-**2** (IC_{50} > 2.0 μM).[29] Taken together, a *para*-substituent on the phenyl ring of the D-amino acid residue at position 2 is suitable for high potency, possibly due to hydrophobic or π interaction between the *para*-substituent on the phenyl ring and the receptor CXCR4.

SAR of analogues with replacement of Arg^{2} of 2 by an aromatic amino acid

Since [D-Tyr^{1}-Phe(4-F)^{2}]-**2** showed high CXCR4 binding activity in the previous paper,[26] analogues having incorporation of an aromatic amino acid into position 2 were synthesized. An L-homophenylalanine (Hph)-substituted analogue **25** showed potent CXCR4 binding activity (IC_{50} = 0.075 μM, Table 4), whereas an L-phenylglycine (Phg)-substituted analogue **27** showed lower CXCR4 binding activity (IC_{50} = 0.17 μM), although a D-phenylglycine (D-Phg)-substituted analogue **26** showed even lower CXCR4 binding activity (IC_{50} = 6.0 μM). Since His has both basic and aromatic character, it would be a useful amino acid substitution at position 2. Practically, L- and D-His-substituted analogues **28** and **29** showed high potency (IC_{50} = 0.037 and 0.035 μM, respectively), indicating that the chirality of L/D-His at position 2 does not affect CXCR4 binding. The potencies are approximately one-fourth of that of **2**. Next, we extended the His-substitution to position 3 (Arg^{3}). Analogues with the sequences of Arg^{2}-His^{3} and His^{2}-His^{3} were synthesized (**30** and **31**, respectively). However, these analogues did not show potent CXCR4 binding activity (IC_{50} = 5.2 and 12 μM, respectively), suggesting that His-substitution for Arg^{3} is not appropriate.

SAR of analogues with Arg^{1}-aromatic-amino acid^{2}

Among analogues with a combination of the sequences of Arg/His^{2}-Arg/His^{3}, **28** having the sequence of His^{2}-Arg^{3} is the most potent compound, with almost the same CXCR4 binding activity as **29** (D-His^{2}-Arg^{3}). Thus, **32** and **33**, where D-Tyr^{1} of **28** and **29** was replaced by Arg, respectively, were synthesized. However, **32** and **33** are more than 10 fold weaker than **28** and **29** (Table 5), indicating that Arg^{1} is not suitable in these analogues. D-Thi, (3*R*)-2,3,4,9-tetrahydro-1*H*-β-carboline-3-carboxylic acid (D-Tpi), D-Hph and L/D-Phg (racemic)-substituted analogues did not show high potency as shown in **34**, **35**, **36** and **37**, respectively. It suggests that these series of analogues with the sequence of Arg^{1}-aromatic-amino acid^{2} are not potent compounds, although **5** and **17** showed high potency.

SAR of analogues with D-Phe(4-F)^{1}-Arg/His^{2}-Arg/His^{3} or Arg/His^{1}-D-Phe(4-F)^{2}-Arg/His^{3}

Since **6**, [D-Phe(4-F)^{1}]-**2**, showed moderate CXCR4 binding activity,[26] a series of analogues with the sequence of D-Phe(4-F)^{1}-Arg/His^{2}-Arg/His^{3} were synthesized (**38**, **39** and **40**). However, significantly potent analogues could not be found (IC_{50} > 10 μM, Table 6). Thus, to interchange the order of positions 1 and 2, a series of analogues with the sequence of Arg/His^{1}-D-Phe(4-F)^{2}-Arg/His^{3} were synthesized (**41**, **42** and **43**). CXCR4 binding

Table 6 Inhibitory activity of the synthetic compounds **38–43** against binding of [^{125}I]-SDF-1α to CXCR4

Compd	*cyclo*(-**Xaa^{1}**- **Xaa^{2}-Xaa^{3}**-Nal^{4}-Gly^{5}-). **Xaa^{1}-Xaa^{2}-Xaa^{3}**	IC_{50}/μM[a]
2	D-Tyr^{1}-Arg^{2}-Arg^{3}	0.0079
38	D-Phe(4-F)^{1}-Arg^{2}-His^{3}	22
39	D-Phe(4-F)^{1}-His^{2}-Arg^{3}	10
40	D-Phe(4-F)^{1}-His^{2}-His^{3}	>100
41	Arg^{1}-D-Phe(4-F)^{2}-His^{3}	>100
42	His^{1}-D-Phe(4-F)^{2}-Arg^{3}	5.7
43	His^{1}-D-Phe(4-F)^{2}-His^{3}	>100

[a] IC_{50} values are the concentrations for 50% inhibition of the [^{125}I]-SDF-1 binding to CXCR4 transfectants of CHO cells. All data are the mean values for at least three experiments.

activities of these analogues are relatively weak (IC_{50} > 5 μM), indicating that incorporation of D-Phe(4-F) at position 1 and His-substitution for Arg^3 are not suitable.

Conclusion

In this paper, SAR of several cyclic pentapeptides having CXCR4 binding activity was studied to discover useful lead compounds. (1) Of the analogues with replacement of D-Tyr^1 of **2** by an aromatic D-amino acid, a D-$Phe(2\text{-}F)^1$-substituted analogue, **13**, and a D-Thi^1-substituted analogue, **16**, have potent CXCR4 binding activity. A para-substituent on the phenyl ring of the D-amino acid residue at position 1 is not favorable for high potency. (2) Among a series of analogues based on shuffling cationic and aromatic amino acids at positions 1 and 2, an [Arg^1-D-$Phe(4\text{-}F)^2$]-containing analogue, **17**, showed the most potent CXCR4 binding activity. A *para*-substituent on the phenyl ring of the D-amino acid residue at position 2 is suitable for high potency. (3) Analogues, where Arg^2 of **2** was replaced by L/D-His which have both basic and aromatic characters, have high CXCR4 binding activity. (4) Arg^1-substituted analogues or His^3-substituted analogues are not potent leads. Taken together, in the present study several new leads were found, and aromatic amino acid residues, Phe(2-F), Phe(4-F), Thi and His, were identified to be new pharmacophore residues in addition to Arg, Nal, Nal(1) and Tyr. The present data will be important for the development of CXCR4 antagonists. In future, the introduction of fluorophenyl, thienyl, imidazoyl groups, *etc.* involving the combinational use of the above groups into the cyclic pentapeptide templates and into low molecular weight linear type scaffolds will bring us the development of new-type leads of CXCR4 antagonists. The present data of preferences of the target CXCR4 such as inclination of aromatic and basic groups will be useful to disclose an unknown detail binding mode of CXCR4 and cyclic pentapeptide-type ligands on the cell membrane.

Experimental

Chemistry

Cyclic peptides were synthesized by Fmoc-based solid-phase synthesis followed by cleavage from the resin, cyclization with the diphenylphosphoryl azide and deprotection, as reported previously.[23]

General. The protected peptide resin (0.100 mmol), which was constructed on H-Gly-(2-chloro)trityl resin manually by Fmoc-based solid phase peptide synthesis (SPPS). *t*-Bu for L/D-Tyr and Pbf for L/D-Arg were used for side-chain protection. Fmoc deprotection was achieved by 20% (v/v) piperidine in DMF (10 mL, 2 × 1 min, 1 × 20 min). Fmoc amino acids were coupled by treatment with five equivalents of reagents [Fmoc-amino acid, *N*,*N*′-diisopropylcarbodiimide (DIPCDI) and $HOBt{\cdot}H_2O$] to free amino group in DMF (5 mL) for 1.5 h. The constructed protected peptide resin was subjected to AcOH/TFE/CH_2Cl_2 (1 : 1 : 3 (v/v/v), 10 mL) treatment at room temperature for 2 h. After filtration of the residual resin, the filtrate was concentrated under reduced pressure to give a crude protected peptide. To a stirred mixture of the protected peptide and $NaHCO_3$ (57.1 mg, 0.680 mmol) in DMF (41 mL) was added diphenylphosphoryl azide (DPPA, 0.0879 mL, 0.408 mmol) at –40 °C. The mixture was stirred for 36 h with warming to room temperature and filtered. The filtrate was concentrated under reduced pressure to give an oily residue, which was subjected to solid phase extraction over basic alumina in $CHCl_3$-MeOH (9 : 1 (v/v)) to remove inorganic salts derived from DPPA. The resulting cyclic protected peptide was treated with 95% TFA solution for 1.5 h at room temperature. Concentration under reduced pressure and purification by preparative HPLC gave a cyclic peptide.

CXCR4 receptor binding assay[30]

Stable CHO cell transfectants expressing CXCR4 were prepared as described previously.[31] CHO transfectants were detached by treatment with trypsin-EDTA, allowed to recover in complete growth medium (MEM-R, 100 μg/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B, 10% FBS (v/v)), and then washed in cold binding buffer (PBS containing 2 mg/mL BSA). For ligand binding, the cells were resuspended in binding buffer at 1×10^7 cell/mL, and 100 μL aliquots were incubated with 0.1 nM of [^{125}I]-SDF-1 (Perkin-Elmer Life Sciences) for 1 h on ice under constant agitation. Free and bound radioligands were separated by centrifugation of the cells through an oil cushion, and bound radioactivity was measured with a gamma-counter (Cobra, Packard, Downers Grove, IL, USA). Inhibitory activity of test compounds was determined based on the inhibition of [^{125}I]-SDF-1 binding to CXCR4 transfectants (IC_{50}).

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