Structure-activity relationship study of CXCR4 antagonists bearing the cyclic pentapeptide scaffold: identification of the new pharmacophore†

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A highly potent CXCR4 antagonist **2** [cyclo (-D-Tyr[1]-Arg[2]-Arg[3]-Nal[4]-Gly[5]-)] has previously been identified by screening cyclic pentapeptide libraries that were designed based on pharmacophore residues of a 14-residue peptidic CXCR4 antagonist **1**. In the present study, D-Tyr and Arg in peptide **2** were replaced by a bicyclic aromatic amino acid and a cationic amino acid, respectively, and their binding activity for CXCR4 was evaluated for identification of the novel pharmacophore.

Introduction

The chemokine receptor CXCR4 is a membrane protein, which belongs to the G-protein coupled receptor family.[1,2] Interaction of CXCR4 with its endogenous ligand stromal-cell derived factor-1α (SDF-1α)/CXCL12 induces various physiological functions: chemotaxis,[3] angiogenesis,[4,5] neurogenesis,[6,7] *etc.* in embryonic stage. On the other hand, CXCR4 is also relevant to multiple diseases: AIDS,[8,9] cancer metastasis,[10] progress of leukemia,[11] rheumatoid arthritis,[12] *etc.* in adulthood. Actually, CXCR4 has been reported to be a potential drug target against these diseases. Thus, CXCR4 antagonists are useful for development of potent therapeutic agents against these diseases.[13–15] To date, various CXCR4 antagonists such as AMD3100[16,17] and KRH-1636[18] have been reported.

A β-sheet-like 14-residue peptide **1** was previously identified by structure optimization of an 18-residue cyclic peptide polyphemusin isolated from horseshoe crabs (Fig. 1).[19,20] In the downsizing of **1**, a cyclic pentapeptide **2** was developed by screening libraries based on four pharmacophore residues [Arg, Arg, 3-(2-naphthyl)alanine (Nal), D-Tyr] found by alanine scanning of **1**.[21]

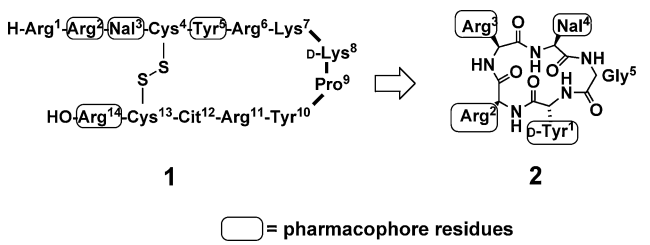

Fig. 1 Development of a cyclic pentapeptide **2** based the pharmacophore of a CXCR4 antagonistic peptide **1**. Cit = L-citrulline, Nal = L-3-(2-naphthyl)alanine.

We have studied structure-activity-relationships of **2** by various modifications.[22,23] In this paper, design of cyclic pentapeptide library based on the previous structure-activity relationship data led to development of novel analogues of **2** to explore new pharmacophore moieties.

Biological results and discussion

Substitution of a large aromatic amino acid for D-Tyr[1] of 2

Our previous data of alanine-scanning of **2** suggested that D-Tyr[1] or Arg[2] was not optimalized.[24] Thus, we attempted to replace these functional groups. According to other previous reports, potent CXCR4 antagonists absolutely contain aromatic and cationic groups.[25] It suggests that these functional groups are involved in binding to CXCR4 mediated by hydrophobic and electrostatic interaction. To evaluate significance of the hydrophobic interaction by aromatic rings, D-Tyr[1] of **2** was replaced by an L/D-bicyclic aromatic amino acid. In addition, four epimers were synthesized to evaluate effects of configuration of amino acids of the 1- and 2- positions (Fig. 2). Compounds **3c** and **3d** with replacement of D-Tyr[1] by D-3-(1-naphthyl)alanine (D-Nal(1)) showed high CXCR4 binding activity (IC_{50} = 0.043 and 0.078 μM, respectively, Table 1), although the potencies were approximately one-third or fifth of that of the parent compound **2** (IC_{50} = 0.015 μM, Table 1). Similarly, compounds **5c** and **5d**, replaced by D-Trp at the 1-position, showed 5–10 fold lower CXCR4 binding activity (IC_{50} = 0.15 and 0.070 μM, respectively, Table 1) than the parent compound **2**. On the other hand, compounds **4c** and **4d** did not show strong CXCR4 binding activity. These data indicates that the spatial position of aromatic ring is essential for the expression of CXCR4 binding activity. In addition, a series of **a** or **b** except for **5a** did not show strong CXCR4 binding activity (all IC_{50} values > 0.3 μM, Table 1). These data indicate that the chirality of L/D-Arg[2] was not important for the expression of CXCR4 binding activity, whereas the chirality of Nal(1)[1] and Trp[1] is influential. The

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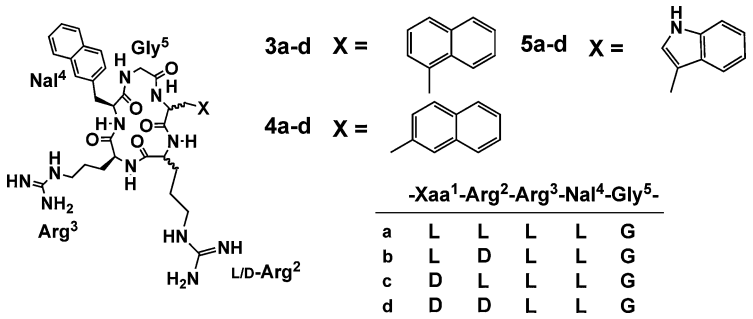

Fig. 2 Structures of compounds having substitution of an L/D- bicyclic aromatic amino acid for Tyr[1].

Table 1 Inhibitory activities of the synthetic compounds against binding of [^{125}I]-SDF-1α to CXCR4

Compound no.	IC_{50}/μM[a]	Compound no.	IC_{50}/μM[a]
2	0.015	**3c**	0.043
3a	0.3–2.0	**4c**	> 2.0
4a	0.3–2.0	**5c**	0.15
5a	0.22	**3d**	0.078
3b	0.3–2.0	**4d**	0.3–2.0
4b	0.3–2.0	**5d**	0.070
5b	> 2.0		

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

Table 2 Inhibitory activities of the synthetic compounds against binding of [^{125}I]-SDF-1α to CXCR4

Compound no.	IC_{50}/μM[a]	Compound no.	IC_{50}/μM[a]
2	0.015	**6c**	0.3–2.0
6a	> 2.0	**7c**	0.3–2.0
7a	0.3–2.0	**8c**	> 2.0
8a	> 2.0	**6d**	> 2.0
6b	> 2.0	**7d**	0.3–2.0
7b	0.045	**8d**	0.3–2.0
8b	> 2.0		

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

dependence of CXCR4 binding activity on the chirality at the 1-position might be caused by a conformational change of the peptide backbone.

Shuffling cationic and aromatic amino acids at the 1- and 2-positions of cyclic pentapeptides

An analogue of **2**, having substitution of Arg[1] and D-4F-phenylalanine[2] for D-Tyr[1] and Arg[2], respectively, was recently found as a strong CXCR4 antagonist.[22] To evaluate effects of the sequential difference of cationic and aromatic groups at the 1- and 2-positions on CXCR4 binding activity, Arg and a large aromatic amino acid (Nal(1), Nal or Trp) were shuffled in the pentapeptide, and four epimers were synthesized in a similar manner (Fig. 3). Synthetic compounds except for **7b** did not show CXCR4 binding activity up to 0.3 μM (Table 2). In particular, a series of **6** and **8** did not show CXCR4 binding activity despite of difference of the chilality of amino acids at the 1- and 2-positions (**6c**, **8d** > 0.3 μM, **6a**, **6b**, **6d**, **8a**, **8b**, **8c** > 2.0 μM). On the other hand, a series of **7**, which introduced L/D-Nal at the 2-position, did not show a serious reduction of CXCR4 binding activity. These data indicated that Nal(1) or Trp might not be appropriate as the amino acid introduced at the 2-position, possibly due to spatial configuration of aromatic rings. **7b** showed the highest CXCR4 binding activity among compounds in this library. Interestingly, **7b** has the opposite chirality and order of the aromatic residue at the 1- and 2-positions compared to the parent compound **2**.

Evaluation of anti-HIV activity and cytotoxicity

Anti-HIV activity and cytotoxicity of compounds **5c**, **5d** and **7b** that showed moderate CXCR4 binding activity and have a characteristic sequence and conformation were evaluated. Since CXCR4 is a coreceptor for an X4-HIV-1 entry, CXCR4 antagonists have anti-HIV activity.[8,9] Anti-HIV activities of compounds **5d** and **7b** (EC_{50} = 0.19 and 0.26 μM, respectively, Table 3) were nearly equal

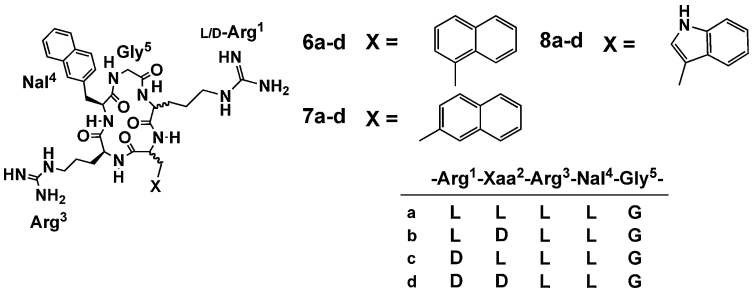

Fig. 3 Structures of compounds having L/D-Arg[1] and an L/D-bicyclic aromatic amino acid.[2]

Table 3 Anti-HIV activity and cytotoxicity of the synthetic compounds

Compound no.	EC_{50}/μM[a]	CC_{50}/μM[b]
AZT	0.077	> 10
1	0.044	> 10
2	0.15	> 10
5c	0.70	> 10
5d	0.19	> 10
7b	0.26	> 10

[a] EC_{50} values are based on the inhibition of HIV-induced cytopathogenicity in MT-4 cells. [b] CC_{50} values are based on the reduction of the viability of MT-4 cells. All data are the mean values for at least three experiments.

to that of **2** (EC_{50} = 0.15 μM, Table 3). Interestingly, CXCR4 binding activity of **5d** (IC_{50} = 0.070 μM, Table 1) was lower than that of **7b** (IC_{50} = 0.045 μM), whereas anti-HIV activity of **5d** (EC_{50} = 0.19 μM, Table 3) was slightly higher than that of **7b** (EC_{50} = 0.26 μM). In addition, all tested compounds did not show significant cytotoxicity (CC_{50} > 10 μM, Table 3).

Conclusion

Our first approach screening cyclic pentapeptides, which have substitution of a bicyclic aromatic amino acid at the 1-position, disclosed that D-3-(1-naphthyl)alanine and D-Trp at the 1-position might be alternative pharmacophore moieties, and that introduction of D-amino acid at the 1-position was required to form an optimal cyclic pentapeptide backbone. In addition, compound **5d** showed high anti-HIV activity, comparable to that of compound **2**.

A cyclic pentapeptide library based on shuffling cationic and aromatic amino acids at the 1- and 2-positions of compound **2** was designed. As a result, the order of a cationic amino acid and an aromatic amino acid is significant to maintain strong CXCR4 binding activity of analogues of **2**. Compound **7b**, however, showed the highest CXCR4 binding activity among the present synthetic cyclic pentapeptides. **7b** was proven to be a new type lead, because of the difference of the order of cationic and aromatic residues, and also showed high anti-HIV activity. Finding of compound **7b** indicated that Arg^1 and D-Nal^2 may be novel pharmacophore moieties in the combination with Nal^4 and Arg^3. To date, pharmacophore functional groups have been identified to be two guanidino, naphthyl and phenol groups derived from two Arg, Nal and D-Tyr in the cyclic pentapeptide scaffolds. In this study, only guanidino and naphthyl groups have been proven to be indispensable for CXCR4 binding activity. The present data will provide useful approaches for simple designs of new low molecular weight CXCR4 antagonists. These results might also give valuable insights for understanding the ligand-receptor interactions.

Experimental

Chemistry

Cyclic peptides were synthesized by Fmoc-based solid-phase synthesis on 2-Chlorotrityl resin followed by cleavage from the resin, cyclization with the diphenylphosphoryl azide and deprotection, as reported previously.[21]

Cell culture

Human T-cell lines, Jurkat cells and MT-4 cells were grown in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum.

Virus

An X4 HIV-1 infectious molecular clone pNL4-3 was obtained from the AIDS Research and Reference Reagent Program.[26] The virus NL4-3 was obtained from the culture supernatant of 293T cells transfected with the pNL4-3. Aliquots of the viral stocks were stored at −80 °C until use. The titer of virus stocks was determined by endpoint titration of 5-fold limiting dilutions in MT-4 cells.

CXCR4 receptor binding assay

Jurkat cells were harvested and centrifugated at 1000 rpm for 5 min. Cells were then resuspended in RPMI buffer (20 mM HEPES, 0.5% bovine serum albumin) and placed in silicone-coated tubes (5.0×10^5 cells/120 μL). Cold SDF-1 (final concentration 1 μM, 15 μL/well) and various concentrations of test compounds (10% DMSO, 15 μL/well) were added to the above tubes followed by addition of [^{125}I]-SDF-1 (Perkin-Elmer Life Sciences, 0.05 nM, 15 μL/well). After 1 h's incubation on ice, 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 counted by γ-counter. Inhibition percentage of FC131 analogs against the binding of [^{125}I]-SDF-1 was calculated by the following equation.[27]

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

Anti-HIV 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 multiplicity of infection (MOI) of 0.001 and placed in wells of a flat-bottomed microtiter tray (2.0×10^4 cells/well). After 5 days' incubation at 37 °C in a CO_2 incubator, the number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method.

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