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Fluorophore Labeling Enables Imaging and Evaluation of Specific CXCR4−Ligand Interaction at the Cell Membrane for Fluorescence-Based Screening

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Development of CXCR4-specific ligands is an important issue in chemotherapy of HIV infection, cancer metastasis, and rheumatoid arthritis, and numerous potential ligands have been developed to date. However, it is difficult to assess their binding mode and specificity because of uncertainties in the structure of the CXCR4−ligand complexes. To address this problem, we have synthesized fluorophore labeled Ac-TZ14011, which is derived from T140, a powerful CXCR4 antagonist. Binding of Ac-TZ14011 to CXCR4 on the cell membrane was observed by fluorescence microscope, and analysis of the binding data produced IC_{50} values of several ligands comparable to those obtained in RI-based assays. This fluorescence-based assay is applicable to explore new pharmacophores of CXCR4-specific ligands with high-throughput screening and also to screening of the other GPCR binding ligands.

The interaction of CXCR4 with ligands causes diverse effects on cellular functions such as metastasis of progenitor cells (*1*−*3*), and a major role of CXCR4 is as the receptor of the chemokine, CXCL12. The interaction of CXCL12 with CXCR4 has been shown to be correlated with cancer progression (*4*) and $CD4^+$ T cell accumulation in the rheumatoid arthritis synovium (*5*). CXCR4 is also known as the second receptor of X4-type HIV-1 (*6*), and numerous ligands for CXCR4 derived from natural and synthetic compounds have been identified as inhibitors of HIV infection and cancer metastasis. [^{125}I]-CXCL12 has been utilized as a competitor in the assays to evaluate the CXCR4-binding activity of synthetic compounds such as T140 (*7*), its derivatives (*8*), KRH-1636 (*9*), and AMD3100 (*10*). Experimental methods utilizing radioisotopes (RI[1]) have advantages in the high resolution of the assays. Recently, molecular probes and fluorescent labeling, an emergent technology in chemical biology, have proved to be very useful for the evaluation in vivo of the functions of proteins and of the biological effect of changing concentrations of Ca^{2+} (*11*), Zn^{2+} (*12*), and NO (*13*), enzyme activity (*14*), and protein phosphorylation (*15*) in cells. Moreover, imaging of living cells by fluorescent probes can be utilized to estimate the accuracy of binding assays under statistically identical conditions. This article describes the synthesis and use of fluorophore labeled Ac-TZ14011 to analyze the CXCR4 binding of ligands at the cell membrane and to determine the IC_{50} values of ligands.

Ac-TZ14011, a derivative of T140 optimized for CXCR4 binding and stability in vivo by functional group substitutions, was synthesized as described previously (*16, 17*). The D-lysine at position 8 was selectively labeled with TAMRA or fluorescein (Figure 1). A hexamethylene group was incorporated into the TAMRA or fluorescein derivative to maintain an appropriate distance between T140 residues and the fluorophore. Residues critical to the CXCR4 binding activity of TZ14011 are Arg2, Nal3, Tyr5, and Arg14, and were assessed by screening of amino acid substitution of T140 (*16*). On the basis of the previously determined interaction between Ac-TZ14011 and CXCR4 (*18*), the fluorophores labeled at D-lysine 8 were assumed not to inhibit binding of fluorescent-Ac-TZ14011 to CXCR4. To investigate if fluorescent labeled Ac-TZ14011 maintains binding activity against CXCR4, the IC_{50} values of peptides were estimated by competitive assays against [^{125}I]-CXCL12 binding. In this assay, the IC_{50} of T140 was 3.7 nM. The IC_{50} values for fluorescein- or TAMRA-labeled Ac-TZ14011 were 11 and 14 nM, respectively. These values indicated that fluorophore labeling does not inhibit binding of Ac-TZ14011 as reported elsewhere about binding of TAMRA-Ac-TZ14011 (*19*).

The binding of TAMRA-Ac-TZ14011 to a cell membrane was observed with a laser-scanning confocal microscope to determine the specific binding of Ac-TZ14011 to CXCR4. The CXCR4-GFP fusion protein was stably expressed in the NP-2 cell line (*20*), and TAMRA-Ac-TZ14011 binding to CXCR4 was clearly observed at the membrane in the absence of competitors (Figure 2A). To assess the specific binding of ligands, excess unlabeled Ac-TZ14011 was added to the medium with TAMRA-Ac-TZ14011. Upon addition of Ac-TZ14011, weak fluorescence intensity was observed on the cell membrane or cytoplasm (Figure 2B). Vesicles observed in the cytoplasm show internalization of CXCR4 receptors induced by binding of the ligands, and signals from GFP and TAMRA showed colocalization in the cytoplasm. These results indicate the specific binding of TAMRA-Ac-TZ14011 to CXCR4. To evaluate the binding specificity for CXCR4 across the different kinds of GPCRs, HeLa cells, which stably express CD4-CCR5, were utilized for microscopy assays. The binding of TAMRA-Ac-TZ14011 was observed as for the NP-2 CXCR4-GFP cell line (Figure 2C). With the addition of excess CXCL12 (Figure

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[1]Abbreviations: Ac, acetyl; Cit, L-citrulline; DIC, differential interference contrast; ESI-MS, electron spray ionization-mass spectrometry; FBS, fetal bovine serum; Fmoc, 9-fluorenylmethoxycarbonyl; GPCR, G-protein-coupled receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Nal, L-3-(2-naphthyl)alanine; Nal(1), L-3-(1-naphthyl)alanine; RI, radioisotope; RP-HPLC, reverse-phase HPLC; TAMRA, tetramethylrhodamine; TFA, trifluoroacetic acid.

10.1021/bc800216p CCC: $40.75
Published on Web 08/16/2008

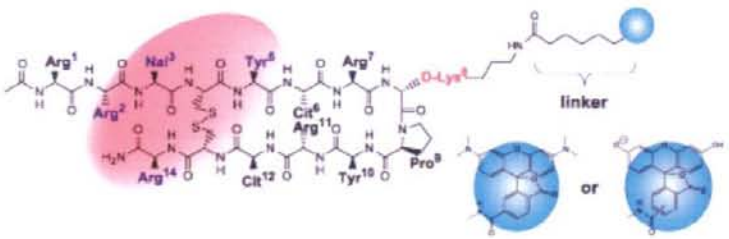


Figure 1. Design of fluorophore-labeled Ac-TZ14011. The amino acid residues in the red area are critical to CXCR4 binding activity. Fluorophores are shown as blue spheres.

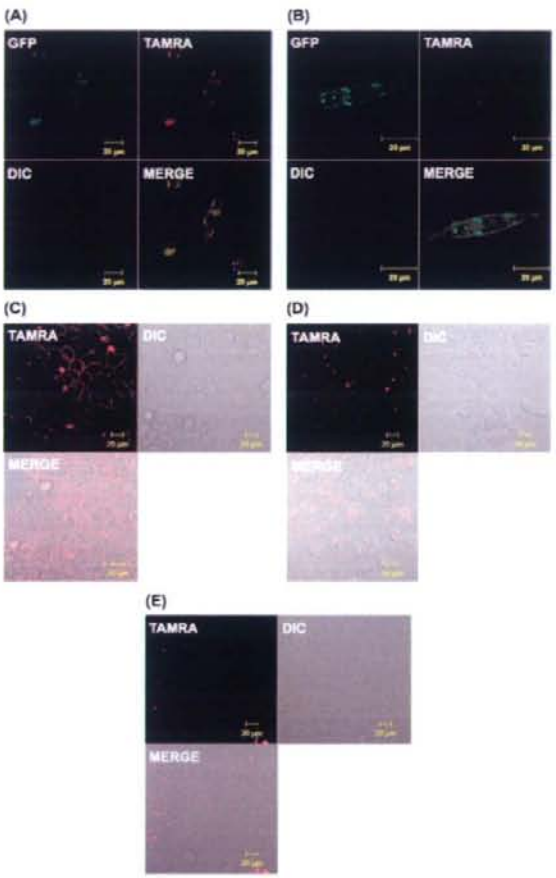


Figure 2. Confocal microscopy assays of TAMRA-Ac-TZ14011 binding to CXCR4. The signals of GFP and TAMRA are displayed in green and red, respectively. (A) Binding to NP2-GFP-CXCR4 cells. (B) Competitive binding to NP2 cells with excess amount of Ac-TZ14011. (C) Binding to HeLa-CD4-CCR5 cells. (D) Competitive binding to HeLa-CD4-CCR5 cells with excess CXCL12. (E) Competitive binding to HeLa-CD4-CCR5 cells with excess Ac-TZ14011. Descriptions of images are indicated in the pictures.

2D) or Ac-TZ14011 (Figure 2E), the fluorescence intensity on the cell membrane was decreased. These results show that TAMRA-Ac-TZ14011 binds specifically to CXCR4 but not to CCR5.

To investigate the utility of fluorescein-labeled Ac-TZ14011, cell-based binding assays were performed. In this binding assay, fluorescein-Ac-TZ14011 was utilized as a competitor to derivatives of FC131 (*8*) and the dipicolylamine-*p*-xylene Zn(II) complex [Zn^{2+}-(Dpa)-*p*-Xyl] that were developed as CXCR4 antagonists (Figure 3) (*21*). The binding constants of these compounds were previously estimated by competitive assays with [^{125}I]-CXCL12. As a result, larger values of IC_{50} than those in the previous assays were observed (Table 1). The difference of the binding constants of competitors was assumed to be a reflection of the difference of IC_{50} values in the assays. It is especially interesting that the values of IC_{50} as determined by fluorescent- and RI-competition assays are clearly correlated. It was clearly indicated that binding activity of compounds can be estimated by binding inhibition assays conducted at a constant concentration of compounds. Indeed, in the detailed binding assays, a significant correlation was observed in IC_{50} values measured by both methods for T140, TC13, and TC22.

Table 1. K_d Values Determined by RI-Competition and Fluorescent Probe Competition Assays

	IC_{50} (nM)		
	[^{125}I]-CXCL12 competition (IC_{50}C)	fluorescein-Ac-TZ14011 competition (IC_{50}F)	IC_{50}F/IC_{50}C
T140	3.93	24.7	6.3
Zn^{2+}-(Dpa)-*p*-Xyl	47[a]	291	6.2
FC131	14.6	109	7.5

[a] This value is derived from ref *18*.

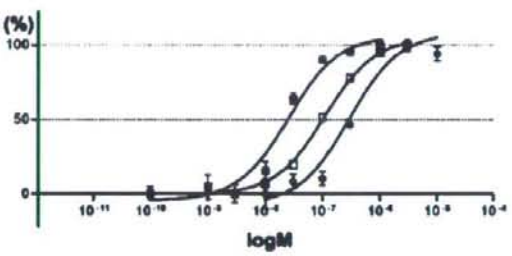


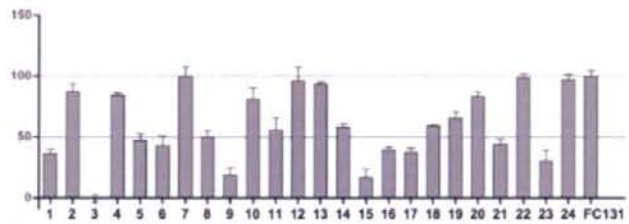
Figure 3. Curve fitting for CXCR4 binding of T140 (■), Zn^{2+}-(Dpa)-*p*-Xyl (●), and FC131 (□) in competitive assays by fluorescein-Ac-TZ14011. The *x* and *y* axes show concentrations and inhibition percentages of the binding of test compounds, respectively.

In the application of high-throughput screening for pharmacophores of CXCR4 ligands, it is important to be able to rapidly determine IC_{50} values. To test whether fluorescein-Ac-TZ14011 could be useful as a ligand in high-throughput screening, binding inhibition analyses at constant compound concentrations were performed. Twenty-four derivatives of a cyclic pentapeptide, FC131, were prepared for the analyses as described previously (Figure 4A) (*8*). The conditions used were the same as in the binding experiments shown in Figure 3 except that the compound concentration was kept constant at 2 μM. Nine compounds were found to induce >75% inhibition at this concentration (Figure 4B). The IC_{50} values of compounds that showed high inhibitory scores in the screening analyses were examined

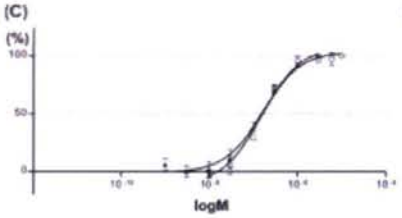
(A)

compound	Xaa^3	Xaa^4	compound	Xaa^3	Xaa^4
1	Arg	Nal	13	Arg	D-Nal
2	Nal	Arg	14	Nal	D-Arg
3	Arg	Trp	15	Arg	D-Trp
4	Trp	Arg	16	Trp	D-Arg
5	Arg	Nal(1)	17	Arg	D-Nal(1)
6	Nal(1)	Arg	18	Nal(1)	D-Arg
7	D-Arg	Nal(1)	19	D-Arg	D-Nal
8	D-Nal	Arg	20	D-Nal	D-Arg
9	D-Arg	Trp	21	D-Arg	D-Trp
10	D-Trp	Arg	22	D-Trp	D-Arg
11	D-Arg	Nal(1)	23	D-Arg	D-Nal(1)
12	D-Nal(1)	Arg	24	D-Nal(1)	D-Arg

(B)



(C)



(D)

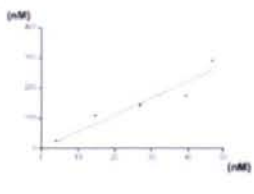


Figure 4. (A) Design of cyclic pentapeptides. Xaa^3 and Xaa^4 (red area) were manually randomized. (B) Results of single concentration point assays for determination of the binding activity of library compounds at one time. The *x* and *y* axes show concentrations and inhibition percentages of binding of test compounds, respectively. Data were measured in triplicate, and error bars show the SEM. (C) Curve fitting for CXCR4 binding of TC13 (○) and TC22 (▲) in competitive assays by fluorescein-Ac-TZ14011. The *x* and *y* axes show concentrations and inhibition percentages of binding of test compounds, respectively. (D) Correlation between IC_{50} values determined by RI-competition assays (*x*-axis) and fluorescein-Ac-TZ14011 competition assays (*y*-axis). The compound and IC_{50} values are shown in Tables 1 and 2. The *P* value determined from correlation analysis was 0.012.

Table 2. K_d Values Determined by RI-Competition and Fluorescent Probe Competition Assays

	IC_{50} (nM)		
	$[^{125}I]$-CXCL12 competition ($IC_{50}C$)	fluorescein-Ac-TZ14011 competition ($IC_{50}F$)	$IC_{50}F/IC_{50}C$
T140	3.93	24.7	6.3
TC13	27.0	143	5.3
TC22	39.4	176	4.5

further (Table 2). The IC_{50} values of TC13 and TC22 were determined to be 143 and 176 nM, respectively (Figure 4C). The IC_{50} values determined in this assay showed a clear correlation with those in RI-competition assays (Figure 4D, manuscript in preparation).

Advantages of the fluorescence-based analyses include their utility in high-throughput screening and direct observation of the binding state on cell membrane by fluorescence microscope; binding assays and confocal microscopy study were performed to evaluate these advantages. The binding of T140 was previously assessed with site-directed mutagenesis of CXCR4, which indicated that the extracellular loop 2 of the receptor is the main target for this peptide (*22*). The observation of Ac-TZ14011 binding to cell membranes provided convincing evidence of specificity for the target receptor. Competition with excess unlabeled Ac-TZ14011 and CXCL12 showed clear inhibition of TAMRA-Ac-TZ14011 binding. There has been concern that CXCR4 ligands could bind nonspecifically to other GPCRs. A binding study utilizing CCR5-CD4-HeLa cells showed evidence of a high degree of specificity of the ligands. HeLa cells naturally express CXCR4 (*23*), and in the event of overexpression of CCR5-CD4 on the membrane, the binding of TAMRA-Ac-TZ14011 was prevented by the addition of competitors. These results indicate that these peptides bind to the same target site on the cell membrane, CXCR4. Internalization of CXCR4 stimulated by binding of ligands was clearly observed, particularly in the presence of competitors indicating that ligands bound to CXCR4 are simultaneously incorporated in the cytoplasm. Interestingly, on the basis of the numbers and size of vesicles observed, CXCL12 showed stronger induction of CXCR4 internalization than Ac-TZ14011. Promotion of CXCR4 internalization is one of the important mechanisms for inhibition of HIV entry (*24*). The difference of ligand-dependent effects on CXCR4 internalization will be studied further in our laboratory.

In conclusion, the structure−activity relationships of ligands for CXCR4 have been well studied, but relatively few known ligand pharmacophores have been studied because of the difficulty associated with the analysis of receptor−ligand interactions. Our results strongly indicate that fluorescence-based ligand binding assays could be useful in the exploration of novel pharmacophores for CXCR4 ligands and that such compounds have promise as therapeutic agents for AIDS, breast cancer metastasis, and rheumatoid arthritis. Furthermore, this methodology is applicable to the design of ligands for other GPCRs.

ACKNOWLEDGMENT

We are grateful to Professor Kazunari Akiyoshi for his generous cooperation in experiments. This research was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and Health and Labour Sciences Research Grants.

Supporting Information Available: Detailed materials and methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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BC800216P

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

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Received 14th July 2008, Accepted 28th August 2008
First published as an Advance Article on the web 17th October 2008
DOI: 10.1039/b812029c

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]

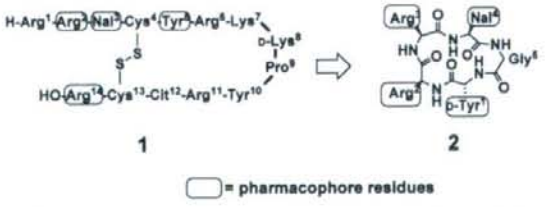

Fig. 1 Development of a cyclic pentapeptide **2** based the pharmacophore of a CXCR4 antagonistic peptide **1**. Cit = L-citrulline, Nal = L-3-(2-naphthyl)alanine.

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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

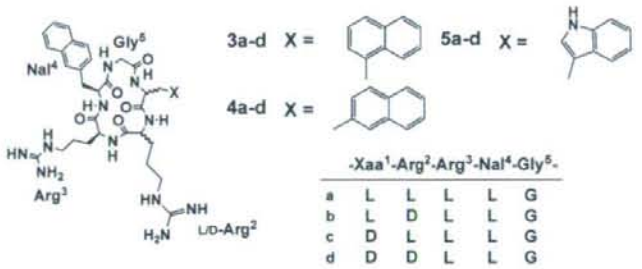

Fig. 2 Structures of compounds having substitution of an L/D- bicyclic aromatic amino acid for Tyr[1].

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

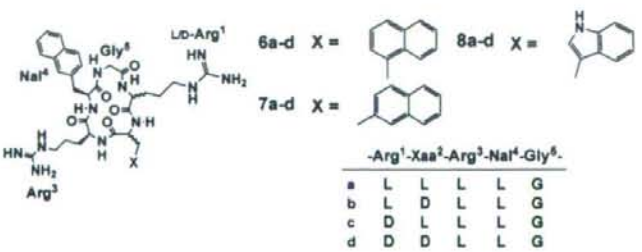

Fig. 3 Structures of compounds having L/D-Arg[1] and an L/D-bicyclic aromatic amino acid.[2]

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^5. 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.

Acknowledgements

This work was supported by Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and Health and Labour Sciences Research Grants from Japanese Ministry of Health, Labor, and Welfare.

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Expert Opinion

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A future perspective on the development of chemokine receptor CXCR4 antagonists

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Background: In the postgenome era, G-protein-coupled receptor families have been recognized as significant drug targets in medicinal chemistry. A specific chemokine receptor, CXCR4, has multiple critical functions in normal physiologies including embryonic development of the cardiovascular, hemopoietic and central nervous systems, and underlies problematic pathologies such as HIV infection, cancer metastasis, leukemia progression and rheumatoid arthritis. *Methods and results*: A tetradecamer peptide, T140, derived from the horseshoe crab, and its biologically stable derivative, 4F-benzoyl-TN14003, were found to be powerful CXCR4 antagonists that block HIV entry to cells. These peptides have also shown remarkable inhibitory activity against cancer metastasis and progression in a variety of cancers. Slow release administration of 4F-benzoyl-TN14003, for example, was found to significantly reduce pulmonary metastasis of breast cancer cells in severe combined immunodeficient mice. This peptide also shows inhibitory effects against melanoma metastasis and Epstein–Barr virus-associated lymphoproliferation in mice, suppresses the delayed-type hypersensitivity response induced by sheep red blood cells and reduced collagen-induced arthritis in both mouse models of arthritis. *Conclusion*: T140 analogues have the potential to become promising agents for chemotherapy of AIDS, cancer and rheumatoid arthritis. This review summarizes the development of low molecular weight CXCR4 antagonists based on pharmacophore identification in T140 analogues and also provides an opinion on the future of the development of CXCR4 antagonists.

Keywords: AIDS, cancer metastasis, chemokine receptor, CXCR4 antagonist, FC131, HIV infection, leukemia, rheumatoid arthritis, T140, T22

Expert Opin. Drug Discov. (2008) 3(10):1-12

1. Introduction

Proteomics and chemical biology have prospered as postgenome projects and specific ligands related to protein networks have been valuable and useful in these studies. Selective antagonists against G-protein-coupled receptors (GPCR) are much sought after, as the GPCR family is a very promising target for drug discovery [1]. Chemokines comprise a chemotactic cytokine family that induces migration of leukocytes, whereas chemokine receptors, which transduce the signals of the corresponding chemokines, are classified into different GPCR families. The correlations between chemokines and their receptors are highly interconnected and complex: most commonly, a single chemokine receptor recognizes a plurality of chemokines, one chemokine recognizes several chemokine receptors and most of the chemokines lack receptor selectivity.

10.1517/17460440802373379 ISSN 1746-0441

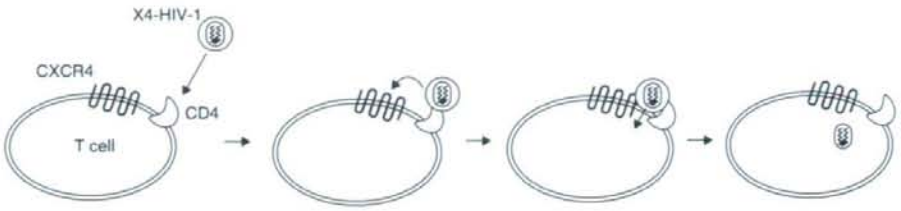

Figure 1. Correlation of CXCR4 to X4-HIV-1 infection. X4-HIV-1 strains enter T cells through association with the first receptor, CD4, and the second receptor, CXCR4.

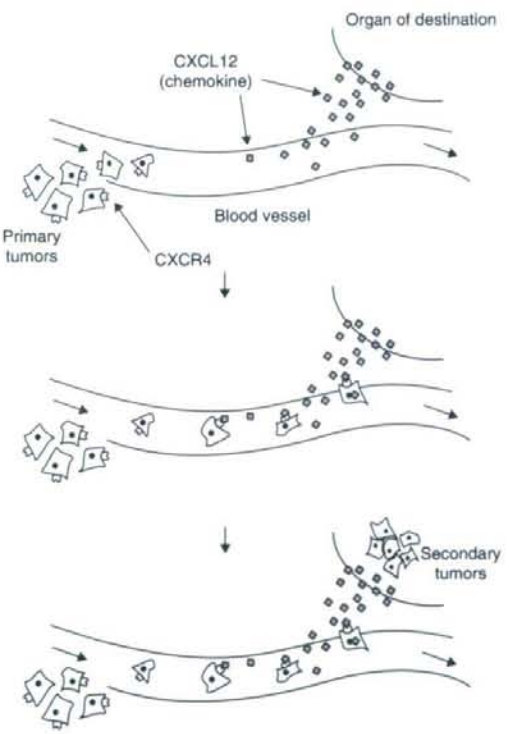

Figure 2. Correlation of the CXCL12–CXCR4 axis to cancer cell metastasis. A metastatic destination of tumor cells might be determined by the CXCL12–CXCR4 axis.

An exception is found, however, in the chemokine CXCL12/stromal cell-derived factor-1 whose chemokine receptor is CXCR4 [2-5]. Interaction between CXCL12 and CXCR4 is essential for the migration of progenitor cells during embryonic development of the cardiovascular, intestine vascular, hemopoietic and central nervous systems. The details of its physiological roles in adults remain obscure but recently it has been found that the CXCR4–CXCL12 axis is involved in multiple intractable disorders such as AIDS [6], cancer cell metastasis [7-24], progression of acute and chronic leukemias [25-28] and rheumatoid arthritis (RA) [29]. It has also been found that CXCL12 binds to and signals through CXCR7 [30], and that ligand activation of CXCR7 does not cause Ca^{2+} mobilization or cell migration but rather cell survival and tumor development [31].

Initially, CXCR4 was identified as a co-receptor, the second receptor of T-cell-line-tropic (X4) HIV-1 entry through its association with the first receptor, CD4 (**Figure 1**). Macrophage-tropic (R5) HIV-1 strains, which use the chemokine receptor CCR5 as a different co-receptor, are major in the early stages of HIV infection [32-36] whereas X4-HIV-1 strains become dominant in the later stages. Recently, it has also been reported that CXCL12 is highly expressed in several internal organs that are the primary targets of cancer cell metastasis, and that CXCR4 is overexpressed on the surfaces of several types of cancer cells. Thus, it is clear that the CXCL12–CXCR4 axis is associated with metastasis of several types of cancer including cancer of pancreas, breast, lung, kidney and prostate as well as non-Hodgkin's lymphoma, neuroblastoma, melanoma, ovarian cancer, multiple myeloma and malignant brain tumors (**Figure 2**). This axis is also correlated to the progression of chronic lymphocytic leukemia (CLL), acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (**Figure 3**). In addition, RA is caused mainly by $CD4^+$ memory T-cell accumulation in the inflamed synovium. It has been reported that the CXCL12 concentration is extremely elevated in the synovium of RA patients and that CXCR4 is highly expressed on the surface of memory T cells. In addition, CXCL12 stimulates migration of the memory T cells thereby inhibiting T-cell apoptosis (**Figure 4**). This indicates that the CXCR4–CXCL12 interaction plays an essential role in the accumulation of T cells in the RA synovium. As a consequence, CXCR4 would appear to be an attractive therapeutic target for these diseases, and our recent research about the development of CXCR4 antagonists is discussed in this review.

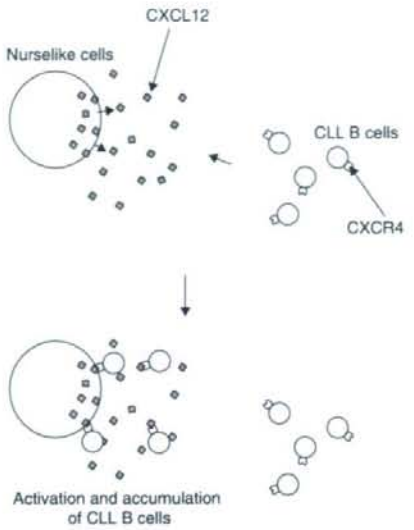

Figure 3. Correlation of the CXCL12–CXCR4 axis to CLL.
CLL B-cells are rescued from apoptosis through their activation by CXCL12 and thereby are accumulated.
CLL: Chronic lymphocytic leukemia.

2. Anti-HIV activity of CXCR4 antagonists as selective inhibitors of X4-HIV-1 entry

Antibacterial and antiviral peptides, the tachyplesins and the polyphemusins, isolated from the hemocyte debris of the Japanese horseshoe crab (*Tachypleus tridentatus*) and the American horseshoe crab (*Limulus polyphemus*), are heptadecamer and octadecamer peptides, respectively (**Figure 5**) [37,38]. Through our structure–activity relationship studies of these peptides, T22 ([Tyr5,12, Lys7]-polyphemusin II) [39,40], and its downsized tetradecamer peptide, T140 [41], have been developed as effective anti-HIV agents (**Figure 5**). They have been shown to suppress X4-HIV-1 entry into cells by binding specifically to CXCR4 and to inhibit Ca^{2+} mobilization resulting from CXCL12 stimulation of CXCR4 [42-44]. Structural analysis revealed that T140 forms an antiparallel β-sheet structure supported by a disulfide bridge between Cys4 and Cys13, which is connected by a type II′ β-turn [45], and four amino-acid residues in T140, Arg2, L-3-(2-naphthyl)alanine (Nal)3, Tyr5 and Arg14, were identified as residues essential for significant activity [46]. T140 analogues have a significant advantage in clinical chemotherapy, as they show a suppressive effect against drug-resistant strains. In passage experiments using cell cultures *in vitro* T140 analogues exhibit a remarkable and significant delay in the appearance of drug-resistant strains of HIV [47].

T140 has been shown to be biologically unstable and is biodegraded in mouse/feline serum or in rat liver homogenate [48,49]. Deletion of essential amino-acid residues, Arg14 (in serum) and Arg2, Nal3 and Arg14 (in liver homogenates) from the N and the C termini of T140 caused a dramatic reduction of the potency of the parent peptide. Modification of T140 analogues at both termini efficiently suppressed this biodegradation and led to development of novel compounds that show high CXCR4-antagonistic activity as well as increased biological stability. In additional, it was found that an electron-deficient aromatic ring such as a 4-fluorobenzoyl moiety at the N terminus might participate in a novel pharmacophore associated with anti-HIV activity. The novel T140 analogues, 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011, which possess an N-terminal 4-fluorobenzoyl moiety, have enhanced biostability in serum/liver homogenates and anti-HIV activity that is two orders of magnitude higher than that of T140 (**Figure 5**) [50].

3. Anticancer metastatic activity of CXCR4 antagonists

CXCR4 is expressed in malignant cells in at least 23 different types of cancers [51] and CXCL12 is highly expressed in some destination organs of cancer cell metastasis, suggesting that the CXCL12–CXCR4 axis may be relevant to cancer metastasis. CXCR4 and another chemokine receptor, CCR7, are highly expressed on the surface of human breast cancer cells, whereas CXCL12 and a CCR7 ligand, CCL21, are highly expressed in lymph nodes, bone marrow, lung and liver, which are common metastatic targets of breast cancer. The metastatic destination of tumor cells is arguably determined by the CXCL12–CXCR4/CCL12–CCR7 axis, which could lead to organ-preferential metastasis [9]. Neutralizing CXCR4 with anti-CXCR4 antibodies in mice significantly inhibited metastasis of breast cancer cells to the lung. The inhibitory activity of our T140 analogues against the migration of breast cancer cells *in vitro* and against metastasis of breast cancer cells *in vivo* has been investigated [20]. These compounds dose-dependently inhibit the migration of a CXCR4-positive human breast carcinoma cell line MDA-MB-231 induced by CXCL12. Experimental metastasis models of breast cancer were adopted, in which MDA-MB-231 cells were injected intravenously into the tail vein of severe combined immunodeficient mice and then trapped in the lung through the heart and the pulmonary artery. When 4F-benzoyl-TN14003, a bio-stable T140 analogue, was injected subcutaneously with an Alzet osmotic pump (DURECT Corp., Cupertino, CA, USA), effective suppression of tumor accumulation resulting from MDA-MB-231 metastasis was shown on the lung surface, compared with the control PBS injection. These results suggest that small molecule CXCR4 antagonists, such as T140 analogues, might be useful as antimetastatic agents, possibly replacing

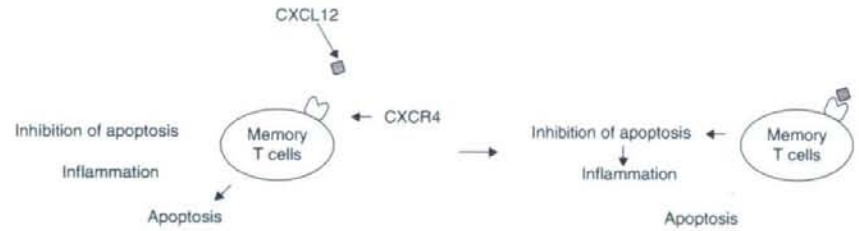

Figure 4. Correlation of the CXCL12–CXCR4 axis to rheumatoid arthritis. CXCL12 stimulates migration of the memory T cells and thereby inhibits T-cell apoptosis.

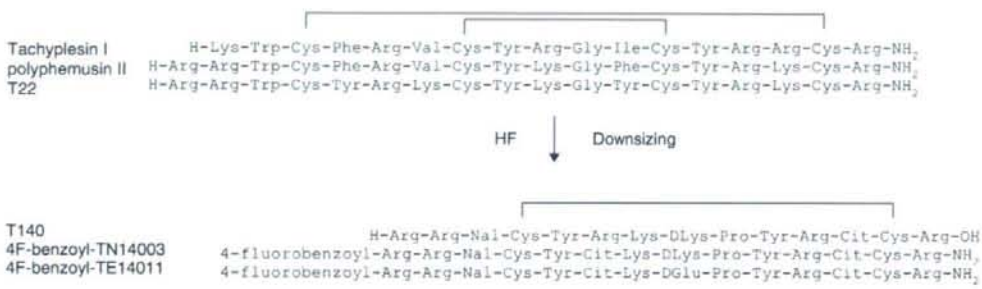

Figure 5. Structures of tachyplesin I, polyphemusin II and their analogues. Disulfide bridges of these peptides are shown by solid lines.
Cit: L-Citrulline; DGlu: D-Glutamic acid; DLys: D-Lysine; Nal: L-3-(2-Naphthyl)alanine.

anti-CXCR4 antibodies as neutralizers of metastasis of breast cancer.

The second example concerns melanoma. It has been reported that CXCR4-transduced B16 melanoma cells cause metastatic cell accumulation in the lungs of mice and that T22, a CXCR4 antagonist, blocks pulmonary metastasis of B16 cells [52]. We investigated whether T140 analogues inhibit pulmonary metastasis in mice injected with B16 cells, which were not transduced with CXCR4 [21]. In this experiment, biodegradable poly-D,L-lactic acid (PLA) microcapsules containing 4F-benzoyl-TE14011 were injected subcutaneously into mice with experimental metastatic models of CXCR4-positive B16–BL6 melanoma cells. The PLA microcapsules release 4F-benzoyl-TE14011 in a controlled fashion for a lengthy period *in vivo* maintaining the level of the 4F-benzoyl-TE14011 concentration in the blood. As a result, such a single s.c. injection of 4F-benzoyl-TE14011-PLA significantly decreases pulmonary metastasis of B16–BL6 cells. Thus, a controlled release of CXCR4 antagonists might be useful for effective suppression of cancer metastasis.

The third example of cancer metastasis concerns pancreatic cancer. The mRNA of CXCR4 is expressed both in pancreatic cancer tissues and in the pancreatic cancer cell lines, AsPC-1, BxPC-3, CFPAC-1, HPAC and PANC-1. CXCL12 mRNA is expressed in pancreatic cancer tissues [7]. We found CXCL12 activates both migration and invasion of pancreatic cancer cells, AsPC-1, PANC-1 and SUIT-2, dose-dependently *in vitro*, suggesting that the interaction between CXCL12 and CXCR4 can be correlated with pancreatic cancer cell progression and metastasis. We also found that T140 analogues suppress CXCL12-mediated migration and invasion of these cells dose-dependently [22] and that CXCL12 treatment of PANC-1 cells causes a drastic increase in actin polymerization (cytoskeleton), which is effectively inhibited by T140 analogues.

In addition, metastasis of several types of cancer cells is relevant to the CXCL12–CXCR4 axis, such as small cell lung cancer [18] and multiple myeloma [24]. Thus, the blockade of this axis might become an effective chemotherapy against these disorders and CXCR4 antagonists such as the T140 analogues might be useful lead compounds for anticancer metastatic agents.

4. Antileukemia activity of CXCR4 antagonists

Mutual contact with bone marrow stromal layers through adhesive interactions between leukemia cells expressing

CXCR4 along with integrins and stromal cells expressing CXCL12 and integrin ligands might cause growth and survival of ALL pre-B cells. Constitutively secreted at high levels from marrow stromal cells, CXCL12 stimulates migration of these cells into stromal layers, as CXCR4 is highly expressed in the pre-B cells. T140 blocks CXCL12-activated migration of the pre-B cells and reduces their migration into bone marrow stromal layers. In addition, T140 analogues enhance the cytotoxic and antiproliferative effects of other anticancer agents such as vincristine and dexamethasone. This suggests that T140 analogues might be useful to overcome cell adhesion-mediated drug resistance (CAM-DR) in ALL chemotherapy [26].

B-cell CLL, the most common leukemia in adults in Western countries, is caused by the accumulation of long-lived, monoclonal, malignant B cells in blood, secondary lymphoid organs and bone marrow. CXCL12 that is released from marrow stromal cells or nurse-like cells stimulates CLL B cells that express CXCR4 highly. CLL B cells are rescued from apoptosis through their activation by CXCL12, and accumulate. Thus, the CXCL12–CXCR4 axis might also be a therapeutic target of B-cell CLL [27]. As a result, chemotaxis of CLL B cells induced by CXCL12, their migration beneath marrow stromal cells and actin polymerization are all suppressed by T140 analogues in a dose-dependent manner *in vitro* [27]. T140 analogues reduce the antiapoptotic effect of CXCL12, thereby preventing stromal cells from inhibiting the spontaneous apoptosis of CLL B cells. Cocultivation of CLL B cells with marrow stromal cells causes stromal CAM-DR, which prevents fludarabine from inducing apoptosis of CLL B cells. The T140 treatment can resensitize these B cells towards fludarabine. T140 analogues might be also useful for the clinical CLL chemotherapy involving anti-CAM-DR.

5. Anti-RA activity of CXCR4 antagonists

The CXCR4–CXCL12 interaction plays a fundamental role in the accumulation of memory T cells in the RA synovium [29]. 4F-benzoyl-TN14003, evaluated by the anti-RA assay, was shown to inhibit CXCL12-mediated migration of human Jurkat cells and mouse splenocyte in a dose-dependent manner *in vitro*. The mouse delayed-type hypersensitivity (DTH) reaction induced by sheep red blood cells was adopted as an *in vivo* experimental model of the cellular immune response [53]. 4F-benzoyl-TN14003, injected subcutaneously using an Alzet osmotic pump, was shown to induce effective suppression of the footpad swelling (the DTH response) in a dose-dependent manner, compared with a control PBS injection. Collagen-induced arthritis (CIA) in mice was adopted as a second *in vivo* experimental RA model. Several RA symptom markers including score increase, body weight loss, ankle swelling and limb weight gain were remarkably suppressed by subcutaneous injection of 4F-benzoyl-TN14003 using an Alzet osmotic pump. An increase in the level of serum antibovine CII IgG2a antibody was apparently suppressed in mice treated with 4F-benzoyl-TN14003 following treatment with the bovine type II collagen (CII) emulsion booster, suggesting that 4F-benzoyl-TN14003 has an inhibitory effect on the humoral immune response to CII. Until now, the development of biological drugs such as monoclonal antibodies, which target inflammatory cytokines and include TNF-α, IFN-γ and IL-1, IL-6, has yielded useful results in clinical RA therapy but complete curative effects have not been achieved. At present, other drugs, which are not relevant to the functions of these cytokines, are used to improve RA chemotherapy and T140 analogues might prove to be useful leads for anti-RA agents.

6. Reduction of the molecular size of T140 analogues based on cyclic pentapeptides

The crucial amino-acid residues of T140 are Arg2, Nal3, Tyr5 and Arg14, which according to NMR analysis and molecular dynamics calculations are located in close proximity to each other in space [45]. To achieve reduction of the molecular size of T140 analogues, a pharmacophore-based strategy was adopted using cyclic pentapeptide libraries, which involve two L/D-Arg, L/D-Nal, L/D-Tyr and a spacer Gly. This strategy led to discovery of FC131 [*cyclo*(-Arg1-Arg2-Nal3-Gly4-D-Tyr5-)], which has strong CXCR4-antagonistic activity comparable to that of T140 (**Figure 6**) [54]. NMR analysis and molecular dynamics calculations revealed that FC131 forms the near-symmetrical pentagonal backbone structure, suggesting that owing to its cyclic pentapeptide template, it is relatively rigid compared with T140 analogues. In addition, an *N*-methylated analogue FC122 [*cyclo*(-D-MeArg1-Arg2-Nal3-Gly4-D-Tyr5-)] has potent antagonistic activity comparable to that of FC131 [55]. Conformational analysis suggests that FC131 and FC122 favor the same backbone conformation and that the orientation of the backbone amide bonds contributes to the pronounced CXCR4-antagonistic activity.

7. Development of FC131 analogues based on cyclic pentapeptides with an additional pharmacophore moiety

As described in the previous section, a 4-fluorophenyl moiety is considered to be an additional and critical part of the pharmacophore and was introduced into cyclic pentapeptides such as FC131 as part of a lead discovery effort. FC401, [Phe(4-F)1]-FC131, shows significant CXCR4-binding activity (**Figure 6**) [56] and FC602, [D-Phe(4-F)1, Arg5]-FC131, shows potent activity, which is 10-fold stronger than that of [D-Tyr1, Arg5]-FC131 (**Figure 6**). These peptides are novel leads, which involve a pharmacophore different from that of FC131.

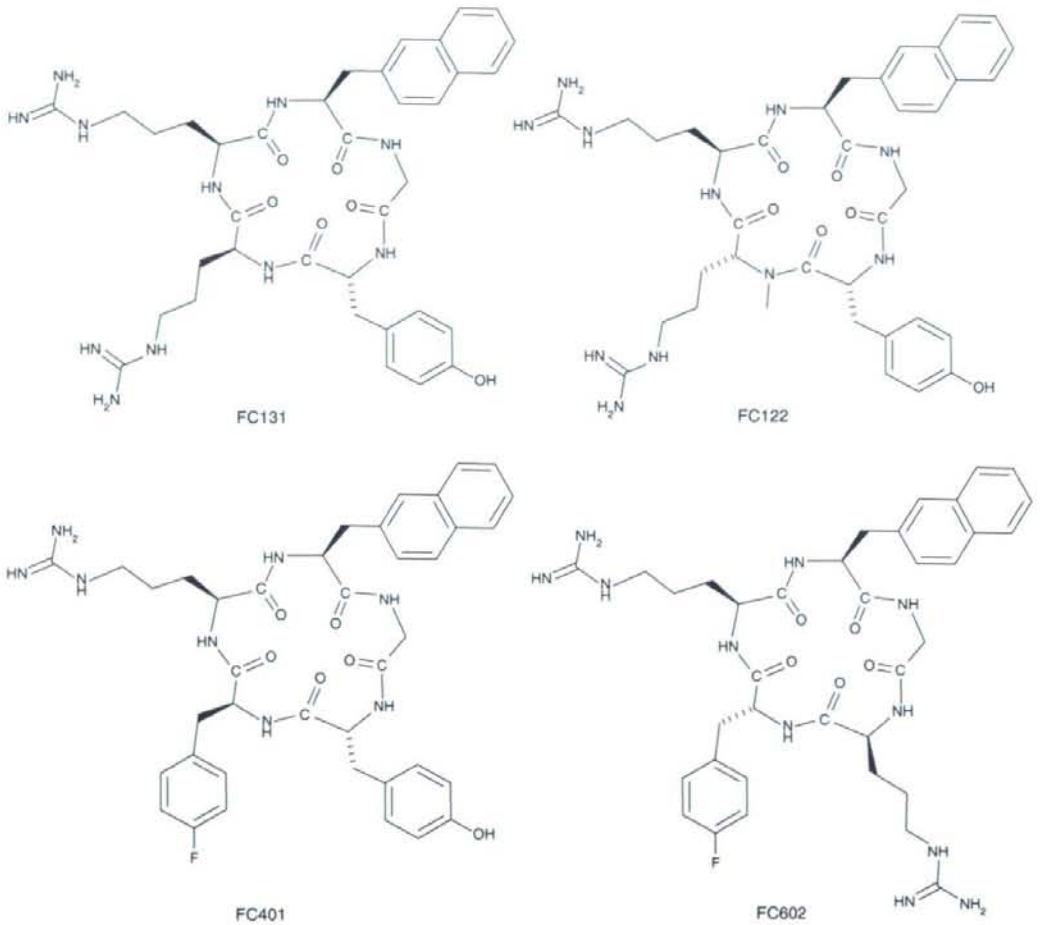

Figure 6. Structures of cyclic pentapeptides FC131, FC122, FC401 and FC602.

8. Development of linear small molecules with CXCR4 antagonistic activity

Development of small linear molecules with CXCR4 antagonistic activity was investigated based on identification of the T140 pharmacophore groups involving Arg, Nal and Tyr together with a novel pharmacophore moiety for CXCR4 antagonism, such as a 4-fluorophenyl moiety. Combination of substructure units of the pharmacophore moieties led to the design and synthesis of several compounds using combinatorial chemistry. As a result, compounds **1** – **4** shown in **Figure 7**, linear-type moderate CXCR4 antagonists, were found [57]. These compounds are generally less potent than the cyclic pentapeptide FC131, suggesting that conformational restriction implicit in the cyclic pentapeptide template is essential for potency. Furthermore, introduction of pharmacophores involving guanidine and aromatic groups into constrained and drug-like scaffolds, such as benzodiazepine, indole and quinoxaline, has provided a new type of nonpeptide CXCR4 antagonist such as **5** [56].

It has recently been reported that anthracene derivatives containing two sets of zinc(II)-2,2′-dipicolylamine (Dpa) complex are useful chemosensors for phosphorylated peptide surfaces [59]. Several low molecular weight compounds involving the above complex structure were identified as selective CXCR4 antagonists lacking significant affinity for any other GPCRs (**Figure 7**) [60]. Overlay of the structure of the zinc(II)-2,2′-dipicolylamine complex compound **6** on

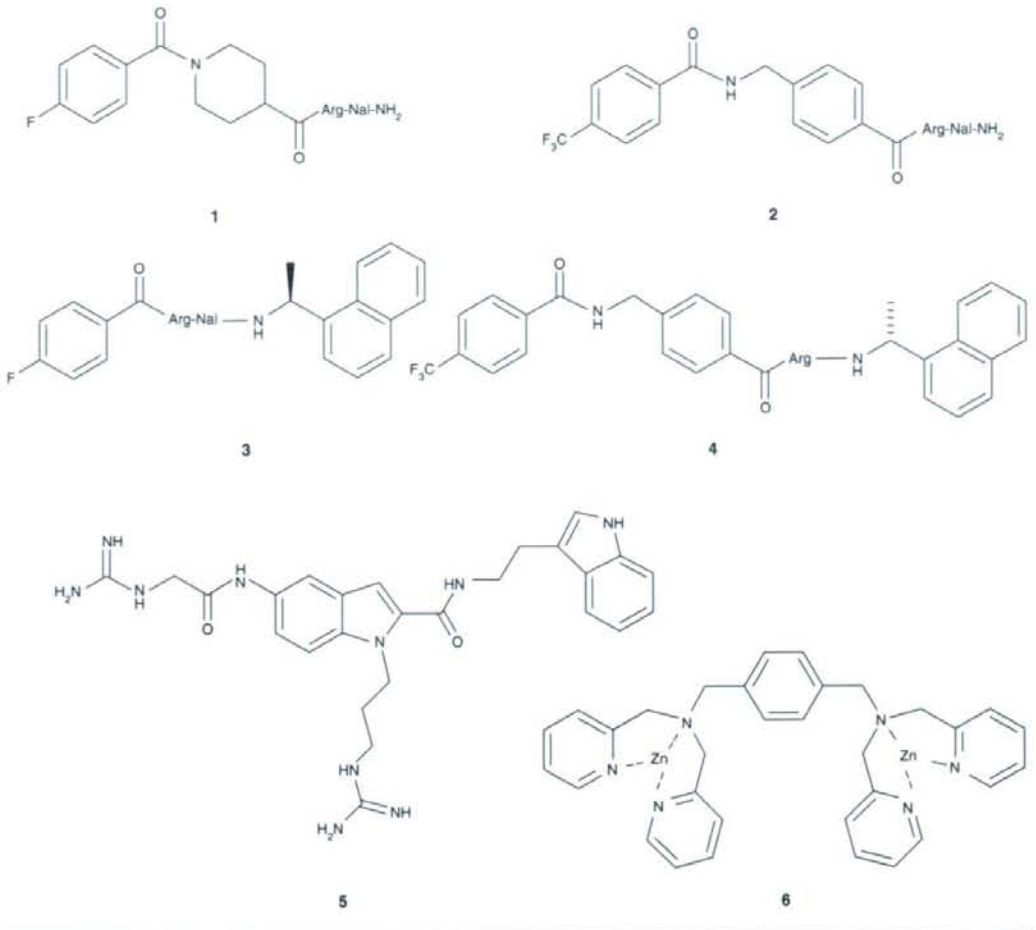

Figure 7. Structures of a linear type of low molecular weight CXCR4 antagonists.

that of the cyclic pentapeptide FC131 provided the best fit with the maintenance of local energy minima of these structures and suggests that the distance between two dipicolylamine moieties of compound 6 is nearly equal to that between the two Arg guanidine groups of FC131, and that the distance of these functional groups might be critical for expression of CXCR4 antagonistic activity.

9. Other CXCR4 antagonists

A peptidomimetic strategy involving β-hairpin protein epitope mimetics has been applied to β-turn moiety of polyphemusin II and T22, providing the macrocyclic peptides POL2438 and POL3026 [61]. POL3026 is a potent CXCR4 antagonist with biostability towards proteolysis in plasma and favorable pharmacokinetic properties in dogs, and has the potential to be a therapeutic agent for anti-AIDS, anticancer and stem cell mobilization. Several low molecular weight CXCR4 antagonists, which are not correlated to T140, have been reported until now [62,63]. The discovery of AMD3100 bearing two cyclam groups (Genzyme) [64] as CXCR4 antagonists has encouraged the development of small molecules that block CXCR4. Although the development of AMD3100 as an anti-AIDS drug was discontinued owing to its cardiovascular effects, its development as a drug for stem cell mobilization is being continued. An *N*-pyridinylmethylene cyclam (monocyclam) AMD3465 (Genzyme) [65], which contains one cyclam moiety of

AMD3100 and a picolylamine group in place of the other cyclam moiety, has almost the same potency as AMD3100. AMD070 (Genzyme) [66] is a tetrahydroquinoline-benzimidazole-based CXCR4 antagonist with anti-X4-HIV-1 activity but clinical trials of AMD070 are now on hold as a result of hepatotoxicity. Synthesis of AMD3100 substituted with a metal ion such as Cu^{2+}, Zn^{2+} or Ni^{2+} revealed a remarkable increase in binding affinity for CXCR4, possibly through enhanced interaction with the carboxylate group of Asp262, which is located at the transmembrane VI region of CXCR4 [67]. In addition, AMD8665 without a cyclam group (Genzyme) [68], ALX40-4C (Ac-[D-Arg]9-NH2; NPS Allelix) [69], CGP64222 [70], R3G [71], NeoR [72], a distamycin analogue, NSC651016 [73], a dipyridine containing xylenediamine compound WZ811 [74] and a flavonoid compound, ampelopsin [75], have also been identified as CXCR4 antagonists. Conjugates of AMD3100 and galactosyl-ceramide (GalCer) analogues have also been found to act as bifunctionalized drugs [76]. KRH-1636/CS-3955 (Kureha Chemical & Daiichi-Sankyo) is an orally bioavailable agent possessing *N*-pyridinylmethylene, Arg and naphthalene moieties [77]. An alkyl amine analogue of KRH1636, KRH2731, which has high bioavailability (37% through oral administration in rat), possesses potent CXCR4 antagonistic activity [78]. Recently, several antagonists related to KRH2731 have been reported [79].

10. Conclusion

An octadecamer peptide, T22, and its downsized analogue, T140, have been found to be strong anti-HIV agents that inhibit entry into T cells by X4-HIV-1 through their specific binding to the co-receptor CXCR4. The T140 analogues, 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011, have been developed as potent and biostable CXCR4 antagonists. These peptides have been found to have not only anti-HIV but also anticancer metastasis, antileukemia and anti-RA activities. Downsizing of T140 analogues led to the discovery of a cyclic pentapeptide FC131, which is common to several new low molecular weight CXCR4 antagonists. A linear type of low molecular weight CXCR4 antagonist containing aromatic compounds bearing a zinc(II)-2,2′-dipicolylamine structure has also been developed. These antagonists are promising agents for clinical chemotherapy of multiple disorders such as HIV infection, cancer metastasis, leukemia and RA.

11. Expert opinion

The discovery of CXCR4 has provoked vigorous research on drug development with its correlation to a co-receptor for HIV entry. However, blocking of the CXCL12–CXCR4 axis might be risky because CXCR4 is constitutively expressed in several organs and tissues, and CXCR4 plays a critical role in embryogenesis, homeostasis and inflammation in the fetus especially in the embryonic development of hemopoietic, cardiovascular and central nervous systems. CXCR4 also plays a role in the homing of immune cells in inflammation. Knockout of CXCL12 or CXCR4 is known to be embryonically lethal [80] and one must carefully consider the risks associated with blockade of the CXCL12–CXCR4 axis. As anti-HIV agents, CXCR4 antagonists play a critical role in HIV-infected patients who have X4-HIV-1 strains that emerge late in the HIV infectious disease process. CXCR4 antagonists might suppress the appearance of X4 or dual-tropic strains in patients who have R5 strains that constitute a majority in the early stages of HIV infection. Combinational use of CXCR4 antagonists with CCR5 antagonists has shown potent synergism against a 1:1 mixture of X4 and R5 strains *in vitro* [81]. In addition, combination of CXCR4 antagonists with fusion inhibitors might improve clinical chemotherapy, and their possible time in AIDS therapy is a critical question. Highly active antiretroviral therapy (HAART) involving the use of a cocktail of reverse transcriptase inhibitors and protease inhibitors should be the first choice in therapy, although other drugs such as a fusion inhibitor, an integrase inhibitor and a CCR5 antagonist have been developed recently. Loss of efficacy of HAART owing to the emergence of multi-drug resistant strains requires change of regimens of the drug combination and monitoring of the virus and CD4 in blood including cellular tropism testing. In this situation, new and potent anti-HIV drugs that target cellular proteins used by HIV as it enters the cell might be promising for chemotherapy following HAART. Entry inhibitors, such as CCR5/CXCR4 antagonists and fusion inhibitors, might be optional agents for an expansion of the drug repertoire available to patients at all stages of HIV infection. CCR5/CXCR4 antagonists are also worthy of attention as the first anti-HIV drugs that act on host cells, rather than on viral components.

As anticancer agents, CXCR4 antagonists that block the CXCL12/CXCR4 interactions might represent a novel and useful chemotherapy of cancer metastasis and leukemia. CXCR4 antagonists have a characteristic advantage in that they can overcome CXCL12-mediated CAM-DR.

As anti-RA agents, CXCR4 antagonists are highly promising and might be useful for alternative clinical RA therapy, which does not target inflammatory cytokines that are correlated to the CXCL12–CXCR4 axis. CXCR4 antagonists might suppress RA by the blockade of the homing of inflammatory cells such as memory T cells to arthritis joints.

CXCR4 antagonists might be useful as agents for mobilization of hemopoietic stem cells from the bone marrow [82]. The interaction between CXCL12 and CXCR4 is involved in the retention of stem cells in the bone marrow, and blocking this axis results in mobilization of stem cells. AMD3100 induces rapid mobilization of mouse and human hemopoietic stem cells [83] and also adverse cardiovascular effects. Its use as an anti-AIDS drug has been discontinued but its development as an agent for stem cell mobilization continues [84]. T140 related compounds function as inverse

agonists against CXCR4, whereas AMD3100 is a partial agonist. The T140 analogues have no CXCL12-like activity and thus do not migrate or activate various cancer cells and rheumatoid T cells that highly express CXCR4. Thus, these analogues might be suitable as anticancer metastasis, anti-leukemia and anti-RA agents. Tetradecamer peptides such as the T140 analogues might be promising drugs for s.c. injection if drug delivery systems such as PLA microcapsules can be improved. Reduction of the molecular size and peptide character has been investigated to develop FC131 analogues and linear small molecules involving Zinc-Dpa compounds. Further downsizing and reduction of the peptide character of these compounds are thought to be critical for the development of orally bioavailable drugs. Large antagonists seem to cover wide extracellular regions of CXCR4, compared with small antagonists, and thus, might be responsible for the difficulty of HIV entry and the rarity of appearance of drug-resistant viruses [47]. Linear and cyclic antagonists of either type benefit from conformational restriction among the pharmacophoric fragments, although cyclic compounds may have too high a molecular weight. CXCR4 antagonists such as T140, FC131, Zinc-Dpa compounds, AMD3100 and KRH-1636 have positively charged areas in their structures, which allow electrostatic interactions with negative-charged regions of CXCR4, and they have aromatic moieties as common features. Hence, hydrophobic interactions with CXCR4 may also be important. Although docking of CXCR4 with T140 or AMD3100 has been provided [85], precise complex structures are required for the design of new leads based on combination of the above common features. The structures common to these known antagonists will be useful in the design of more effective agents.

Acknowledgements

The authors acknowledge their collaborators: N Yamamoto (National Institute of Infectious Diseases), H Nakashima (St. Marianna University), H Mitsuya (Kumamoto University), T Hattori (Tohoku University), M Waki (Kyushu University), R Doi (Kyoto University), M Imamura (Kyoto University), Y Tanaka (University of the Ryukyus), A Otaka (The University of Tokushima), I Hamachi (Kyoto University), LJ Bendall (University of Sydney), JO Trent (University of Louisville), SC Peiper (Medical College of Georgia), T Murakami (National Institute of Infectious Diseases), T Mori (Kyoto University), M Takenaga (St. Marianna University), R Igarashi, (St. Marianna University), Z Wang (Medical College of Georgia), JA Burger (Freiburg University), M Burger (Freiburg University), ACW Zannettino (University of Adelaide), E Piovan (University of Padua), JG Cyster (University of California San Francisco), J Zheng (University of Nebraska Medical Center), N Heveker (Universite de Montreal), H Xiong (University of Nebraska Medical Center), M Retz (University of California San Francisco), S Kusano (St. Marianna University), S Terakubo (St. Marianna University), A Ojida (Kyoto University), S Oishi (Kyoto University), S Ueda (Kyoto University), J Komano (National Institute of Infectious Diseases), K Ohba (National Institute of Infectious Diseases), K Hiramatsu (Kyoto University), T Araki (Kyoto University), B Evans (Medical College of Georgia), Y Tanabe (Tokyo Medical and Dental University), A Omagari (Kyoto University), A Esaka (Kyoto University) and N Ohashi (Tokyo Medical and Dental University).

Declaration of interest

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and the Ministry of Health, Labor and Welfare, Japan, and a 21st Century COE Program 'Knowledge Information Infrastructure for Genome Science'.