ARTICLE

OBC
www.rsc.org/obc

Structure–activity relationship studies on CXCR4 antagonists having cyclic pentapeptide scaffolds†

Hirokazu Tamamura,*[a,b] Ai Esaka,[b] Teppei Ogawa,[b] Takanobu Araki,[b] Satoshi Ueda,[b] Zixuan Wang,[c] John O. Trent,[d] Hiroshi Tsutsumi,[a] Hiroyuki Masuno,[a] Hideki Nakashima,[e] Naoki Yamamoto,[f] Stephen C. Peiper,[c] Akira Otaka[b,g] and Nobutaka Fujii*[b]

[a] *Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, Chiyoda-ku, Tokyo, 101-0062, Japan. E-mail: tamamura.mr@tmd.ac.jp; Fax: +81 3 5280 8039; Tel: +81 3 5280 8036*

[b] *Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto, 606-8501, Japan. E-mail: nfujii@pharm.kyoto-u.ac.jp; Fax: +81 75 753 4570; Tel: +81 75 753 4551*

[c] *Medical College of Georgia, Augusta, GA, 30912, USA*

[d] *James Graham Brown Cancer Center, University of Louisville, Louisville, KY, 40202, USA*

[e] *St. Marianna University, School of Medicine, Miyamae-ku, Kawasaki, 216-8511, Japan*

[f] *AIDS Research Center, National Institute of Infectious Diseases, Shinjuku-ku, Tokyo, 162-8640, Japan*

[g] *Graduate School of Pharmaceutical Sciences, The University of Tokushima, Tokushima, 770-8505, Japan*

Received 19th September 2005, Accepted 26th October 2005
First published as an Advance Article on the web 15th November 2005

Structure–activity relationship studies on CXCR4 antagonists, which were previously found by using cyclic pentapeptide libraries, were performed to optimize side-chain functional groups, involving conformationally constrained analogues. In addition, a new lead of cyclic pentapeptides with the introduction of a novel pharmacophore was developed.

Introduction

Chemokine receptors belong to a superfamily of seven transmembrane G-protein coupled receptors (7TM-GPCRs). An axis of a chemokine receptor, CXCR4, and its endogenous ligand, stromal cell-derived factor-1 (SDF-1/CXCL12),[1] has multiple important functions in normal physiology involving the migration of progenitors during embryologic development of the cardiovascular, hemopoietic and central nervous systems. This axis has been also recognized to be involved in several pathological conditions, such as HIV infection,[2] cancer metastasis/progression[3] and rheumatoid arthritis (RA).[4] Initially, CXCR4 was identified as a co-receptor that is used in the entry of T cell line-tropic (X4-) HIV-1 into T cells.[2] Subsequently, several papers reported that malignant cells from different types of cancer express CXCR4,[5] and that CXCL12 is highly expressed in the major metastatic destinations of the corresponding cancer,[3] suggesting that the interaction between CXCR4 and CXCL12 might determine the metastatic destination of cancer cells and cause organ preferential metastasis. Furthermore, Nanki *et al.* reported that CXCL12, which is highly expressed in the synovium of RA patients, stimulates migration of the memory T cells, which highly express CXCR4, thereby inhibits T cell apoptosis and leads to T cell accumulation in the RA synovium.[4a] Thus, CXCR4 is thought to be a great therapeutic target. A 14-mer peptide T140 and its analogues were previously found to be specific CXCR4 antagonists that were characterized as HIV-entry inhibitors,[6] anti-cancer-metastatic agents[3c] and anti-RA agents.[4b] The utilization of cyclic pentapeptide libraries involving the critical residues of T140, which were previously identified to be Arg^2, L-3-(2-naphthyl)alanine $(Nal)^3$, Tyr^5 and Arg^{14},[7] led to the finding of a cyclic pentapeptide FC131 [*cyclo*(-Arg^1-Arg^2-Nal^3-Gly^4-D-Tyr^5-)], which has strong CXCR4 antagonistic activity, comparable to that of T140 (Fig. 1).[8] Several FC131 analogues constrained or modified in Arg^1 were synthesized to find useful leads.[9] In this paper, we describe structure–activity relationship (SAR) studies on FC131 based on several synthetic analogues, which involve substitution for Arg^2, Nal^3 and D-Tyr^5. In addition, we attempt to incorporate a new pharmacophore such as a 4-fluorophenyl moiety, which was previously identified by the *N*-terminal modification of T140 analogs,[10] into cyclic pentapeptides.

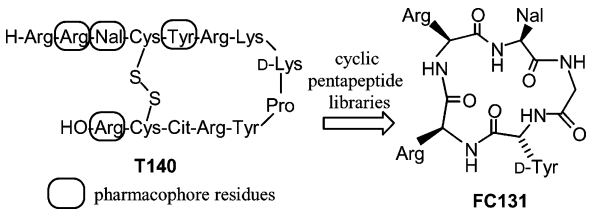


Fig. 1 Development of a low molecular weight CXCR4 antagonist FC131 based on cyclic pentapeptide libraries. Cit = L-citrulline.

Chemistry

Each peptide was synthesized in a general manner.[8] In the synthesis of compounds **6**, **7**, **9** and **10**, after cyclization and deprotection, *N*-guanylation of the resulting free side-chain amino group was performed with 1*H*-pyrazole-1-carboxamidine hydrochloride and DIPEA.[9]

Biological results and discussion

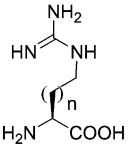
Several FC131 analogues, which have substitution for Arg^2, Nal^3 and D-Tyr^5, were prepared and assessed for CXCR4-binding activity based on inhibitory activity against CXCL12 binding to CXCR4.[11] First, analogues modified in the peripheral region of

† Electronic supplementary information (ESI) available: Characterization data (MS) of novel synthetic compounds. See DOI: 10.1039/b513145f

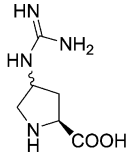
DOI:10.1039/b513145f

Table 1 Inhibitory activity of cyclic pentapeptides involving substitution for Arg^2 in FC131 against CXCL12 binding to CXCR4

Compd	*cyclo*(-Arg^1-**X**-Nal^3-Gly^4-D-Tyr^5-) X	IC_{50}/μM[a]
1 (FC131)	Arg	0.0079
2	Ala	>1
3	Dab	0.44
4	Orn	0.69
5	Lys	>1
6	g-Dab	1.1
7	g-Lys	0.033
8	Glu	>1
9	*trans*-4-Guanidino-Pro	>1
10	*cis*-4-Guanidino-Pro	>1



n = 1 γ-*N*-amidino-Dab (g-Dab)
n = 3 ε-*N*-amidino-Lys (g-Lys)



trans/*cis*-4-guanidino-Pro

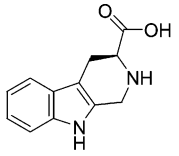
[a] IC_{50} values are based on the inhibition of [^{125}I]-CXCL12 binding to CXCR4 transfectants of CHO cells. All data are mean values for at least three independent experiments.

Arg^2 were assayed (Table 1). Ala-substitution for Arg^2 in FC131 completely diminished the activity of the parent compound, whereas Ala-substitution for Arg^1 did not cause a severe decrease in potency,[9] suggesting that the side-chain of Arg^2 is very important for strong activity. Thus, optimization of the side-chain of Arg^2 was attempted by the synthesis of several analogues, where Arg^2 was replaced by Arg/Lys mimetics having various lengths of alkyl chains. L-2,4-Diaminobutyric acid (Dab)/L-ornithine (Orn)-substituted analogues, **3** and **4**, showed moderate CXCR4-binding activity, which is two orders of magnitude less potent than that of FC131, while a Lys-substituted analogue **5** did not show any significant activity until 1 μM. An ε-*N*-amidino-Lys (g-Lys)-substituted analogue, **7**, which has the side-chain with a one-carbon elongation compared to Arg^2, showed significant CXCR4-binding activity, which is 4-fold weaker than FC131. A γ-*N*-amidino-Dab (g-Dab)-substituted analogue, **6**, which has the side-chain with a one-carbon reduction compared to Arg^2, showed very low activity. It suggests that Arg is the most suitable at position 2 among the Arg/Lys mimetics used in this study. A Glu-substituted analogue, **8**, did not show any significant activity until 1 μM, suggesting that a basic functional group, such as an amino or guanidino group, in the side-chain of the amino acid at position 2 is indispensable for binding to CXCR4. In our previous study, analogues, in which a conformationally constrained Arg mimetic, *trans*- or *cis*-4-guanidino-Pro, was incorporated at position 1, showed higher CXCR4-binding activity than a g-Dab-substituted analogue, having the same length of the linear-type side chain of the amino acid at position 1.[9] Thus, in this study, analogues, in which *trans*- or *cis*-4-guanidino-Pro was incorporated at position 2, were prepared and assessed for CXCR4-binding activity. However, the conformationally constrained analogues, **9** and **10**, did not show any significant activity until 1 μM. This proved that fixing the backbone and the side-chain of Arg^2 is not suitable.

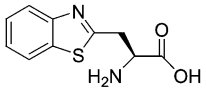
Second, analogues modified in the peripheral region of Nal^3 were assayed (Table 2). Ala-substitution for Nal^3 in FC131 completely diminished the activity of the parent compound. Since the side-chain of Nal^3 is indispensable for strong activity, optimization of the side-chain of Nal^3 was attempted by the synthesis of several analogues, where Nal^3 was replaced by Trp mimetics. A Trp-substituted analogue, **12**, showed strong CXCR4-binding activity, which is slightly less potent than

Table 2 Inhibitory activity of cyclic pentapeptides involving substitution for Nal^3 in FC131 against CXCL12 binding to CXCR4

Compd	*cyclo*(-Arg^1-Arg^2-**X**-Gly^4-D-Tyr^5-) X	IC_{50}/μM
1 (FC131)	Nal	0.0079
11	Ala	>1
12	Trp	0.013
13	Tpi	>1
14	Bth	0.018
15	D-Bth	0.26



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



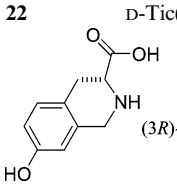
(2*S*)-2-amino-3-benzothiazol-2-yl-propionic acid (Bth)

that of FC131. This is compatible with our previous result: T140 is more potent than T134 [Trp^3-T140].[6] A (3*S*)-2,3,4,9-tetrahydro-1*H*-β-carboline-3-carboxylic acid (Tpi)-substituted analogue, **13**, which is conformationally constrained in the backbone and the side-chain of the amino acid at position 3, did not show any significant activity until 1 μM, suggesting that fixing the backbone and the side-chain of Trp (or Nal^3) is not suitable. A (2*S*)-2-amino-3-benzothiazol-2-yl-propionic acid (Bth)-substituted analogue, **14**, showed strong CXCR4-binding activity, which is almost the same as that of the Trp-substituted analogue, **12**. D-Bth-substituted analogue, **15**, is 14-fold less potent than **14**. This is also compatible with our previous result: D-Nal^3-FC131 is 20-fold less potent than FC131.[8] Taken together, Nal is more suitable at position 3 than any other Trp-mimetics.

Third, analogues modified in the peripheral region of D-Tyr^5 were assayed (Table 3). D-Ala-substitution for D-Tyr^5 in FC131 also diminished the activity of the parent compound. Optimization of the side-chain of D-Tyr^5 was attempted by the synthesis of several analogues, where D-Tyr^5 was replaced by D-Tyr/Phe mimetics. A D-Phe(4-NH_2)-substituted analogue, **17**, and a D-Phe(4-OMe)-substituted analogue, **18**, which have electron-donating substituents on the aromatic ring of the amino acid at position 5, showed remarkably less potent CXCR4-binding activity than FC131, which also has an electron-donating substituent on the aromatic ring. The analogues, **17** and **18**, were weaker than a D-Phe-substituted analogue,

Table 3 Inhibitory activity of cyclic pentapeptides involving substitution for D-Tyr^5 in FC131 against CXCL12 binding to CXCR4

Compd	*cyclo*(-Arg^1-Arg^2-Nal^3-Gly^4-**X**^{5}-) X	IC_{50}/μM
1 (FC131)	D-Tyr	0.0079
16	D-Ala	>1
17	4-Amino-D-phenylalanine [D-Phe(4-NH_2)]	0.10
18	4-Methoxy-D-phenylalanine [D-Phe(4-OMe)]	0.51
19	D-His	0.15
20	D-Phe	0.051
21	4-Fluoro-D-phenylalanine [D-Phe(4-F)]	0.22
22	D-Tic(7-OH)	0.16



(3*R*)-7-Hydroxy-1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid [D-Tic(7-OH)]

Table 4 Inhibitory activity of cyclic pentapeptides involving the incorporation of Phe(4-F)[1] into FC131 against CXCL12 binding to CXCR4

Compd	Sequence	IC_{50}/μM
1 (FC131)	*cyclo*(-Arg[1]-Arg[2]-Nal[3]-Gly[4]-D-Tyr[5]-)	0.0079
23	*cyclo*(-Phe(4-F)[1]-Arg[2]-Nal[3]-Gly[4]-D-Tyr[5]-)	0.057
24	*cyclo*(-Phe(4-F)[1]-Arg[2]-Nal[3]-Gly[4]-Arg[5]-)	0.62
25	*cyclo*(-D-Phe(4-F)[1]-Arg[2]-Nal[3]-Gly[4]-Arg[5]-)	0.035
26	*cyclo*(-Phe(4-F)[1]-Arg[2]-Nal[3]-Gly[4]-D-Arg[5]-)	0.088
27	*cyclo*(-D-Phe(4-F)[1]-Arg[2]-Nal[3]-Gly[4]-D-Arg[5]-)	0.094
28	*cyclo*(-D-Tyr[1]-Arg[2]-Nal[3]-Gly[4]-Arg[5]-)	0.30

20. Thus, an electron-donating substituent on the aromatic ring of the amino acid at position 5 is not always suitable for strong CXCR4-binding activity. On the other hand, a D-Phe(4-F)-substituted analogue, **21**, which has an electron-withdrawing substituent on the aromatic ring, was also less potent than FC131 or the D-Phe-substituted analogue, **20**. A D-His-substituted analogue, **19**, which has a basic/aromatic amino acid at position 5, did not show stronger activity than **20**. A (3*R*)-7-hydroxy-1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid [D-Tic(7-OH)]-substituted analogue, **22**, which is conformationally constrained in the backbone and the side-chain of the amino acid at position 5, showed remarkably less potent CXCR4-binding activity than FC131, suggesting that fixing the backbone and the side-chain of D-Tyr[5] is also not suitable. Taken together, D-Tyr is the most suitable at position 5 among the tested amino acids without any relation to the electron-withdrawing or -donating effect of the substituent on the aromatic ring.

Recently, a novel pharmacophore of T140-related CXCR4 antagonists, such as a 4-fluorophenyl moiety, was found in addition to the original pharmacophores of T140, Arg (x 2), Nal and Tyr.[10] Fourth, since the phenol group of D-Tyr[5] could not be replaced by the 4-fluorophenyl group with maintenance of high activity, as seen in the D-Phe(4-F)-substituted analogue, **21**, we attempted to incorporate the 4-fluorophenyl group into the amino acid at position 1. [Phe(4-F)[1]]-FC131, **23**, showed significant CXCR4-binding activity, which is less potent than that of FC131. Since another Arg residue is thought to be indispensable for high activity and an aromatic residue [L/D-Phe(4-F)] is incorporated into position 1, we tried to replace D-Tyr[5] by L/D-Arg[5]. Four analogues, **24**–**27**, [L/D-Phe(4-F)[1], L/D-Arg[5]]-FC131, were prepared and assayed (Table 4). Among these compounds [D-Phe(4-F)[1], Arg[5]]-FC131, **25**, showed the most potent activity, which is 10-fold more potent than that of [D-Tyr[1], Arg[5]]-FC131, **28**. Thus, it is thought that [D-Phe(4-F)[1], Arg[5]]-FC131, **25**, is useful as a novel lead involving the pharmacophores different from FC131, although **25** is 4-fold less potent than FC131.

Conclusion

In summary, SAR studies on cyclic pentapeptides having CXCR4-antagonistic activity, such as FC131, were performed. Several analogues were synthesized to optimize side-chain functional groups, involving constrained analogues that conformationally fix the backbone and the side-chains. Taken together, Arg, Nal and D-Tyr are the most suitable at position 2, 3 and 5, respectively, than any other corresponding amino acid mimetics that were tested in the present study. Furthermore, a novel lead compound, which contains a 4-fluorophenyl group as the new pharmacophore, was found.

Acknowledgements

This work was supported in part by a 21st Century COE Program "Knowledge Information Infrastructure for Genome Science", a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan, the Japan Health Science Foundation and Philip Morris USA Inc. and Philip Morris International. S. U. is grateful for a Research Fellowship from the Japan Society for the Promotion of Science for Young Scientists.

References

1 (*a*) T. Nagasawa, H. Kikutani and T. Kishimoto, *Proc. Natl. Acad. Sci. U. S. A.*, 1994, **91**, 2305; (*b*) C. C. Bleul, M. Farzan, H. Choe, C. Parolin, I. Clark-Lewis, J. Sodroski and T. A. Springer, *Nature*, 1996, **382**, 829; (*c*) E. Oberlin, A. Amara, F. Bachelerie, C. Bessia, J.-L. Virelizier, F. Arenzana-Seisdedos, O. Schwartz, J.-M. Heard, I. Clark-Lewis, D. F. Legler, M. Loetscher, M. Baggiolini and B. Moser, *Nature*, 1996, **382**, 833; (*d*) K. Tashiro, H. Tada, R. Heilker, M. Shirozu, T. Nakano and T. Honjo, *Science*, 1993, **261**, 600.

2 Y. Feng, C. C. Broder, P. E. Kennedy and E. A. Berger, *Science*, 1996, **272**, 872.

3 (*a*) T. Koshiba, R. Hosotani, Y. Miyamoto, J. Ida, S. Tsuji, S. Nakajima, M. Kawaguchi, H. Kobayashi, R. Doi, T. Hori, N. Fujii and M. Imamura, *Clin. Cancer Res.*, 2000, **6**, 3530; (*b*) A. Müller, B. Homey, H. Soto, N. Ge, D. Catron, M. E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S. N. Wagner, J. L. Barrera, A. Mohar, E. Verastegui and A. Zlotnik, *Nature*, 2001, **410**, 50; (*c*) H. Tamamura, A. Hori, N. Kanzaki, K. Hiramatsu, M. Mizumoto, H. Nakashima, N. Yamamoto, A. Otaka and N. Fujii, *FEBS Lett.*, 2003, **550**, 79; (*d*) N. Tsukada, J. A. Burger, N. J. Zvaifler and T. J. Kipps, *Blood*, 2002, **99**, 1030; (*e*) J. Juarez, K. F. Bradstock, D. J. Gottlieb and L. J. Bendall, *Leukemia*, 2003, **17**, 1294.

4 (*a*) T. Nanki, K. Hayashida, H. S. EI-Gabalawy, S. Suson, K. Shi, H. J. Girschick, S. Yavuz and P. E. Lipsky, *J. Immunol.*, 2000, **165**, 6590; (*b*) H. Tamamura, M. Fujisawa, K. Hiramatsu, M. Mizumoto, H. Nakashima, N. Yamamoto, A. Otaka and N. Fujii, *FEBS Lett.*, 2004, **569**, 99.

5 F. Balkwill, *Semin. Cancer Biol.*, 2004, **14**, 171.

6 H. Tamamura, Y. Xu, T. Hattori, X. Zhang, R. Arakaki, K. Kanbara, A. Omagari, A. Otaka, T. Ibuka, N. Yamamoto, H. Nakashima and N. Fujii, *Biochem. Biophys. Res. Commun.*, 1998, **253**, 877.

7 H. Tamamura, A. Omagari, S. Oishi, T. Kanamoto, N. Yamamoto, S. C. Peiper, H. Nakashima, A. Otaka and N. Fujii, *Bioorg. Med. Chem. Lett.*, 2000, **10**, 2633.

8 N. Fujii, S. Oishi, K. Hiramatsu, T. Araki, S. Ueda, H. Tamamura, A. Otaka, S. Kusano, S. Terakubo, H. Nakashima, J. A. Broach, J. O. Trent, Z. Wang and S. C. Peiper, *Angew. Chem., Int. Ed.*, 2003, **42**, 3251.

9 H. Tamamura, T. Araki, S. Ueda, Z. Wang, S. Oishi, A. Esaka, J. O. Trent, H. Nakashima, N. Yamamoto, S. C. Peiper, A. Otaka and N. Fujii, *J. Med. Chem.*, 2005, **48**, 3280.

10 H. Tamamura, K. Hiramatsu, M. Mizumoto, S. Ueda, S. Kusano, S. Terakubo, M. Akamatsu, N. Yamamoto, J. O. Trent, Z. Wang, S. C. Peiper, H. Nakashima, A. Otaka and N. Fujii, *Org. Biomol. Chem.*, 2003, **1**, 3663.

11 H. Tamamura, K. Hiramatsu, S. Ueda, Z. Wang, S. Kusano, S. Terakubo, J. O. Trent, S. C. Peiper, N. Yamamoto, H. Nakashima, A. Otaka and N. Fujii, *J. Med. Chem.*, 2005, **48**, 380.