(i)

(ii)

(iii)

Figure 1. Various disorders relevant to the CXCL12-CXCR4 axis, such as HIV infection (i), cancer cell metastasis (ii) and rheumatoid arthritis (iii).

(Alkhatib et al. 1996; Choe et al. 1996; Deng et al. 1996; Doranz et al. 1996; Dragic et al. 1996). However, X4 HIV-1 strains become dominant in the late 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 has been shown that the CXCL12-CXCR4 axis is associated to metastasis of several types of cancer including cancer of the pancreas, breast, lung, kidney, and prostate and non-Hodgkin's lymphoma, neuroblastoma, melanoma, ovarian cancer, multiple myeloma and malignant brain tumors. Furthermore, this axis is also correlated to the progression of chronic lymphocytic leukemia (CLL) and acute lymphoblastic leukemia (ALL), and acute myeloid leukemia (AML). In addition, rheumatoid arthritis (RA) is caused mainly by CD4$^+$ memory T cell accumulation in the inflamed synovium. It was reported that CXCL12 concentration is extremely elevated in the synovium of RA

patients, and that CXCR4 is highly expressed on the surface of memory T cells. Further, CXCL12 stimulates migration of the memory T cells and thereby inhibits T cell apoptosis. This indicates that the CXCR4-CXCL12 interaction plays an essential role in the accumulation of T cells in the RA synovium. Taken together, CXCR4 is an attractive therapeutic target for these diseases, and our recent research concerning the development of several CXCR4 antagonists including downsizing is discussed in this review article.

Development of CXCR4 Antagonists as Selective Inhibitors of X4-HIV-1 Entry

Self-defense peptides with antibacterial and antiviral activities, tachyplesins and polyphemusins, have been isolated from the hemocyte debris of the Japanese horseshoe crab (*Tachypleus tridentatus*) and the American horseshoe crab (*Limulus polyphemus*), which are 17-mer and 18-mer peptides, respectively (Fig. 2) (Nakamura et al. 1988; Miyata et al. 1989). Our preliminary structure-activity relationship studies of these peptides led to the development of T22 ([Tyr5,12, Lys7]-polyphemusin II) (Masuda et al. 1992; Nakashima et al. 1992) and its downsized 14-mer peptide, T140, which possess strong anti-HIV activity (Fig. 2) (Tamamura et al. 1998). T22 and T140 effectively

block X4-HIV-1 entry into cells by binding specifically to CXCR4, and inhibit Ca2+ mobilization caused by CXCL12 stimulation against CXCR4 (Murakami et al. 1997; Xu et al. 1999; Murakami et al. 1999). In addition, a T140 analog exhibited a remarkable and significant delaying of the appearance of drug-resistant strains of HIV in passage experiments using cell cultures *in vitro* (Kanbara et al. 2001), and it was presumed that the T140 analogs would be useful for its suppressive effect against drug-resistant strains. 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 (Tamamura, Sugioka et al. 2001). Four amino acid residues that were contained in T140, Arg2, L-3-(2-naphthyl)alanine (Nal)3, Tyr5 and Arg14, were identified as residues indispensable for significant activity (Tamamura et al. 2000).

However, T140 is proven to be biologically unstable, and biodegradable in mouse/feline serum or in rat liver homogenate (Tamamura, Omagari et al. 2001; Tamamura, Hiramatsu, Kusano et al. 2003). When indispensable amino acid residues (Arg[14] in serum; Arg[2], Nal[3] and Arg[14] in liver homogenate) are deleted from the N- and the C-termini, the efficacy of degraded peptides is dramatically reduced. Modification of T140 analogs at both termini efficiently suppresses the above biodegradations and leads to development of novel
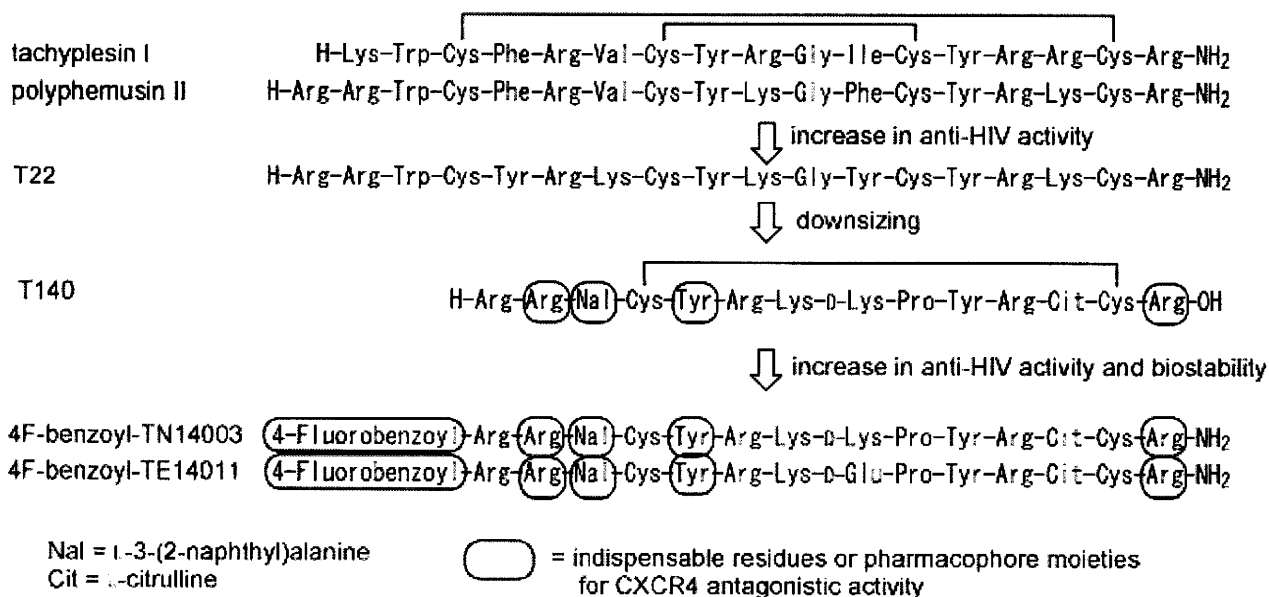

Figure 2. Structures of tachyplesin I, polyphemusin II, its analog T22, its downsized analog T140, its biostable analogs 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011.

and effective compounds that show highly CXCR4-antagonistic activity as well as increased biological stability. Further studies on the *N*-terminal modification found an electron-deficient aromatic ring such as a 4-fluorobenzoyl moiety at the *N*-terminus to constitute a novel pharmacophore for strong anti-HIV activity. The T140 analogs, which contain an *N*-terminal 4-fluorobenzoyl moiety, 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011, have anti-HIV activity two orders of magnitude higher than that of T140 and enhanced biostability in serum/liver homogenates (Fig. 2) (Tamamura, Hiramatsu, Mizumoto et al. 2003).

Cyclic Peptides with CXCR4 Antagonistic Activity Derived from T140

Arg^2, Nal^3, Tyr^5 and Arg^{14} of T140, which are located in close proximity to each other in space,

are indispensable to high antagonistic activity against CXCR4 as described above. For downsizing of T140 analogs, a pharmacophore-guided approach was performed using cyclic pentapeptide libraries, which were composed of two L/D-Arg, L/D-Nal and L/D-Tyr in addition to Gly as a spacer. This approach led to FC131 [$cyclo$(-Arg^1-Arg^2-Nal^3-Gly^4-D-Tyr^5-)], which showed strong CXCR4-antagonistic activity comparable to that of T140 (Fig. 3) (Fujii et al. 2003). Structural analysis of FC131 by NMR and simulated annealing molecular dynamics revealed the near-symmetrical pentagonal backbone structure.

A 4-fluorophenyl moiety found as a pharmacophoric moiety as described in the above section was introduced into cyclic pentapeptides. Since replacement of the phenol group of D-Tyr^5 by a 4-fluorophenyl group did not cause the maintenance of high potency, the 4-fluorophenyl group
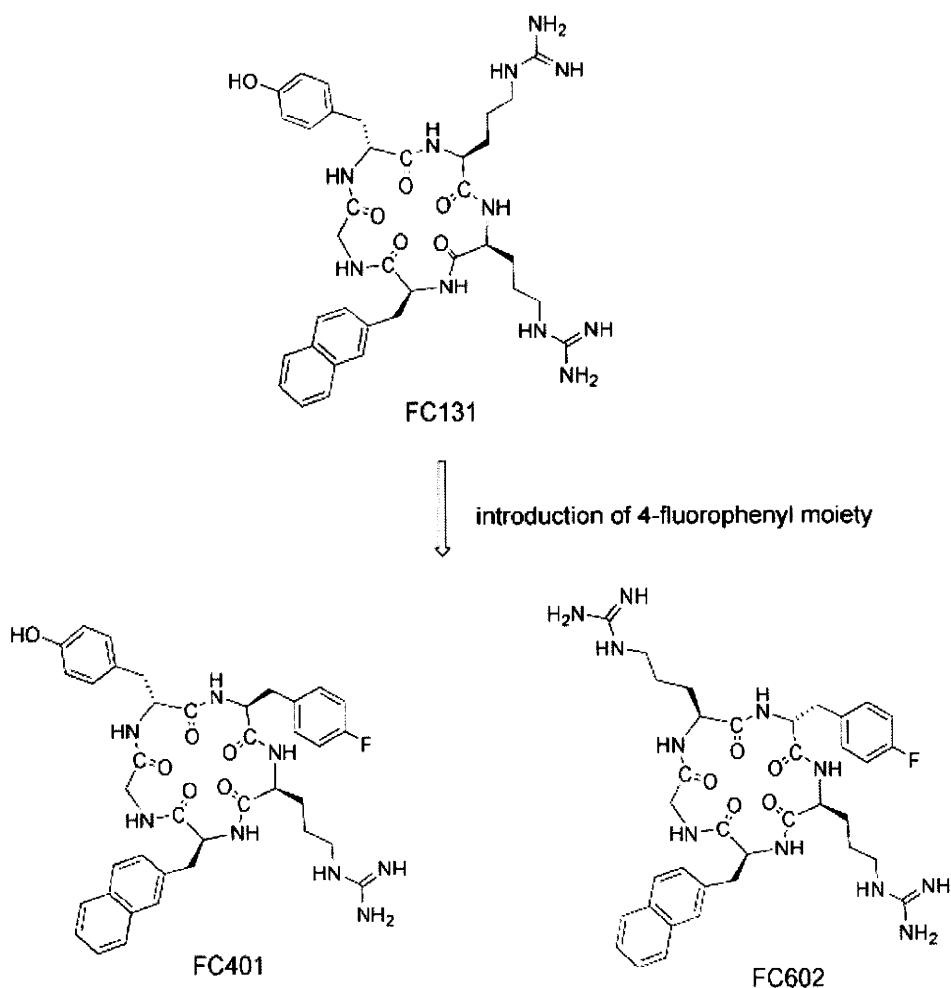

FC131

introduction of 4-fluorophenyl moiety

FC401

FC602

Figure 3. Structures of cyclic pentapetides FC131, FC401 and FC602.

was incorporated into position 1. The resulting compound, FC401 ([Phe(4-F)[1]]-FC131), shows significant CXCR4-binding activity (Fig. 3) (Tamamura et al. 2005). Next, since a second Arg residue is thought to be indispensable for high potency and an aromatic residue [L/D-Phe(4-F)] has been incorporated into position 1, four analogs [L/D-Phe(4-F)[1], L/D-Arg[5]]-FC131 were synthesized based on replacement of D-Tyr[5] by L/D-Arg[5]. Among these analogs, FC602, which is [D-Phe(4-F)[1], Arg[5]]-FC131, shows the most potent activity, which is 10-fold greater than that of [D-Tyr[1], Arg[5]]-FC131 (Fig. 3). Thus, FC602 is a novel lead, which involves a pharmacophore moiety different from the pharmacophore groups of FC131.

A Linear Type of Low Molecular Weight CXCR4 Antagonists

Identification of a novel pharmacophore for CXCR4 antagonism, such as a 4-fluorobenzoyl or 4-fluorophenyl moiety, prompted us to develop a linear type of low molecular weight CXCR4 antagonists. By combining substructure units of the T140 pharmacophore and new pharmacophore moieties, several compounds were designed and synthesized using combinatorial chemistry. As a result, several linear compounds were found as moderate CXCR4 antagonists, such as compounds 1–3 shown in Figure 4 (Tamamura, Tsutsumi et al. 2006). These compounds are relatively weaker than a cyclic pentapeptide FC131. Thus, it is thought that conformational constriction based on a cyclic pentapeptide scaffold is critical for strong potency.

Anthracene derivatives possessing two sets of zinc(II)-2,2'-dipicolylamine complex were previously found as useful chemosensors that can selectively bind to phosphorylated peptide surfaces (Ojida et al. 2004). Several low molecular weight compounds bearing the complex structure were identified as selective CXCR4 antagonists (Tamamura, Ojida et al. 2006). Molecular superposition of structures of the zinc(II)-2,2'-dipicolylamine complex compound 4 and the cyclic pentapeptide FC131 was investigated as it provided the best fit with the maintenance of local energy minimizations of both of the structures (Fig. 4). The distance between two dipicolylamine moieties of compound 4 is estimated to be nearly equal to that between the two Arg side chains of FC131. Thus, the distance of these functional groups is thought to be
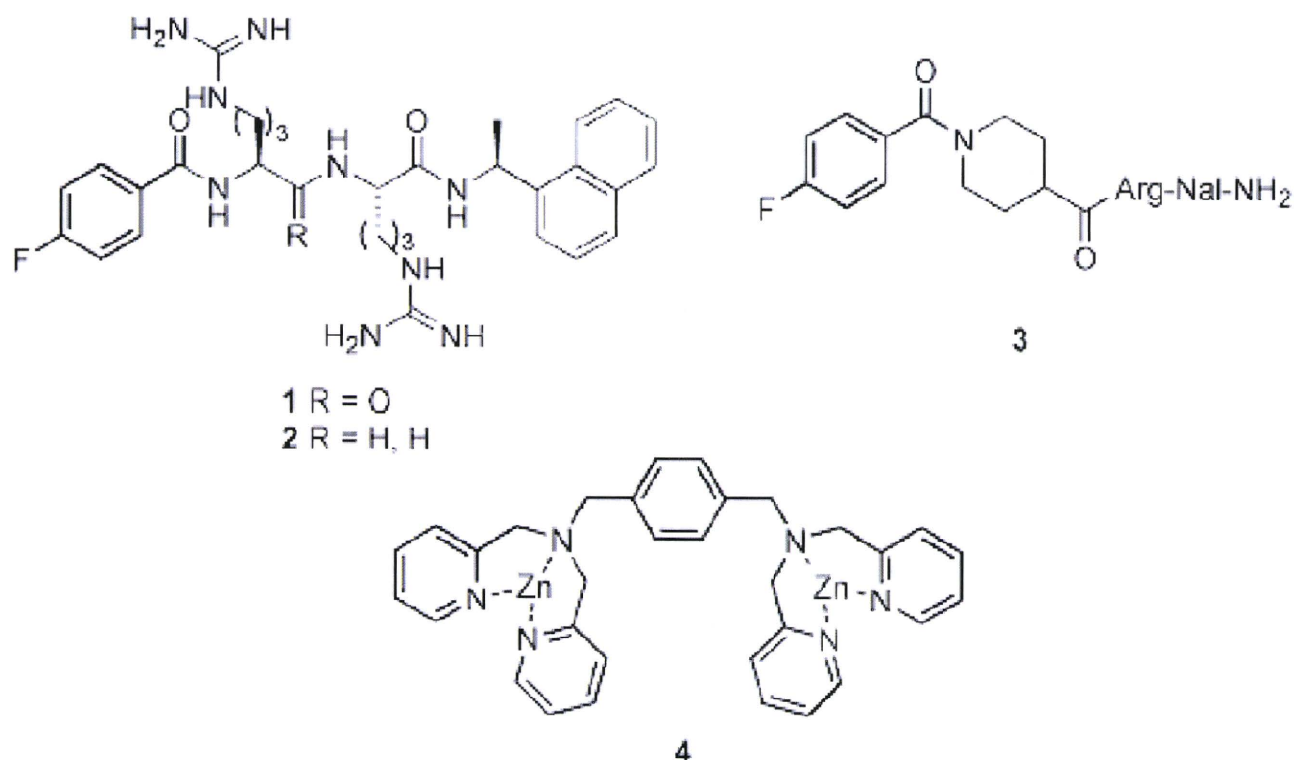

Figure 4. Structures of a linear type of low molecular weight CXCR4 antagonists.

essential for expression of CXCR4 antagonistic activity.

Anti-Metastasis Activity of T140 Analogs Against Breast Cancer, Melanoma and Pancreatic Cancer

While CXCR4 and another chemokine receptor, CCR7, are highly expressed on the surface of human breast cancer cells, CXCL12 and a CCR7 ligand, CCL21, are highly expressed in lymph nodes, bone marrow, lung and liver, which form the common metastatic destinations of breast cancer. The CXCL12-CXCR4/CCR7-CCL12 axis might determine the metastatic destination of tumor cells and cause organ-preferential metastasis (Müller et al. 2001). Metastasis of breast cancer cells to the lung in mice was inhibited by neutralizing CXCR4 with anti-CXCR4 antibodies. We evaluated the inhibitory activity of our T140 analogs against the migration of breast cancer cells *in vitro* and metastasis of breast cancer cells *in vivo* (Tamamura, Hori et al. 2003). T140 analogs inhibited in dose-dependent manners the migration of a CXCR4-positive human breast carcinoma cell line MDA-MB-231 induced by CXCL12. Furthermore, the inhibitory effect of a bio-stable T140 analog, 4F-benzoyl-TN14003, was confirmed using experimental metastasis models of breast cancer, in which MDA-MB-231 cells were injected intravenously into the tail vein of SCID mice and trapped in the lung to which they migrated through the heart and the pulmonary artery. 4F-benzoyl-TN14003 was subcutaneously injected using an Alzet osmotic pump (DURECT Corp., Cupertino, CA, U.S.A.), and an effective suppression of tumor accumulation was then shown on the lung surface as a result of MDA-MB-231 metastasis. This suggests that small molecule CXCR4 antagonists, such as T140 analogs, can replace anti-CXCR4 antibodies as neutralizers of metastasis of breast cancer.

It was reported that an excessive expression of CXCR4 on B16 melanoma cells enhances the metastatic accumulation of the cells in mouse lung, and that a CXCR4 antagonist T22 blocks pulmonary metastasis in mice injected with CXCR4-transduced B16 cells (Murakami et al. 2002). We found that T140 analogs inhibited pulmonary metastasis in mice injected with B16 cells, which are not transduced with CXCR4 (Takenaga et al. 2004). Poly D,L-lactic acid (PLA) microcapsules containing a T140 analog, 4F-benzoyl-TE14011,

was subcutaneously injected in experimental metastatic models of CXCR4-positive B16-BL6 melanoma cells. 4F-benzoyl-TE14011 is released in a controlled fashion from the PLA microcapsules for a long period *in vivo* with the maintenance of the level of the 4F-benzoyl-TE14011 concentration in bloods. A single subcutaneous administration of 4F-benzoyl-TE14011-PLA significantly decreases the number of colonies ascribed to pulmonary metastasis of B16-BL6 cells. Thus, a controlled release of CXCR4 antagonists might lead to effective suppression of cancer metastasis.

While CXCL12 mRNA is expressed in pancreatic cancer tissues, CXCR4 mRNA is expressed both in pancreatic cancer tissues and in pancreatic cancer cell lines (AsPC-1, BxPC-3, CFPAC-1, HPAC and PANC-1) (Koshiba et al. 2000). CXCL12 stimulates induction of both migration and invasion of pancreatic cancer cells, AsPC-1, PANC-1 and SUIT-2, in dose-dependent manners *in vitro*. Thus, it suggests that the interaction between CXCL12 and CXCR4 is relevant to pancreatic cancer cell progression and metastasis. CXCL12-induced migration and invasion of these cells are suppressed by T140 analogs in dose-dependent manners (Mori et al. 2004). CXCL12 treatment of PANC-1 cells induces a drastic increase in actin polymerization (cytoskeleton), which causes the invasion and subsequent metastasis of malignant cells into tissues. T140 analogs effectively inhibit this phenomenon.

Furthermore, the CXCL12-CXCR4 axis is relevant to metastasis of SCLC and osteolysis in multiple myeloma. Thus, the blockade of this axis might be an effective remedy against these diseases (Hartmann et al. 2005; Zannettino et al. 2005).

CXCR4 is expressed in malignant cells in at least 23 different types of cancers (Balkwill 2004), including those discussed above. Antagonists of CXCR4 such as the T140 analogs might be useful lead compounds for the development of anti-metastatic agents in several types of cancer.

Effect of T140 Analogs Against ALL and CLL

Growth and survival of ALL precursor B (pre-B) cells might be caused by 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. Migration of these

cells into stromal layers is stimulated by CXCL12, which is constitutively secreted at high levels by marrow stromal cells, since CXCR4 is highly expressed in the pre-B cells. T140 suppresses CXCL12-induced chemotaxis of the cells and attenuates their migration into bone marrow stromal layers. Furthermore, since T140 analogs enhance the cytotoxic and anti-proliferative effects of other anti-cancer agents such as vincristine and dexamethasone, T140 analogs might overcome cell adhesion-mediated drug resistance (CAM-DR) in ALL chemotherapy (Juarez et al. 2003).

On the other hand, B cell CLL, which is the most frequent 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. CLL B cells that highly express CXCR4 are activated by CXCL12 released from marrow stromal cells or nurselike cells. CXCL12-stimulation rescues the CLL B cells from apoptosis and contributes to their accumulation. Consequently, the CXCL12-CXCR4 axis should also be a therapeutic target of B cell CLL (Burger et al. 2000). Practically, T140 analogs inhibit chemotaxis of CLL B cells induced by CXCL12, their migration beneath marrow stromal cells and actin polymerization in dose-dependent manners, in vitro (Burger et al. 2005). Furthermore, T140 analogs attenuate the anti-apoptotic effect of CXCL12 and prevent stromal cells from protecting against spontaneous apoptosis of CLL B cells. Co-cultivation of CLL B cells with marrow stromal cells causes stromal CAM-DR, protecting CLL B cells from undergoing fludarabine-induced apoptosis. Treatment with T140 analogs re-sensitizes these B cells towards fludarabine-induced apoptosis T140 analogs might overcome CAM-DR which is a serious problem in the clinical CLL chemotherapy.

Anti-RA Activity of T140 Analogs

The development of biological drugs such as monoclonal antibodies, which target inflammatory cytokines: tumor necrosis factor, TNF-α, interferon, IFN-γ, the interleukins, IL-1, IL-6, etc., has brought useful results in clinical RA therapy. However, complete curative effects have not yet been achieved. Thus, other drugs, which are not relevant to the functions of these cytokines, are required to improve RA chemotherapy. Since the CXCR4-CXCL12 axis plays a critical role in the accumula-

tion of memory T cells in the RA synovium (Nanki et al. 2000), anti-RA activity of 4F-benzoyl-TN14003 was evaluated. Delayed-type hypersensitivity (DTH) reaction induced by sheep red blood cells (SRBC) was performed as an in vivo experimental model of the cellular immune response (Tamamura et al. 2004). Subcutaneous injection of 4F-benzoyl-TN14003 using an Alzet osmotic pump significantly suppressed the footpad swelling (the DTH response) in dose-dependent manners. Collagen-induced arthritis (CIA) in mice was adopted as the other in vivo experimental RA model. Several symptoms of arthritis: score increase, body weight loss, ankle swelling, limb weight gain, etc. were remarkably suppressed. Furthermore, the increase in levels of serum anti-bovine CII IgG2a antibody was apparently suppressed in mice treated with 4F-benzoyl-TN14003 subcutaneously using an Alzet osmotic pump after treatment with the bovine type II collagen (CII) emulsion booster. 4F-benzoyl-TN14003 exhibits an inhibitory effect towards the humoral immune response to CII. Thus, CXCR4 antagonists such as T140 analogs might also be useful leads for anti-RA agents.

Other CXCR4 Antagonists

Several low molecular weight CXCR4 antagonists, which are not correlated to T140, have been reported to date (Mastrolorenzo, Scozzafava and Supuran, 2001; Scozzafava, Mastrolorenzo and Supuran, 2002). AMD3100 bearing two cyclam groups (Genzyme) (Schols et al. 1997), an N-pyridinylmethylene cyclam (monocyclam) AMD3465 (Genzyme) (De Clercq, 2002), AMD8665 without a cyclam group (Genzyme) (Seibert and Sakmar, 2004), AMD070 (Genzyme) (Vermeire et al. 2004), ALX40-4C (Ac-[D-Arg]$_9$-NH2; NPS Allelix) (Doranz et al. 1997), CGP64222 (Cabrera et al. 2002), R3G (Cabrera et al. 2000), NeoR (Daelemans et al. 2000), a distamycin analog, NSC651016 (Howard et al. 1998) and a flavonoid compound, ampelopsin (Liu et al. 2004), have been identified as CXCR4 antagonists. Conjugates of AMD3100 and galactosylceramide (GalCer) analog have also been found as doubly-functionalized drugs (Daoudi et al. 2004). KRH-1636 (Kureha Chemical and Sankyo) is an orally bioavailable agent possessing N-pyridinylmethylene, Arg and naphthalene moieties (Ichiyama et al. 2003). A review of non-T140-related CXCR4 antagonists has been published elsewhere (Tamamura and Fujii, 2005).

Conclusion

We have found strong anti-HIV agents, T22 and its downsized analog T140, identified as entry inhibitors that bind specifically to the chemokine receptor CXCR4 and thus inhibit entry of X4-HIV-1 to T-cells. T140 and its analogs also show effective activity against cancer metastasis, leukemia and rheumatoid arthritis. Cyclic pentapeptide FC131 was developed as a new low molecular weight CXCR4 antagonist by downsizing of T140. Furthermore, a linear type of low molecular weight CXCR4 antagonists involving aromatic compounds having the zinc(II)-2,2'-dipicolylamine complex structure have been found. These antagonists might be promising agents for clinical chemotherapy of multiple disorders such as HIV infection, cancer metastasis, leukemia and RA. However, blocking of the CXCL12-CXCR4 axis might be a risky procedure because 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. Use of CXCR4 antagonists combined with CCR5 antagonists/fusion inhibitors might lead to a useful candidate for clinical AIDS chemotherapy.

Acknowledgements

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Fluorescent Labeling for PKC delta C1b Domain and Its Application to Sensing Biology

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In finding drug lead compounds, development of screening methods, which would be applicable to high-throughput screenings, is an interesting topic. Furthermore, these techniques have potential, which would be used for sensing biological phenomena. This study is focused on the development of an assay method using fluorescence, which targets for PKC that plays important roles in cell signaling pathways and interacts with tumor promoters such as phorbol esters. Herein, the synthesis of ligand binding domains with fluorescent-labeling and their applications are reported.

Keywords: C1 domain, fluorescent-labeling, ligand screening, native chemical ligation, protein kinase C

Introduction

Protein kinase C (PKC) is phospholipid-dependent serine/threonine-specific protein kinases. In physiological conditions, PKC plays fundamental roles in signaling pathways that regulate cell cycle progression, differentiation, and apoptosis. PKC is also recognized as a receptor for tumor promoters such as phorbol esters. To date, eleven isozymes have been identified for PKC. Each isozyme comprises a catalytic domain and a regulatory domain that contains C1 domains, which form zinc finger structures. PKC activation depends on the binding of ligands such as a diacylglycerol (DAG) and phorbol esters [1]. C1b domains are fairly conserved among the family and known as targets for these ligands. To study the binding properties of currently synthetic ligands or to screen the suitable ligand candidates from vast numbers of compound libraries, a new system for detection of the ligand binding with efficiency and safety (without radio-isotopes) is demanded. Recently, fluorescent dyes are recognized as convenient markers to study the biological functions of proteins or cellular functions. In this study, we have synthesized fluorescent-labeled PKCδ C1b domain analogs by normal Fmoc solid phase peptide synthesis (Fmoc-SPPS) and

native chemical ligation (NCL). These fluorescent-labeled domains were characterized as molecular markers for ligand bindings (Fig. 1).
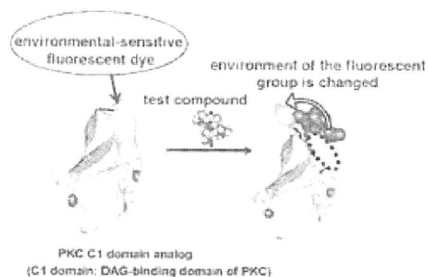

Fig. 1. Plausible detection of ligand binding by fluorescent-labeled PKC C1b domain analogs.

Results and Discussion

For the C1b domain of PKCδ (221-281), three amino acids (tyrosine at 238, serine at 240, and threonine at 242) were selected as positions for introduction of a fluorescent dye. As a fluorescent dye for this study, a dansyl group was adopted because of its high sensitivity to environmental change and small molecular size. To introduce the dansyl group by Fmoc-SPPS, Fmoc-Lys(dansyl-Gly)-OH was prepared. Each fluorescent-labeled C1b domain analog was successfully synthesized. Ligand bindings of these synthetic C1b domain analogs were evaluated by utilizing [^{3}H]-Phorbol 12,13-dibutylate (PDBu). The C1b domain analog, T242K(Dns-G), showed similar binding affinity with that of wild-type. The fluorescence spectra of T242K(Dns-G) in a titration of PDBu are shown in Fig. 2. As an increase of PDBu amount, the fluorescence intensity was increased and fluorescence emission maximum showed blue-shift. This result suggests that the environment of the dansyl group was altered by binding of PDBu to the synthetic C1b domain analog. In conclusion, the present fluorescent-labeled C1b domains enabled us to detect the binding of ligands by an increase of fluorescence intensity and a change of fluorescence emission maximum. The fluorescent-labeled C1b domain analogs would be useful for ligand screening and might be developed as effective biosensors.
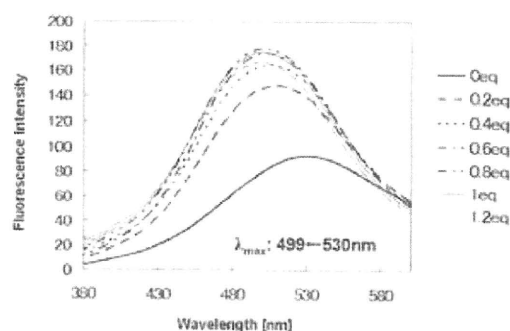

Fig. 2. Fluorescence spectra of the fluorescent-labeled C1b domain analog, T242K(Dns-G) in a titration of PDBu (0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.2 eq.).

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Structure-Activity Relationship Study of CXCR4 Antagonists on the Cyclic Pentapeptide Scaffold: Identification of New Pharmacophore Moieties

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A highly potent CXCR4 antagonist 2 [cyclo (-D-Tyr1-Arg2-Arg3-Nal4-Gly5-)] 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.

Keywords: CXCR4 antagonists, cationic aminoacid, bicyclic aromatic aminoacid, FC131

Introduction

The chemokine receptor CXCR4 is a membrane protein, which belongs to the G-protein coupled receptor family. Interaction of CXCR4 with its endogenous ligand stromal-cell derived factor-1α (SDF-1α)/CXCL12 induces various physiological functions: chemotaxis, angiogenesis, neurogenesis etc. in embryonic stage. On the other hand, CXCR4 is also relevant to multiple diseases: AIDS, cancer metastasis, progress of leukemia, rheumatoid arthritis, 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. 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. In the downsizing of 1, a cyclic pentapeptide 2 was developed by screeninglibraries based on four pharmacophore residues [Arg, Arg, 3-(2-naphthyl)alanine (Nal), D-Tyr] found by alanine scanning of 1. (Fig. 1)
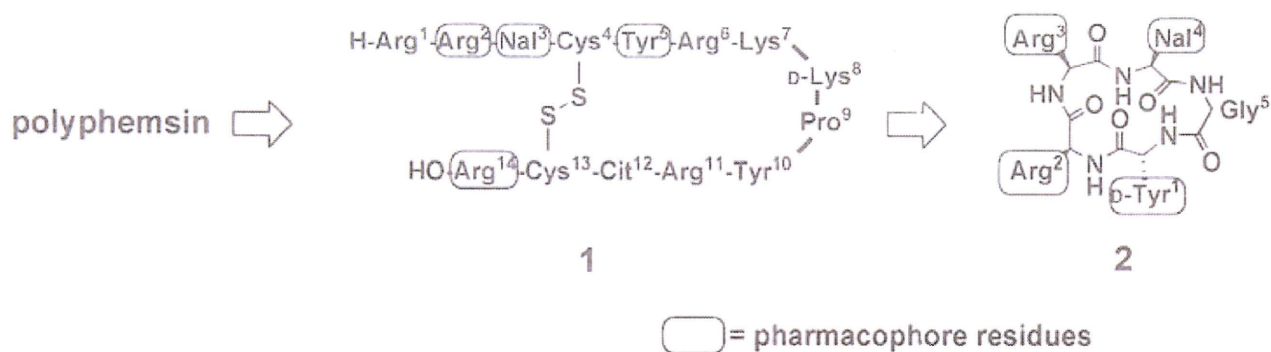
Fig. 1. Development of a cyclic pentapeptide 2 based the pharmacophore of a CXCR4 antagonistic peptide 1. Cit = L-citrulline, Nal = L-3-(2-naphthyl)alanine.

Results and Discussion

Our previous data of alanine-scanning of 2 suggested that D-Tyr[1] or Arg[2] was not optimalized [1]. Thus, we attempted to replace these functional groups. According to other previous reports, potent CXCR4 antagonists absolutely contain aromatic and

	-Xaa[1]-Arg[2]-Arg[3]-Nal[4]-Gly[5]-				
a	L	L	L	L	G
b	L	D	L	L	G
c	D	L	L	L	G
d	D	D	L	L	G
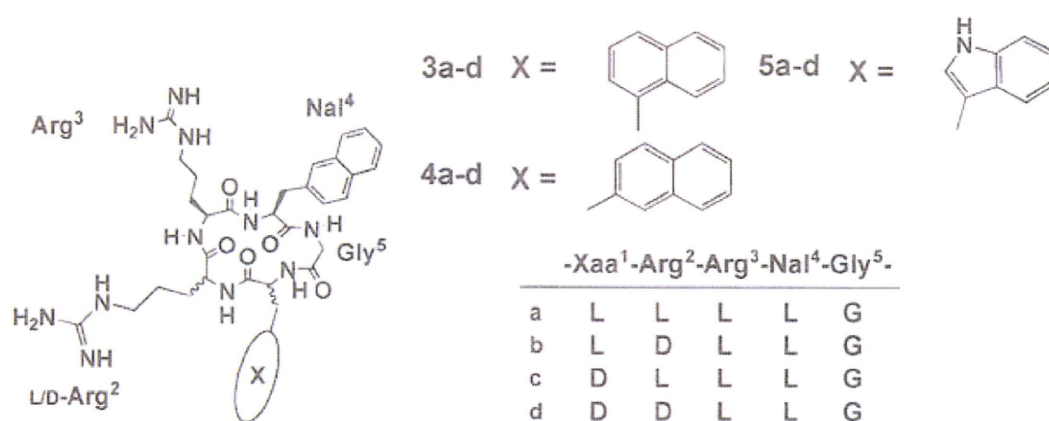
Fig. 2. Structures of compounds having substitution of an L/D- bicyclic aromatic amino acid for D-Tyr[1].

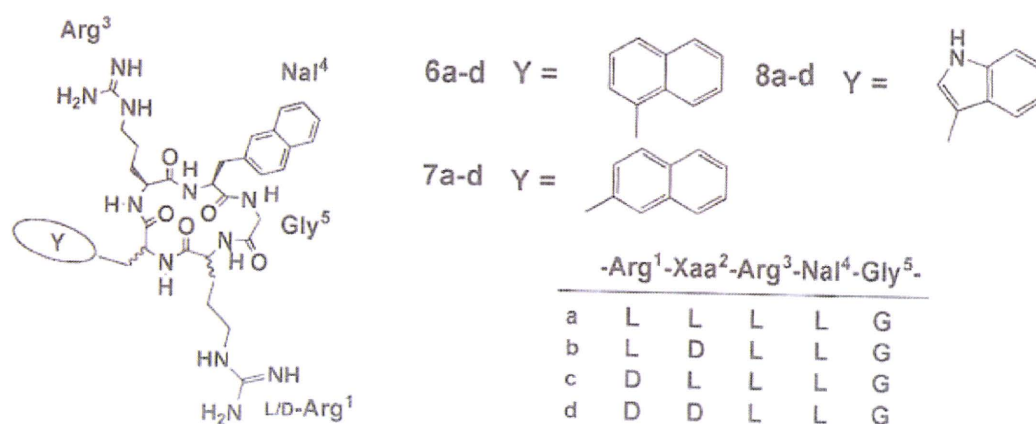|	-Arg[1]-Xaa[2]-Arg[3]-Nal[4]-Gly[5]-				
a	L	L	L	L	G
b	L	D	L	L	G
c	D	L	L	L	G
d	D	D	L	L	G

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

cationic groups. It suggests that these functional groups are involved in binding to CXCR4 mediated by hydrophobic and electrostatic interaction. In our first approach, D-Tyr[1] of 2 was replaced by an L/D-bicyclic aromatic amino acid to evaluate significance of the hydrophobic interaction by aromatic rings. 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.

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 [2]. 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 1). 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 mM, 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.

Each synthetic compounds were evaluated to CXCR4 binding (IC_{50}) by competition assay utilized radio-isotope labeled SDF-1α (Table 1). In addition, several compound that showed high CXCR4 binding activity were evaluated anti-HIV activity and cytotoxicity.(Table 2)

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

Table 1 *inhibitory activity of the synthetic compounds against binding of [125I]-SDF-1α to CXCR4*

Compound	IC_{50}/μM	Compound	IC_{50}/μM	Compound	IC_{50}/μM	Compound	IC_{50}/μM
2	0.015	3c	0.043	6a	> 2.0	6c	0.3 - 2.0
3a	0.3 - 2.0	4c	> 2.0	7a	0.3 - 2.0	7c	0.3-2.0
4a	0.3-2.0	5c	0.15	8a	> 2.0	8c	> 2.0
5a	0.22	3d	0.078	6b	> 2.0	6d	> 2.0
3b	0.3 - 2.0	4d	0.3 - 2.0	7b	0.045	7d	0.3 - 2.0
4b	0.3 - 2.0	5d	0.07	8b	> 2.0	8d	0.3 - 2.0
5b	> 2.0						

Table 2 *Anti-HIV activity and cytotoxicity of the synthetic compounds*

Compound	EC_{50}/mM^{a}	$CC_{50}/\mu M^{b}$
AZT	0.077	>10
1	0.044	>10
2	0.15	>10
5c	0.7	>10
5d	0.19	>10
7b	0.26	>10

introduction of D-amino acid at the 1-position was required to form an optimal cyclic pentapeptide backbone. 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 $D\text{-}Nal^{2}$ may be novel pharmacophoremoieties in the combination with Nal^{4} and Arg^{3}. The present data will provide useful approaches for simple designs of new lowmolecular weight CXCR4 antagonists. These results might also give valuable insights for understanding the ligand-receptor interactions.
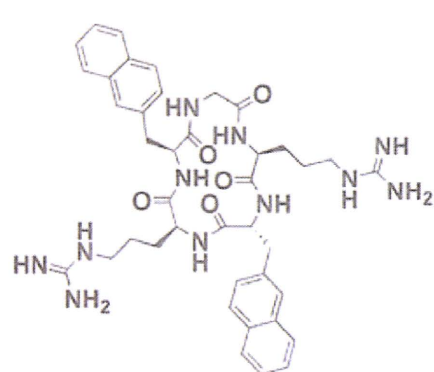
7b
$IC_{50} = 45$ nM
$EC_{50} = 260$ nM
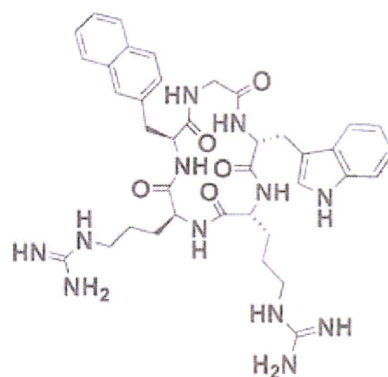
5d
$IC_{50} = 70$ nM
$EC_{50} = 190$ nM

IC_{50}: SDF-1a binding
EC_{50}: anti HIV activity

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Small-Sized CD4 Mimics Targeted for Dynamic Supramolecular Mechanism of HIV-1 Entry

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The elucidation of the dynamic supramolecular mechanism in HIV entry has provided insights into new type of drugs that can block HIV infection. In this study, we focused on CD4 mimic small compounds, such as NBD-556, and performed their structure-activity relationship study. In addition, it was found that CD4 mimic compounds have a great effect on the dynamic supramolecular mechanism of HIV entry, and that their combinational use with CXCR4 antagonists or neutralizing antibodies shows a synergic effect.

Keywords: CD4 mimic, CXCR4 antagonists, dynamic supramolecular mechanism, HIV entry, synergic effect

Introduction

Recently, as the clinical treatment of HIV-infected and AIDS patients, "highly active anti-retroviral therapy (HAART)", which involves a combination of two or three agents that are usually adopted from two categories composed of reverse transcriptase inhibitors and protease inhibitors, has brought us a tremendous success. Furthermore, a molecular mechanism involved in HIV-entry and -fusion into host cells has been disclosed in detail (Fig. 1). A fusion inhibitor, enfuvirtide (fuzeon, Trimers & Roche), a CCR5 antagonist, maraviroc (Pfizer) and an integrase inhibitor, raltegravir (Merck) have also been clinically used in USA. However, serious problems still remain even with the chemotherapy, such as the emergence of viral strains with multi-drug resistance (MDR), considerable adverse effects and high costs. Thus, development of novel drugs possessing action mechanisms different from the above inhibitors would be currently required. We have previously developed selective antagonists against the chemokine receptor CXCR4, which is the co-receptor of an X4-HIV-1 entry [1]. In the present study, we focus on the development of CD4 mimic compounds based on NBD-556 [2] (Fig. 2). In addition, we investigate an effect of CD4 mimic compounds on conformational change of an HIV surface protein, gp120 (Fig. 1).
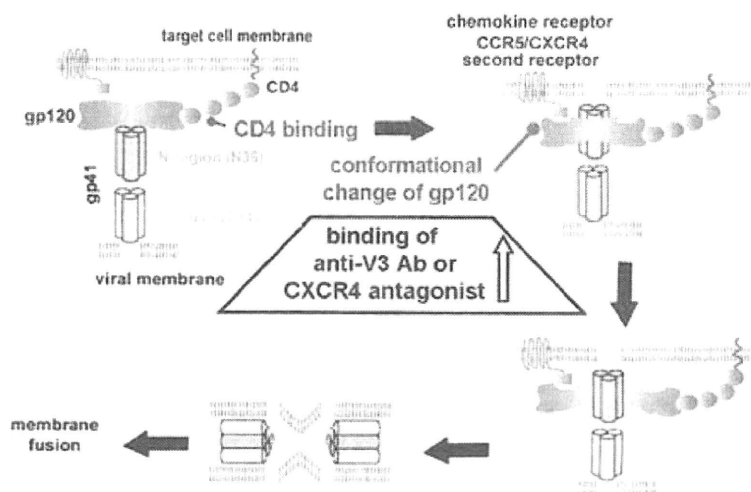
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Fig. 1. Plausible mechanism for the dynamic supramolecular mechanism in HIV-entry and –fusion. The binding of a cell surface protein CD4 to an HIV surface protein gp120 causes a conformational change of gp120 and an increase in binding affinity of anti-gp120-V3 antibody for gp120.
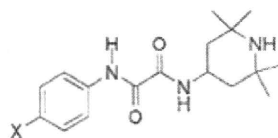

Fig. 2. A structure of CD4 mimic compounds. X = halogen, alkyl or nitro group (X = Cl: NBD-556).

Results and Discussion

It is speculated that there is a large space around the p-position of the phenyl ring of NBD-556, which might interact with a cavity of a viral surface protein gp120. Thus, NBD analogues having various substituents on the phenyl ring were synthesized (Fig. 2). Several compounds showed potent anti-HIV activity (data not shown). In addition, NBD analogues showed highly synergic effects not only with an anti-V3 antibody but also with T140. FACS analysis showed that in the presence of NBD analogues the anti-V3 antibody strongly binds to the HIV envelope.

CD4 mimic compounds might expose the correceptor-binding region of gp120, which was hidden inside. These compounds would be important probes directed to the dynamic supramolecular mechanism of HIV Entry, and useful lead compounds for cocktail therapy of AIDS.

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Development of Inhibitory Peptides against HIV-1 Integrase

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Wataru Nomura[1], Emiko Urano[3,4], Yan Han[3], Hideyoshi Fuji[5], Makiko
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*Several effective compounds that inhibit HIV-1 integrase activity have been found
by screening overlapping peptide libraries derived from HIV-1 gene products.
Among these compounds, two peptides showed significant anti-HIV activity by
fusing an octa-arginine sequence, which added the cell membrane permeability. In
addition, higher potent inhibitors against the integrase were obtained by
introducing intramolecular salt bridges to stabilize their α-helical structures,
suggesting that these peptides are novel lead compounds of anti-HIV agents.*

Keywords: α-helical structure, anti-HIV, integrase inhibitor, octa-arginine, Vpr

Introduction

A great success in the chemotherapy of AIDS or HIV-1-infected patients has
been brought by highly active anti-retroviral therapy (HAART), which involves a
combination of two or three different agents among reverse transcriptase inhibitors and
protease inhibitors. However, there still remain several serious problems such as the
emergence of the viral strains with multi-drug resistance (MDR), considerable adverse
effects and high costs. It is strongly desirable to develop novel drugs possessing action
mechanisms different from the above inhibitors. Recently, enfuvirtide (fuzeon, T-20,
Trimers & Roche) has been used as a fusion inhibitor in USA. Selective antagonists
against the chemokine receptor CXCR4, which plays a role of the co-receptor of an
X4-HIV-1 entry, have been developed in our previous study. HIV-1 integrase, which
catalyses the integration step in which viral cDNA is inserted into the host
chromosome, has been focused as a next target protein (Fig. 1). Raltegravir (Isentress,
Merck) has been used as the integrase inhibitor in the clinical field. In our previous
study, several inhibitory peptides against HIV-1 integrase were successfully found by
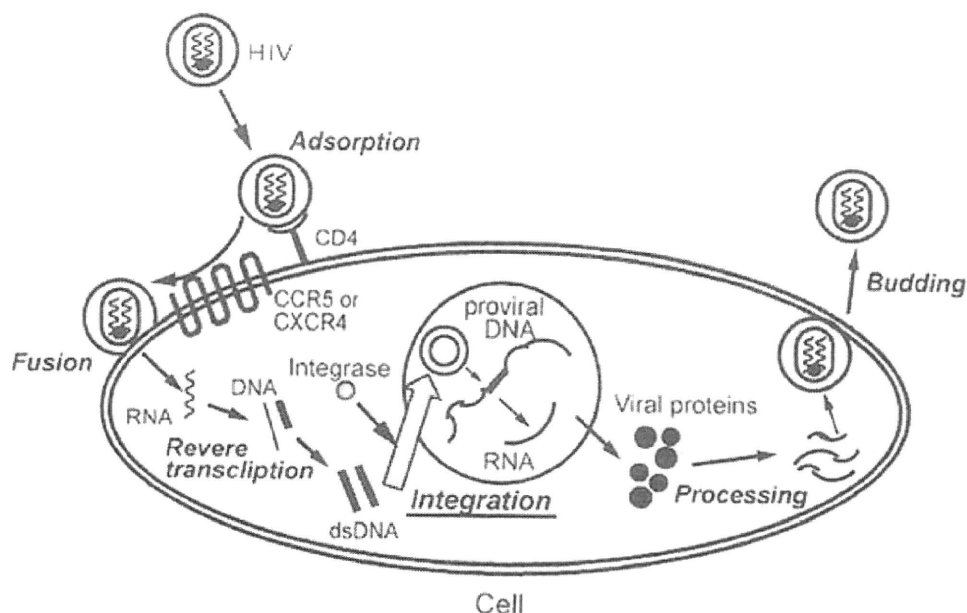
Fig. 1. Life cycle of HIV-1. The integration step is represented by a bold arrow.

screening peptide libraries of HIV-1 gene products. Among these peptides. Vpr-derived peptides have an essential hexapeptidic motif (Vpr 64-69). In addition. Vpr-derived peptides with an octa-arginyl sequence, which added the cell membrane permeability, showed significant anti-HIV-1 activity. In this study, we have developed higher active inhibitors against HIV-1 integrase based on the Vpr-derived peptides.

Results and Discussion

The essential hexapeptidic motif for the inhibitory activity is included in an α-helical structure of the parent Vpr protein. Then, the intramolecular salt bridges between Glu and Lys are introduced into the Vpr-derived peptides to stabilize the α-helical structure. Seven Vpr-derived peptide analogs, in which salt bridges were systematically introduced, were synthesized. In CD spectra, four analogs showed more stable α-helical structures than the original Vpr-derived peptide, however, other analogs showed less stable α-helical structures. However, interestingly, Vpr-peptide analogs with less stable α-helical structures showed the same or slightly higher inhibitory activity against the integrase compared with the original Vpr-derived peptide in the strand transfer assay *in vitro*. On the other hand, the Vpr-derived peptide analogs with enhanced α-helicity showed lower activity than that of the original peptide. Furthermore, a peptide analog with a less stable α-helical structure showed higher inhibitory activity against HIV-1 replication than the original Vpr-derived peptides, indicating that α-helical structures might not be required or that Glu and/or Lys residues introduced to form salt bridges might play an important role for the inhibitory activity against HIV-1 replication.

In conclusion, we have developed Vpr-peptide analogs with the intramolecular salt bridges and obtained the analogs with the remarkable anti-integrase activity. In addition, a Vpr-peptide analog showed higher inhibitory activity against HIV-1 replication than the original peptide. These Vpr-peptide analogs will be new lead compounds with the inhibitory activity against HIV-1 replication.

Development of a Novel Tag-Probe System for Fluorescent Imaging of Proteins in Living Cells

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In the fluorescent imaging of proteins in living systems, several pairs of peptide tags and their complementary probes have been used as useful tools. New tag and probe pairs with fluorogenic activity based on leucine zipper peptides have been developed. In this study, fluorescent colors applicable to our tag-probe pair have been extended by using a different solvatochromic fluorescent dye or a fluorescent resonance energy transfer system.

Keywords: fluorescent probe, FRET, leucine zipper, protein imaging, tag

Introduction

Time lapse imaging of proteins in living cells gives us detail information about dynamics and functions of target proteins. In general, target proteins are genetically fused with fluorescent proteins. However, the labeling method using fluorescent proteins is not suitable for pulse chase labeling of target proteins. Recently, a tag-probe system has been reported as a new helpful imaging tool, which can label proteins by fluorescent probes specifically bound to tag peptides genetically incorporated into target proteins [1]. In this system, the timing of labeling and the type of fluorescent dyes can be chosen arbitrarily. In addition, the binding of the probe to a target protein via its complement tag with a fluorescent color change and/or an increase in fluorescent intensity might enable us to clearly distinguish the labeled proteins from the free probes. The fluorogenic tag-probe pairs based on leucine zipper peptides have been successfully developed in our previous study. Thus, the expansion of fluorescent colors in our leucine zipper type tag-probe pairs has been performed by using other solvatochromic fluorescent dyes or fluorescence resonance energy transfer (FRET) systems.

Results and Discussion

A probe peptide is designed as an α-helix peptide, which has a solvatochromic fluorescent dye on the side chain of L-α-2,3-diaminopropionic acid (Dap). Tag peptides are designed as an antiparallel 2α-helix peptide and the amino acids, leucines (L2), which are located on the complementary position against a fluorescent dye of the probe peptide, are replaced by alanines (A2) or glycines (G2) to form a hydrophobic pocket. Previously, 4-nitrobenzo-2-oxa-1,3-diazole (NBD), which shows a green

fluorescence, was used as a solvatochromic fluorescent dye in a probe peptide. Instead of an NBD unit, 7-diethylaminocoumarin-3-carboxylic acid (DEAC) is adopted as another solvatochromic dye, which shows a blue fluorescent color, in this study (Fig 1(a)). In addition, an FRET system using an NBD unit and a tetramethylrhodamine (TAMRA) unit is constructed, in which NBD acts as a fluorescent donor and TAMRA acts as a fluorescent acceptor (Fig 1(b)). All peptides were synthesized by Fmoc solid phase peptide synthesis, purified by reversed phase HPLC and identified with ESI-TOFMS.

In the titration of the tag peptides into the probe peptide having a DEAC unit, the A2 tag and probe peptide pair showed the wavelength shift from 480 nm to 471nm. The fluorescent intensity of the tag-probe complex at 471 nm is approximately 11-fold higher than that of the probe peptide alone. These results are compatible with those of the pair of A2 tag and the probe peptide having an NBD unit. Interestingly, the G2 tag and the probe peptide with a DEAC unit showed the largest fluorescent change. The emission maximum of the probe peptide with a DEAC unit shifted from 480 nm to 459 nm, and the fluorescent intensity at 459 nm showed an approximately 60-fold increase in the presence of the G2 tag. These results suggest that the probe peptide with a DEAC unit can be used as a blue-color fluorogenic probe. Furthermore, the FRET type probe peptide showed a remarkable increase in the fluorescent intensity due to the TAMRA unit in the presence of the tag peptides when the NBD unit was excited at 456 nm. This result indicated that FRET from the NBD unit to the TAMRA unit effectively occurred and that the FRET type probe peptide can be used as a red-color fluorogenic probe.

In conclusion, we have demonstrated that the probe peptide with the DEAC unit and the FRET type probe peptide are new probe molecules available for the fluorescent imaging of proteins using our tag-probe system. Three types of probe peptides with blue (DEAC), green (NBD) and red (NBD-TAMRA FRET system) fluorescent colors are available in combination with several tag peptides. These tag-probe pairs will be potent tools for pulse chase imaging of target proteins.
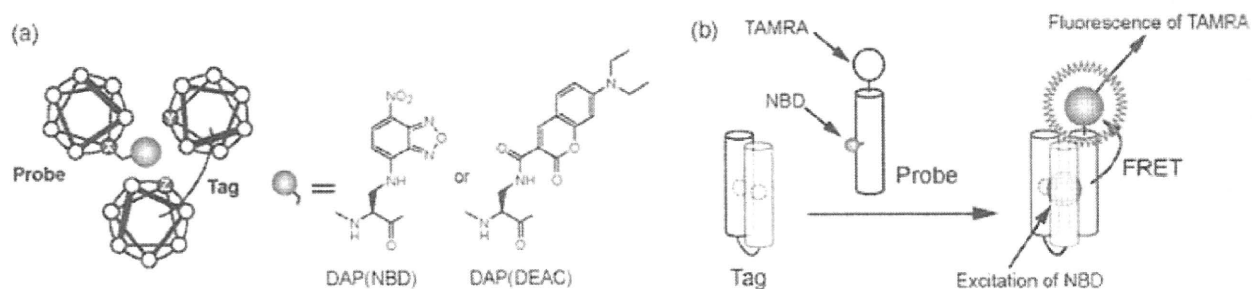

Fig. 1. (a) Helical wheel model and chemical structures of fluorescent amino acids. (b) The represented scheme of the FRET type tag-probe system.

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