The Chemokine Receptor CXCR4 as a Therapeutic Target for Several Diseases

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Abstract: CXCR4 is the receptor for a chemokine, CXCL12 (stromal cell-derived factor-1, SDF-1). The CXCL12-CXCR4 axis has been proven to be involved in several problematic diseases, including AIDS, cancer cell metastasis, leukemia cell progression and rheumatoid arthritis (RA). Thus, CXCR4 is thought to be an important therapeutic target to overcome the above diseases. We have developed several specific CXCR4 antagonists.

Key Words: Leukemia cell progression, cancer metastasis, chemokine receptor, HIV infection, low molecular weight CXCR4 antagonist, rheumatoid arthritis, T140, T22.

INTRODUCTION

Since elucidation of human genome, proteomics has been prosperous in biology and life science. Seven transmembrane (7TM) G-protein-coupled receptor (GPCR) families are important targets in proteomics. The chemokine receptor CXCR4 is a 7TM-GPCR, which transduces signals of the chemokine, CXCL12/stromal cell-derived factor-1 (SDF-1) that is a solitary ligand for CXCR4 [1-4]. The CXCL12-CXCR4 axis plays an important role in the migration of progenitor cells during embryonic development of the cardiovascular, hemopoietic, central nervous systems and so on. This system has also been shown to be involved in several problematic diseases such as HIV infection [5], cancer cell metastasis [6-23], leukemia cell progression [24-26] and rheumatoid arthritis (RA) [27] (Fig. **1**): First, CXCR4 was identified as a co-receptor that is utilized by T cell line-tropic (X4-) HIV-1 entry in association with CD4 [5]. X4 HIV-1 strains constitute majority in the late stage of HIV infection and AIDS. On the other hand, macrophage-tropic (R5-) HIV-1 strains, which utilize the chemokine receptor CCR5 as another co-receptor, do in the early stage of HIV infection [28-32]. Second, ample papers have reported that several types of cancers express CXCR4 on the surface of cells, and that CXCL12 is highly expressed in internal organs that represent the primary metastatic destinations of the corresponding cancer cells [6-26]. The CXCL12-CXCR4 axis has been recognized to be involved in the metastasis of several cancers, such as breast cancer, pancreatic cancer, melanoma, prostate cancer, kidney cancer, neuroblastoma, non-Hodgkin's lymphoma, lung cancer, ovarian cancer, multiple myeloma and malignant brain tumor [6-23]. This axis is also relevant to the progression of chronic lymphocytic leukemia (CLL) B-cells and pre-B acute lymphoblastic leukemia (ALL) cells [24-26]. Third, RA is a disorder, which is mainly caused by the accumulation of $CD4^+$ memory T cells in the inflamed synovium. Nanki *et al.* reported that the memory T cells highly express CXCR4 on the surface and the CXCL12 concentration is extremely high in the synovium of RA patients, and that CXCL12 stimulates migration of the memory T cells and inhibits T cell apoptosis, indicating that the CXCL12-CXCR4 interaction plays a critical role in T cell accumulation in the RA synovium [27]. Thus, CXCR4 is thought to be an attractive therapeutic target for the above diseases. This mini-review article focuses on our recent researches on the development of CXCR4 antagonists.

DISCOVERY OF SELECTIVE INHIBITORS AGAINST X4-HIV-1 ENTRY

As the clinical treatment of AIDS or HIV-1-infected patients, "Highly active anti-retroviral therapy (HAART)", which uses a combination of two (or three) different agents that are usually chosen from two drug categories (reverse transcriptase inhibitors and protease inhibitors), has brought us a significant success. However, there still remain several serious problems even with HAART, which involve the emergence of viral strains with considerable adverse effects, multi-drug resistance (MDR) and high costs [33,34]. An ideal therapeutic approach would block the HIV entry/fusion processes. A dynamic supramolecular mechanism relevant to the HIV entry/fusion process during the HIV-1 replication has been elucidated in detail. Initially, an envelope protein gp120 binds to a cell surface protein CD4, which causes a conformational change of gp120 and its subsequent binding to the second cellular receptor (co-receptor), CCR5 [28–32] or CXCR4 [5]. The binding causes penetration of an another envelope protein, gp41, which anchors HIV envelope into membrane, to the cell membrane from the *N*-terminus end

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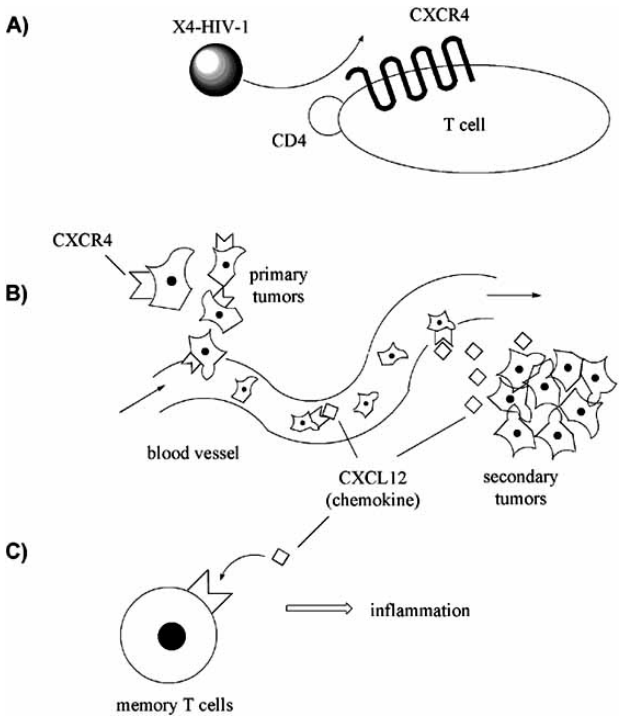

Fig. (1). Mechanistic presentation of problematic diseases relevant to CXCR4: A) HIV infection and AIDS, B) cancer cell metastasis, C) rheumatoid arthritis (RA).

followed by subsequent formation of the gp41 trimer-of-hairpins structure in the center region, which leads to membrane fusion of HIV cells and consequently results in completion of the infection [35]. Since elucidation of the above dynamic molecular machinery, the development of effective inhibitors blocking HIV-entry/fusion that are directed to the second receptors, CCR5 and CXCR4, and the dynamic process involving formation of the gp41 trimer-of-hairpins structure has been performed. Practically, clinical use of enfuvirtide (DP-178, T-20, Fuzeon, Trimeris & Roche), which is a 36-mer peptide derived from *C*-terminal helical region of gp41, has brought us a great hope toward fusion inhibitors as a new class of anti-HIV drugs against MDR HIV-1 strains [36]. Thus, our research has focused on drug discovery targeting CXCR4. A useful review article referring to CCR5 antagonists and gp41-targeting fusion inhibitors are reported elsewhere [37].

Tachyplesins and polyphemusins, which are 17-mer and 18-mer self-defense peptides that were isolated from the hemocyte debris of the Japanese horseshoe crab (*Tachypleus tridentatus*) and the American horseshoe crab (*Limulus polyphemus*), respectively, show antimicrobial activity against several strains of bacteria and viruses (Fig. **2**) [38,39]. Based on these peptides, an anti-HIV peptide, T22 ([$Tyr^{5, 12}$, Lys^{7}]-polyphemusin II) [40,41] and its shortened 14-mer peptide, T140, were developed (Fig. **2**) [42]. T22 and T140 were proven to strongly block an X4-HIV-1 entry through their specific binding to CXCR4 and to inhibit Ca^{2+} mobilization induced by CXCL12 stimulation through CXCR4 [43-45]. The T140 analog exhibited remarkable delaying effect against the generation of drug-resistant strains in *in vitro* passage experiments using cell cultures [46]. The difficulty of the generation of drug-resistant strains would be a useful advantage for development of T140 analogs in clinical chemotherapy. T140 forms an antiparallel β-sheet structure that is maintained by a disulfide bridge between Cys^{4} and Cys^{13} and connected by a type II' β-turn [47]. Four amino acid residues in T140, Arg^{2}, L-3-(2-naphthyl)alanine $(Nal)^{3}$, Tyr^{5} and Arg^{14}, are indispensable for strong activity [48].

BIO-STABLE LEAD COMPOUNDS OF CXCR4 ANTAGONISTS DERIVED FROM T140 DERIVATIVES

T140 is not stable in mouse/feline serum or in rat liver homogenate [49,50]. Degradative deletion of indispensable residues (Arg^{14} in serum; Arg^{2}, Nal^{3} and Arg^{14} in liver homogenate) from *N*-/*C*-terminus caused drastic diminishment of the efficacy of T140. *N*- And *C*-terminal modifications of T140 analogs suppressed the biodegradations and led to development of novel effective compounds, which showed high CXCR4-antagonistic activity and increased biostability. In addition, the *N*-terminal modification studies found that an electron-deficient aromatic ring such as a *p*-fluorobenzoyl moiety at the *N*-terminus constitutes a novel pharmacophore for strong anti-HIV activity. *p*-Fluorobenzoyl moiety-containing analogs, 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011, are promising leads to date, which have two orders of magnitude higher anti-HIV activity than T140 and enhanced stability in serum/liver homogenate (Fig. **2**) [51].

ANTI-METASTATIC ACTIVITY OF A BIO-STABLE T140 ANALOG AGAINST BREAST CANCER

Müller *et al.* revealed that CXCR4 and another chemokine receptor, CCR7, are highly expressed in the surface of human breast cancer cells, while CXCL12 and a CCR7 ligand, CCL21, are highly expressed in lymph nodes, bone marrow, lung and liver, which constitute the common metastatic destinations of breast cancer, suggesting that the CXCL12/CXCR4 axis might determine the metastatic destination of tumor cells and cause organ preferential metastasis [6]. Practically, pulmonary metastasis of breast cancer cells was inhibited by neutralization using anti-CXCR4 antibodies in mice. To evaluate the potency of CXCR4 antagonists as anti-cancer-metastatic agents, we investigated whether T140 analogs inhibit migration of breast cancer cells *in vitro* and metastasis of breast cancer cells *in vivo* [22]. In cell migration assays using cell culture chambers, T140 analogs inhibited CXCL12-induced migration of a CXCR4-positive human breast carcinoma cell line MDA-MB-231 in dose-dependent manners. Next, effect of the bio-stable CXCR4 antagonist, 4F-benzoyl-TN14003, was investigated by using experimental metastasis models of breast cancer. In the models, MDA-MB-231 cells were injected intravenously (i.v.) into the tail vein of SCID mice and trapped in the lung through heart and pulmonary artery. An effective suppression of tumor accumulation on lung surface derived from the MDA-MB-231 metastasis was observed in mice treated by subcutaneous (s.c.) injection of 4F-benzoyl-TN14003 using an Alzet osmotic pump

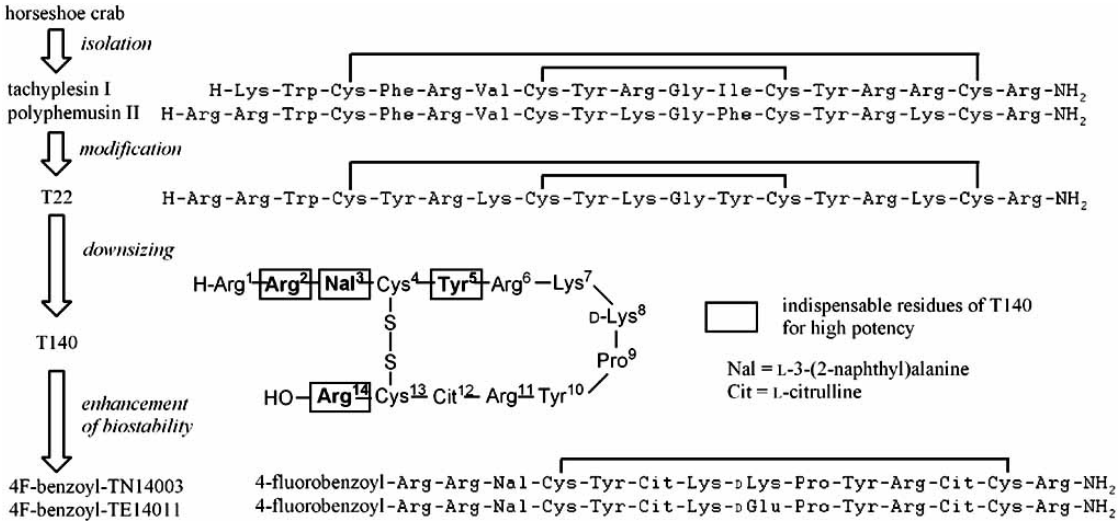

Fig. (2). Development of the CXCR4 antagonist T140 and its bio-stable analogs, 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011, based on horseshoe crab peptides. Disulfide bridges of tachyplesin I, polyphemusin II, T22, 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011 are shown by solid lines.

(DURECT Corp., Cupertino, CA, USA). Quantitative analyses based on calculations of ratios of tumor area to total lung surface area revealed that 4F-benzoyl-TN14003 significantly reduced pulmonary metastasis of MDA-MB-231 cells in mice. This proved that CXCR4 antagonists, such as T140 analogs, could replace neutralizing anti-CXCR4 antibodies as anti-metastatic agents.

ANTI-METASTATIC ACTIVITY AGAINST MELANOMA

Murakami *et al.* showed that an excessive expression of CXCR4 dramatically enhanced the metastatic accumulation of B16 melanoma cells in mice lungs. They also showed that the CXCR4 antagonist T22, developed in our laboratory, blocked pulmonary metastasis in mice injected with B16 cells transduced with CXCR4 [52]. To evaluate the potency of CXCR4 antagonists against pulmonary metastasis in mice injected with B16 cells, which are not transduced with CXCR4, we investigated whether T140 analogs inhibit pulmonary metastasis *in vivo* [8]. A steady release by poly D,L-lactic acid (PLA) microcapsules containing 4F-benzoyl-TE14011 was performed in experimental models of metastasis of CXCR4-positive B16-BL6 melanoma cells [8]. 4F-benzoyl-TE14011 can be steadily released from the PLA microcapsules for a long period *in vivo*, leading to maintenance of the 4F-benzoyl-TE14011 concentration in bloods. A single subcutaneous administration of 4F-benzoyl-TE14011-PLA significantly reduced the number of colonies derived from pulmonary metastasis of B16-BL6 cells. This result suggests that a controlled release of CXCR4 antagonists might lead to the effective suppression of cancer metastasis.

EFFECTS AGAINST MULTIPLE MYELOMA, PANCREATIC CANCER AND SMALL CELL LUNG CANCER

CXCL12 might play a potential role in the recruitment of osteoclast precursors to the bone marrow and activation, since the CXCL12 level is correlated to the expression of multiple radiological bone lesions in individuals with multiple myeloma. Practically, 4F-benzoyl-TE14011 significantly inhibited both CXCL12-mediated and the myeloma plasma cell line (RPMI-8226) conditioned medium-stimulated osteoclast activity *in vitro* [15], suggesting that blockade of the CXCL12/CXCR4 axis might be an effective therapy against osteolysis in multiple myeloma patients.

CXCL12 mRNA is expressed in pancreatic cancer tissues, and 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) [9]. CXCL12 stimulated both migration and invasion of pancreatic cancer cells, AsPC-1, PANC-1 and SUIT-2, in a dose-dependent manner *in vitro*. This suggests that the CXCL12-CXCR4 interaction is involved in pancreatic cancer cell progression and metastasis. CXCL12-induced migration and invasion of these cells were suppressed by T140 analogs in dose-dependent manners [10]. The treatment of PANC-1 cells with CXCL12 caused a drastic increase in actin polymerization (cytoskeleton), leading to the invasion of malignant cells into tissues and subsequent metastasis. The phenomenon was effectively inhibited by T140 analog.

Small cell lung cancer (SCLC), which constitutes a fourth – fifth of lung cancer, is the leading cause of death in Western countries [53]. Primary tumor cells isolated from SCLC patients highly express CXCR4. CXCL12 is constitutively secreted by marrow stromal cells and plays a critical role in homing of hematopoietic cells to the marrow. Burger *et al.* showed that CXCL12 stimulated SCLC cell invasion into extracellular matrix and firm adhesion to marrow stromal cells, which were effectively suppressed by T140 *in vitro*, confirming involvement of the CXCL12-CXCR4 interaction in SCLC metastasis [17]. Adhesion of SCLC cells to extracellular matrix or accessory cells within the tumor microenvironment confers cell adhesion-mediated drug resistance (CAM-DR) to chemotherapy *via* integrin signaling. CXCL12 was found to induce activation of $\alpha 2$,

α4, α5 and β1 integrins through CXCR4, which was inhibited by T140 analog. They showed that stromal cells protected SCLC cells from anti-cancer drug (etoposide)-induced apoptosis, and that this protection was inhibited by T140 analog [54]. Thus, T140 analogs in combination with anti-cancer drugs might overcome CXCL12-mediated CAM-DR in SCLC. CXCR4 is expressed on malignant cells from at least 23 different types of cancers [55], including the above cancers. CXCR4 antagonists such as T140 analogs might be useful leads for the development of anti-metastatic agents of several types of cancer.

Anti-ALL AND -CLL Activities

Precursor-B (pre-B) cell ALL might be caused by their intimate contact with bone marrow stromal layers using the β_1 integrins. The migration of these pre-B cells into stromal layers is regulated by CXCL12, since CXCR4 is uniformly and highly expressed on the cells. T140 blocked CXCL12-induced chemotaxis and attenuated the migration of pre-B ALL cells into bone marrow stromal layers. Furthermore, T140 analog enhanced the cytotoxic and antiproliferative effects of the other agents, such as vincristine and dexamethasone, suggesting that T140 analogs might overcome CAM-DR in ALL chemotherapy [26].

On the other hand, B cell CLL is the most common leukemia in adults in Western countries. This disease is caused by the accumulation of long-lived, monoclonal B malignant cells in the blood, secondary lymphoid organs and bone marrow. CLL B cells highly express CXCR4, whereas marrow stromal cells or nurselike cells constitutively release CXCL12, which activates CLL B cells, and rescues the cells from apoptosis, leading to their accumulation. The CXCL12-CXCR4 axis would also be a therapeutic target for B cell CLL [24]. Practically, T140 analogs inhibited CXCL12-mediated chemotaxis of CLL cells, their migration beneath marrow stromal cells and actin polymerization in a dose-dependent manner, *in vitro* [25]. In addition, T140 analogs reduced not only the anti-apoptotic effect caused by CXCL12 but also stromal cell-mediated protection of CLL cells from spontaneous apoptosis. Co-culture of CLL cells and marrow stromal cells protected CLL cells from drug (fludarabine)-induced apoptosis, causing stromal CAM-DR. Treatment with T140 analogs re-sensitized the above CLL cells to fludarabine-induced apoptosis. T140 analogs might overcome CAM-DR, which is a serious problem in clinical chemotherapy of CLL.

ANTI-RA ACTIVITY OF A BIO-STABLE T140 ANALOG

Inflammatory cytokines, such as tumor necrosis factor (TNF)-α, IFN-γ, IL-1 and IL-6, play a critical role in RA. [27]. Although the development of biological drugs directed to these cytokines, such as monoclonal antibodies, has produced useful results in clinical RA therapy, the curative effects have not yet reached a perfect stage. The development of other drugs independent on the above cytokine's functions is required for the improvement of chemotherapy. Delayed-type hypersensitivity (DTH) reaction induced by sheep red blood cells (SRBC) was performed as an *in vivo* mouse experimental model of the cellular immune response for evaluation of the activity of 4F-benzoyl-TN14003 [56]. Subcutaneous injection of 4F-benzoyl-TN14003 using an Alzet osmotic pump significantly suppressed the footpad swelling after challenge as the DTH response in a dose-dependent manner. As the second *in vivo* experimental RA model, collagen-induced arthritis (CIA) in mice was adopted. Treatment with 4F-benzoyl-TN14003 using an Alzet osmotic pump (s.c.) after the bovine type II collagen (CII) emulsion booster showed significant suppression of several symptoms of arthritis (score increase, body weight loss, ankle swelling and limbs' weight gain) and apparent suppression of the increase in levels of serum anti-bovine CII IgG2a antibody. 4F-benzoyl-TN14003 also interferes with the humoral immune response to CII. CXCR4 antagonists such as T140 analogs might also be useful leads for anti-RA agents.

OTHER PROFILES OF T140 ANALOGS

Since T140 decreases autonomous signaling through CXCR4 wild type and its constitutively active mutant (CAM), T140 is an inverse agonist, not a partial agonist. Partial agonists of CXCR4, which have CXCL12-like agonistic activity through CXCR4 although weaker than that of CXCL12, would migrate and activate various cancer cells and memory T cells that highly express CXCR4. Thus, inverse agonists such as T140 analogs, which lack agonistic effect on CXCR4, may reduce toxicities and side effects involving migration of several cancer cells and memory T cells and have a great clinical advantage, especially in terms of cancer and RA chemotherapy.

Germinal center (GC) dark and light zones segregate cells undergoing somatic hypermutation and antigen-driven selection. Cyster *et al.* found that GC organization was absent from mice that were deficient in CXCR4, and that GC B cells, which highly express CXCR4, migrated toward the dark zone where CXCL12 was more abundant than that in the light zone [57]. Both genetic ablation of CXCR4 and its pharmacological abrogation by T140 analog disrupted GC compartmentalization into the dark zone, suggesting the presence of CXCR4-mediated GC organization.

Zheng *et al.* showed that CXCR4 is highly expressed on neural progenitor cells (NPCs), and that CXCL12 induced human NPC chemotaxis *in vitro*, which was blocked by T140 [58]. The CXCL12-CXCR4 axis might play essential roles in cerebellar, hippocampal and neocortical neural cell migration during embryogenesis.

SMALL-SIZED CXCR4 ANTAGONISTS BASED ON CYCLIC PENTAPEPTIDES

The four amino acid residues of T140, Arg^2, Nal^3, Tyr^5 and Arg^{14}, are indispensable to express high CXCR4-antagonistic activity as mentioned in the previous section [48]. Thus, the pharmacophore-guided approach was adopted to downsize T140 analogs. Cyclic pentapeptide libraries using two L/D-Arg, L/D-Nal and L/D-Tyr in addition of Gly as a spacer were constructed to lead to efficient discoveryof a hit compound, 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. **3**) [59]. NMR and simulated annealing

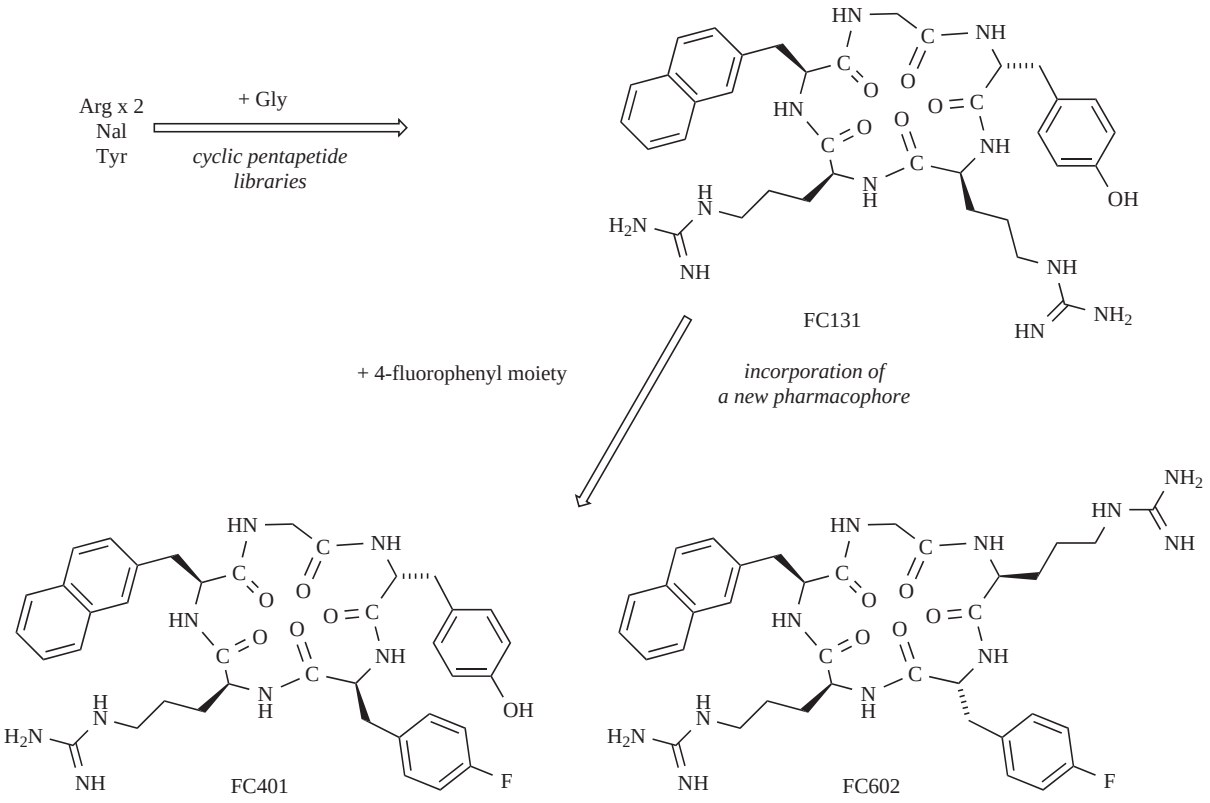

Fig. (3). Structures of a cyclic pentapetide, FC131, and its analogs containing L/D-Phe(4-F), FC401 and FC602.

molecular dynamics (SA-MD) analysis of FC131 showed an almost symmetrical pentagonal backbone structure.

Fourthermore, introduction of (*E*)-alkene dipeptide isosteres (EADIs) [60-63] were adopted for reduction of the peptide character of FC131. Several FC131 analogs, in which the above isosteres were substituted for the backbone amide bonds were developed by the synthetic strategy as reported in the previous paper [64-66]. Structure-activity relationship studies by substitution of these isosteres provided useful information for reduction of peptide character [67].

Several cyclic tetrapeptide-scaffolds have been prepared and investigated for structural tuning of FC131. A γ-amino acid-containing peptide, FC151 (containing 4-amino-5-naphthalen-2-yl-pentanoic acid in the place of the Nal-Gly sequence), disulfide-bridged cyclic peptides, FC205 [*N*-3-guanidinopropanoyl-Cys(S-)-Arg-Nal-D-Cys(S-)-NH_2] and FC225 [N-3-guanidinopropanoyl-Cys(S-)-Arg-Nal-D-Cys(S-)-tyramine], showed significant CXCR4-antagonistic activity. Furthermore, cyclic compounds that were bridged by an olefin using ring-closing metathesis (RCM), FC341 and FC351, exhibited moderate CXCR4-antagonistic activity [68]. Attempt on further downsizing and structural tuning is now in progress.

In addition, incorporation of a new pharmacophore such as a 4-fluorophenyl moiety, which was identified by the *N*-terminal modification of T140 analogs as mentioned in the previous section [51], into cyclic pentapeptides was performed. Since the phenol group of D-Tyr^5 could not be replaced by the 4-fluorophenyl group with maintenance of high activity, the 4-fluorophenyl group was incorporated into the amino acid at positon 1. [$Phe(4\text{-}F)^1$]-FC131, FC401, showed significant CXCR4-binding activity (Fig. **3**) [69]. 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, D-Tyr^5 was replaced by L/D-Arg^5. Among four analogues [L/D-$Phe(4\text{-}F)^1$, L/D-Arg^5]-FC131, [D-$Phe(4\text{-}F)^1$, Arg^5]-FC131, FC602, showed the most potent activity, which is 10-fold more potent than that of [D-Tyr^1, Arg^5]-FC131 [69]. Thus, it is thought that FC602 is useful as a novel lead involving the pharmacophores different from FC131.

Except for T140-related compounds, several CXCR4 antagonists have been reported to date [70,71]: *i.e.* a bicyclam AMD3100 (AnorMED, Inc.) [72], an *N*-pyridinyl-methylene cyclam (monocyclam) AMD3465 (AnorMED, Inc.) [73], a non-cyclam AMD8665 (AnorMED, Inc.) [74], AMD070 (AnorMED, Inc.) [75], ALX40-4C (Ac-[D-Arg]$_9$-NH_2; NPS Allelix) [76], CGP64222, R3G, NeoR [77-79], a distamycin analog, NSC651016 [80], a flavonoid compound, ampelopsin [81] and so on. Double-functional drugs based on AMD3100 and galactosylceramide (GalCer) analog conjugates were also reported [82]. An orally bioavailable agent, KRH-1636 (Kureha Chemical & Sankyo), might be produced by intensive modification of the *N*-terminal tripeptide of T140, Arg-Arg-Nal [83]. A review referring to these CXCR4 antagonists except for T140-related compounds is reported elsewhere in detail [84]. In addition, Schols *et al.* reported a dual CCR5/CXCR4 antagonist

AMD3451, which showed antiviral activity against R5, R5/X4(dual-tropic) and X4-HIV-1 strains in micromolar levels [85].

CONCLUDING REMARKS

HIV-entry inhibitors, T22 and its downsized analog T140, which inhibit entry to T-cells by X4-HIV-1 through their specific binding to the coreceptor CXCR4, have been developed. These peptides were also identified to have anti-cancer-metastasis, anti-leukemia and anti-RA activities. Structural tuning, downsizing and reduction of peptide character based on T140 were explored to develop new leads. It is very important to recognize that CXCR4 plays a critical role in embryogenesis, homeostasis and inflammation in fetus, especially in embryonic development of hemopoietic, cardiovascular and central nervous systems. Thus, researchers must carefully consider risky reactions toward living human bodies derived from blocking the CXCL12-CXCR4 axis. Under the above condition, these CXCR4 antagonists might be promising agents for clinical chemotherapy of HIV infection, cancer metastasis, leukemia progression and RA. A combinational use of CXCR4 antagonists with CCR5 antagonists/fusion inhibitors might improve clinical chemotherapy of HIV infection and AIDS. Further development of optimized CXCR4 antagonists involving investigation on administration routes is thought to become important in the chemotherapy of CXCR4-associated multiple diseases.

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