internal organs that represent the primary metastatic destinations of the corresponding cancer cells [10-30]. 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, small cell lung cancer (SCLC), ovarian cancer, multiple myeloma and malignant brain tumor [10-27]. Third, this axis is also relevant to the progression of chronic lymphocytic leukemia (CLL) B-cells and pre-B acute lymphoblastic leukemia (ALL) cells [28-30]. Fourth, RA is an annoying disorder, which is largely caused by the accumulation of $CD4^+$ memory T cells in the inflamed synovium. Nanki *et al.* found that the memory T cells overexpress CXCR4 on the surface and CXCL12 is highly expressed in the synovium of RA patients, and that CXCL12 stimulates T cell migration and thereby inhibits T cell apoptosis, suggesting that the CXCL12-CXCR4 interaction plays a critical role in memory T cell accumulation in the RA synovium [31]. Fifth, Phillips *et al.* reported that $CD45^+$collagen I^+CXCR4^+ circulating fibrocytes, which migrate in response to CXCL12 and traffic to the lung, contribute to the pathogenesis of pulmonary fibrosis [32]. Taken together, CXCR4 is considered to be an attractive therapeutic target for the above diseases. This review article focuses on our recent researches on the development of CXCR4 antagonists including low molecular weight compounds and structure tuning of these antagonists.

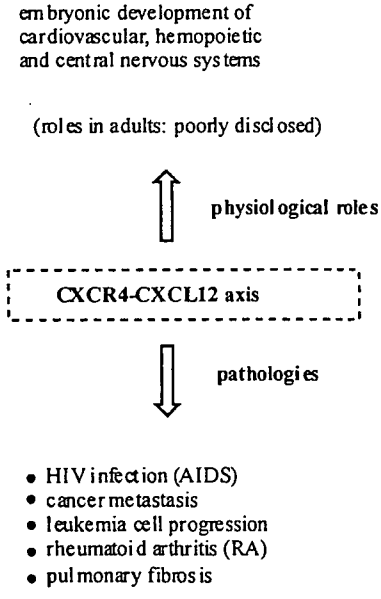

Fig. (1). Physiological roles and problematic diseases correlated to the CXCR4-CXCL12 axis.

DISCOVERY OF SELECTIVE CXCR4 ANTAGONISTS

Tachyplesins and polyphemusins, which were isolated from the hemocyte debris of the Japanese horseshoe crab (*Tachypleus tridentatus*) and the American horseshoe crab (*Limulus polyphemus*), respectively, are 17-mer and 18-mer self-defense peptides that show antimicrobial activity against several strains of bacteria and viruses (Fig. (2)) [39,40]. Through structure-activity relationship studies on these peptides, a polyphemusin analog, T22 ([$Tyr^{5, 12}$, Lys^7]-polyphemusin II) [41,42] and its shortened 14-mer peptide, T140, were developed as anti-HIV peptides (Fig. (2)) [43]. T22 and T140 were proven to strongly block an X4-HIV-1 entry through their specific binding to CXCR4 as well as to inhibit Ca^{2+} mobilization induced by CXCL12 stimulation through CXCR4 [44-46]. 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]. However, T140 is proven not to be 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 more effective compounds, which showed high CXCR4-antagonistic activity and increased bio-stability. 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 new pharmacophore for strong anti-HIV activity. *p*-Fluorobenzoyl moiety-containing analogs, 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011, are useful leads, which have two orders of magnitude higher anti-HIV activity than T140 and enhanced stability in serum/liver homogenate (Fig. (2)) [51]. T140 analogs, such as 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011, have been used widely for studies on elucidation of several physiological functions of CXCR4, which are not known yet, and on the drug development for the chemotherapy of multiple diseases.

IN VITRO AND *IN VIVO* EFFECTS OF T140-DERIVED CXCR4 ANTAGONISTS

1) CXCL12-Mediated CXCR4 Signaling in Neural Progenitor Cells

Peng *et al.* reported that CXCR4 is highly expressed on rat and human neural progenitor cells (NPCs), and that CXCL12 induced human NPC chemotaxis *in vitro*, which was suppressed by T140 [52]. Their studies also showed that the CXCL12-CXCR4 axis plays essential roles in cerebellar, hippocampal and neocortical neural cell migration during embryogenesis, i.e. neural development and repair.

2) CXCR4-Mediated Germinal Center Organization

It is known that germinal center (GC) dark and light zones segregate cells undergoing somatic hypermutation and antigen-driven selection. Allen *et al.* found that GC organization was absent from CXCR4-deficient mice, and that GC B cells, which express CXCR4 in abundance, migrated and trafficked to the dark zone where CXCL12 was more abundant than that in the light zone [53]. Genetic ablation of CXCR4 and its pharmacological abrogation by T140 analogs disrupted GC compartmentalization into the dark zone, suggesting that CXCR4 is indispensable for suitable GC organization. In addition, another chemokine receptor CXCR5 helps direct cells to the light zone.

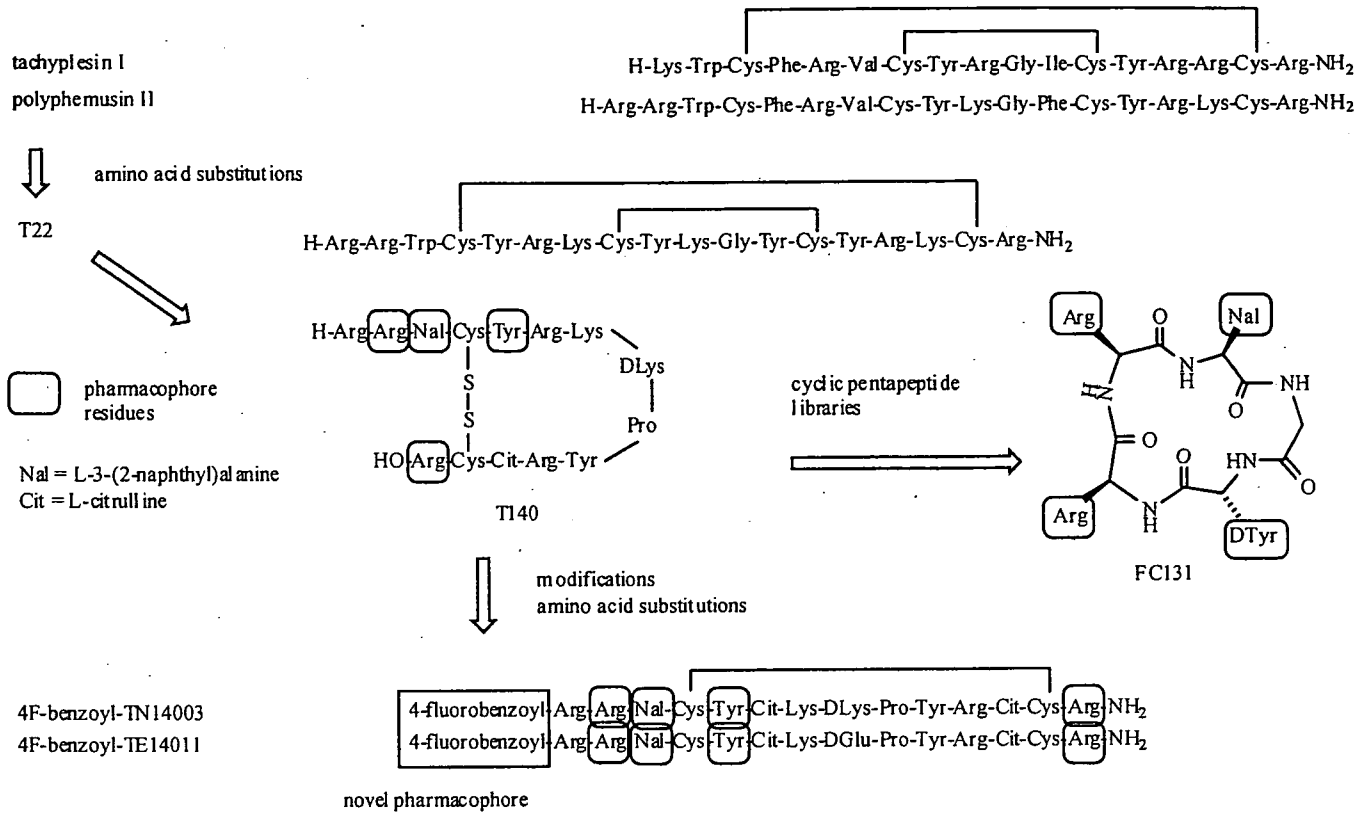

Fig. (2). Development of CXCR4 antagonists based on horseshoe crab peptides. Disulfide bridges are shown by solid lines.

3) Delaying Effecting Against the Generation of Drug-Resistant Strains of HIV

As the clinical chemotherapy of AIDS or HIV-1-infected patients, "Highly active anti-retroviral therapy (HAART)", which adopts two (or three) agents from two conventional drug categories that are composed of reverse transcriptase inhibitors and protease inhibitors, has brought us a great success to date. However, there still remain several serious problems with HAART, involving the emergence of viral strains with multi-drug resistance (MDR), considerable adverse effects and high costs [54,55]. An ideal therapeutic approach would suppress the generation of drug-resistant strains of HIV. The T140 analog also exhibited remarkable delaying effect against the generation of drug-resistant strains in *in vitro* passage experiments using cell cultures [56]. The difficulty of the generation of drug-resistant strains would be a useful advantage for development of T140 analogs in clinical chemotherapy. A useful review article referring to CCR5 antagonists and gp41-targeting fusion inhibitors are reported elsewhere [57,58].

4) Anti-RA Activity

RA is an annoying disease, which requires novel chemotherapy. Inflammatory cytokines, such as tumor necrosis factor (TNF)-α, IFN-γ, IL-1 and IL-6, play critical roles in the synovium of clinical RA patients [31]. The development of biological drugs directed to these cytokines, such as monoclonal antibodies, has produced significant and useful results in clinical RA therapy. However, their curative effects have not yet reached a perfect stage. The development of other drugs that are independent on the above cytokines is desired for the improvement of chemotherapy. Thus, ablation of CXCR4 has become a great expectation. As *in vivo* mouse experimental models of the cellular and humoral immune responses, delayed-type hypersensitivity (DTH) reaction induced by sheep red blood cells (SRBC) and collagen-induced arthritis (CIA) were adopted, respectively, for evaluation of the activity of 4F-benzoyl-TN14003 [59]. Subcutaneous (s.c.) injection of 4F-benzoyl-TN14003 in mice using an Alzet osmotic pump (DURECT Corp., Cupertino, CA, USA) significantly attenuated the footpad swelling after challenge as the DTH response in a dose-dependent manner. Furthermore, treatment of mice with 4F-benzoyl-TN14003 using an Alzet osmotic pump (s.c.) after the bovine type II collagen (CII) emulsion booster showed significant reduction 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 cellular and humoral immune responses. CXCR4 antagonists such as T140 analogs might also be promising leads for anti-RA agents.

5) Anti-Metastatic Activity Against Breast Cancer

Experimental data suggesting that the CXCL12/CXCR4 axis might determine the metastatic destination of tumor cells and cause organ preferential metastasis were initially obtained in experimental metastasis models of breast cancer by Müller *et al.* [10]. They found 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, and that pulmonary metastasis of breast cancer cells was practically inhibited by neutralization using anti-CXCR4 antibodies in mice [10]. Thus, we investigated whether T140 analogs inhibit migration of breast cancer cells *in vitro* and metastasis of breast cancer cells *in vivo* to evaluate the potency of CXCR4 antagonists as anti-cancer-metastatic agents [26]. T140 analogs inhibited CXCL12-induced migration of a CXCR4-positive human breast carcinoma cell line MDA-MB-231 in dose-dependent manners in cell migration assays using cell culture chambers. Furthermore, inhibitory effect of the bio-stable CXCR4 antagonist, 4F-benzoyl-TN14003, was confirmed 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. Treatment of mice with s.c. injection of 4F-benzoyl-TN14003 using an Alzet osmotic pump caused an effective suppression of tumor accumulation on lung surface derived from the MDA-MB-231 metastasis. According to quantitative analyses of tumor area, 4F-benzoyl-TN14003 significantly reduced pulmonary metastasis of MDA-MB-231 cells in mice. This result suggests that CXCR4 antagonists, such as T140 analogs, can be promising anti-metastatic agents.

6) Effects Against Melanoma, Multiple Myeloma, Pancreatic Cancer and Small Cell Lung Cancer

As an alternative administration way, s.c. injection of biodegradable poly D,L-lactic acid (PLA) microcapsules was adopted in place of injection using Alzet osmotic pumps. A controlled release of drugs by PLA microcapsules is more suitable for practical and clinical utilities. A steady release by PLA microcapsules containing 4F-benzoyl-TE14011 was performed in mouse experimental pulmonary metastasis models of CXCR4-positive B16-BL6 melanoma cells [12]. 4F-benzoyl-TE14011 was steadily released from the microcapsules for a long period, leading to maintenance of a sufficient concentration of 4F-benzoyl-TE14011 in bloods. A single s.c. administration of 4F-benzoyl-TE14011-PLA significantly reduced the number of metastatic colonies of B16-BL6 cells. In the case of melanoma metastasis, the PLA microcapsules caused more effective results than Alzet osmotic pumps. As a result, a controlled release of CXCR4 antagonists might cause the efficient suppression of cancer metastasis. Murakami *et al.* previously showed that an excessive expression of CXCR4 remarkably enhanced the metastatic accumulation of B16 melanoma cells in mice lungs, and that our CXCR4 antagonist T22 inhibited pulmonary metastasis in mice injected with B16 cells, which were transduced with CXCR4 [60]. We confirmed the potency of T140 analogs against pulmonary metastasis in mice injected with B16 cells, which are not transduced with CXCR4 [12].

The CXCL12 level is correlated to the expression of multiple radiological bone lesions in patients with multiple myeloma [61]. Thus, CXCL12 is thought to play a potential role in the recruitment of osteoclast precursors to the bone marrow and their activation. 4F-benzoyl-TE14011 significantly blocked both CXCL12-mediated and the myeloma plasma cell line (RPMI-8226) conditioned medium-stimulated osteoclast activity *in vitro* [19], suggesting that blockade of the CXCL12/CXCR4 axis might be an effective chemotherapy against osteolysis in patients with multiple myeloma.

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), whereas CXCL12 mRNA is expressed in pancreatic cancer tissues [13]. CXCL12 stimulated both migration and invasion of AsPC-1, PANC-1 and SUIT-2 (pancreatic cancer cell) in a dose-dependent manner *in vitro*, suggesting that the CXCL12-CXCR4 interaction is involved in cancer cell progression and metastasis. CXCL12-induced migration and invasion of these cells were suppressed by T140 analogs in dose-dependent manners [14]. The CXCL12 treatment of PANC-1 cells caused a remarkable increase in actin cytoskeleton (polymerization), which might lead to the invasion of malignant cells into tissues and subsequent metastasis. The phenomenon was effectively inhibited by T140 analog.

SCLC constitutes a fourth-fifth of lung cancer, which is a leading cause of death in European countries [62]. CXCL12 is constitutively secreted from marrow stromal cells and plays critical roles in homing of hematopoietic cells to the marrow. Primary tumor cells in SCLC patients highly express CXCR4. Burger *et al.* found that CXCL12 stimulated invasion of SCLC cells into extracellular matrix and firm adhesion to marrow stromal cells, which were effectively inhibited by T140 *in vitro* [21]. It suggests that the CXCL12-CXCR4 axis is also involved in SCLC metastasis. Furthermore, 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. The CXCL12-CXCR4 interaction causes activation of $\alpha 2$, $\alpha 4$, $\alpha 5$ and $\beta 1$ integrins, which was suppressed by T140 analog. They also found that stromal cells protected SCLC cells from anti-cancer drug (etoposide)-induced apoptosis, and that this protection was abrogated by T140 analog [63]. Thus, a combinational use of T140 analogs and anti-cancer drugs might be useful for subjugation of CXCL12-mediated CAM-DR in SCLC.

CXCR4 is expressed on various malignant cells in at least 23 different types of cancers including the above cancers at the point of 2004 [64]. CXCR4 antagonists such as T140 analogs might be promising leads for the development of anti-metastatic agents against the above cancers.

7) Anti-CLL and -ALL Activities

In European countries, B cell CLL, which is caused by the accumulation of long-lived, monoclonal B malignant cells in bloods, secondary lymphoid organs and bone marrow, is the most common leukemia in adults. CLL B cells highly express CXCR4. On the other hand, marrow stromal cells or nurselike cells constitutively secrete CXCL12, which activates CLL B cells through CXCR4, and protects the cells from apoptosis, followed by their accumulation. Thus, the CXCL12-CXCR4 axis would also

be a therapeutic target for B cell CLL [28]. Practically, T140 analogs suppressed CXCL12-induced chemotaxis of CLL cells, their migration beneath marrow stromal cells and actin polymerization in a dose-dependent manner, *in vitro* [29]. Furthermore, T140 analogs attenuated not only the anti-apoptotic effect caused by CXCL12 but also stromal cell-mediated protection of CLL cells from spontaneous apoptosis. Co-cultivation of CLL cells and marrow stromal cells protected CLL cells from drug (fludarabine)-induced apoptosis, which caused stromal CAM-DR. Treatment with T140 analogs re-sensitized the above CLL cells toward fludarabine-induced apoptosis. T140 analogs might also overcome CAM-DR.

On the other hand, precursor-B (pre-B) cell ALL is thought to be caused by their contact with bone marrow stromal layers using the β_1 integrins. The invasion of the pre-B cells into stromal layers is controlled by the interaction of CXCL12 with CXCR4, which is constitutively and highly expressed on the cells. T140 blocked CXCL12-induced chemotaxis and attenuated the invasion of pre-B cells into bone marrow stromal layers *in vitro*. In addition, T140 analog enhanced the antiproliferative effects of the other anti-cancer agents, including vincristine and dexamethasone, suggesting that T140 analogs might overcome CAM-DR in ALL [30].

T140 ANALOGS AS INVERSE AGONISTS

Generally, antagonists are classified into two categories that are composed of inverse agonists that show no agonistic activity at all and partial agonists that show weak agonistic activity. Our collaborators, Zhang *et al.*, revealed that T140 decreases autonomous signaling through CXCR4 wild type and its constitutively active mutant (CAM), and that T140 is an inverse agonist although a bicyclam AMD3100 is a partial agonist [65]. Inverse agonists such as T140 analogs,

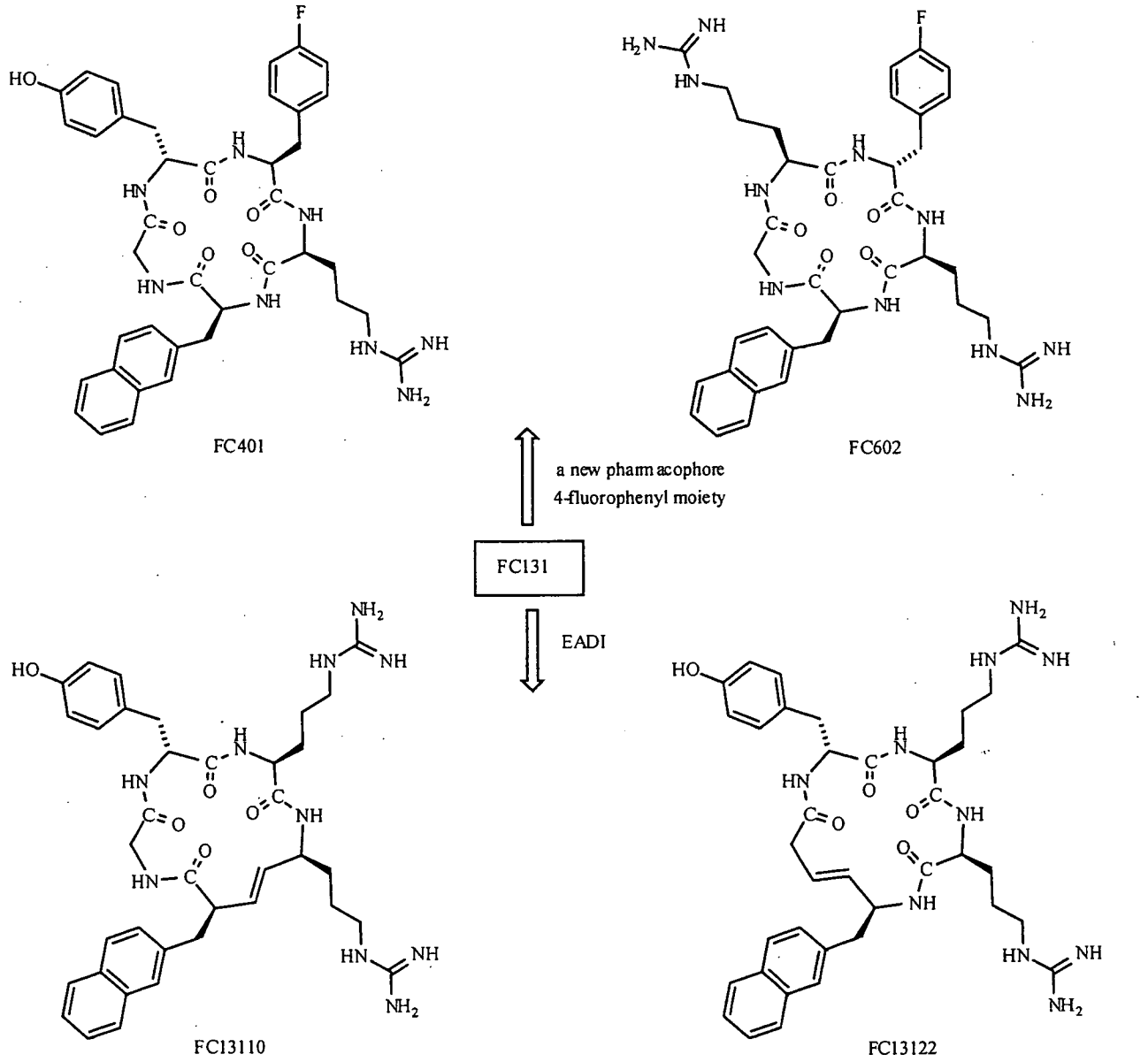

Fig. (3). Structures of L/D-Phe(4-F)-containing FC131 analogs, FC401 and FC602, and EADI-containing analogs, FC13110 and FC13122.

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. On the other hand, 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.

DOWNSIZING OF T140 ANALOGS BASED ON CYCLIC PENTAPEPTIDE TEMPLATES

Since T140 is a 14-mer peptide, its downsizing would be desirable. 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 described in the previous section [48]. These residues are located in close proximity to each other in the spatial structure, as shown in conformational analysis by NMR [47]. As such, the pharmacophore-guided approach can be adopted to downsize T140. Cyclic pentapeptides have been used as useful conformational-constrained templates disposing functional groups by medicinal chemists [66-71]. Thus, 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 bring us efficient discovery of 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. (2)) [72]. NMR and simulated annealing molecular dynamics (SA-MD)

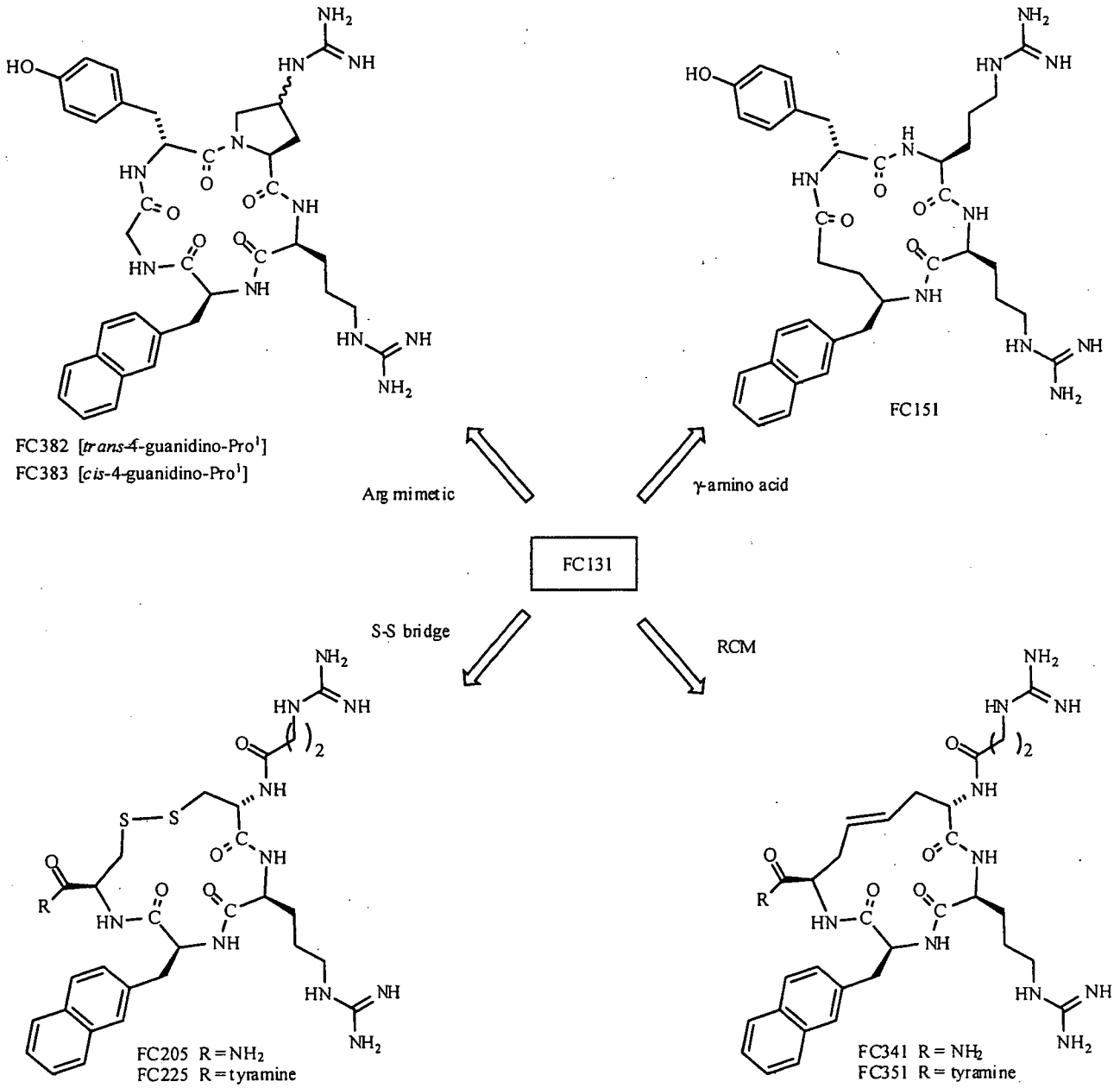

Fig. (4). Structures of cyclic peptides containing conformationally constrained Arg mimetics (FC382 and FC383), a γ-amino acid-containing cyclic peptide (FC151) and cyclic peptides bridged by a disulfide bond (FC205 and FC225) and by an olefin using RCM (FC341 and FC351).

analysis revealed that FC131 assumed an almost symmetrical pentagonal backbone structure.

Furthermore, a novel pharmacophore such as a 4-fluorophenyl moiety, which was identified in the development of 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011 as described in the previous section [51], was introduced into cyclic pentapeptides. Since the 4-fluorophenyl group could not be substituted for the phenol group of D-Tyr^5 with maintenance of high activity, the 4-fluorophenyl group was incorporated into the amino acid at positon 1. [4-Fluorophenylalanine (Phe(4-F))^1]-FC131, FC401, showed significant CXCR4-binding activity (Fig. (3)) [73]. Next, since two Arg residues are thought to be indispensable for high activity and an aromatic residue [L/D-Phe(4-F)] was incorporated into position 1, L/D-Arg^5 was substituted for D-Tyr^5. Among four analogues [L/D-Phe(4-F)^1, L/D-Arg^5]-FC131, [D-Phe(4-F)^1, Arg^5]-FC131, FC602, showed the most potent activity, which is 10-fold more potent than [D-Tyr^1, Arg^5]-FC131 [73]. Thus, FC602 might be useful as a novel lead that is composed of the pharmacophores different from FC131. Development of other promising leads based on cyclic pentapeptide templates is in progress.

In the next step, reduction of the peptide character of the obtained cyclic pentapeptides was performed. Substituition of (*E*)-alkene dipeptide isosteres (EADIs) [74-77] for constituent backbone amide bonds is a common strategy for this purpose. Several FC131 analogs, such as FC13110 and FC13122, in which the above isosteres were substituted for the backbone amide bonds, were synthesized by our reported methodologies, which utilize the combination of stereoselective aziridinyl ring-opening reactions and organozinc-copper-mediated reactions [78-80] (Fig. (3)). In addition, replacement by reduced amide-type dipeptide isosteres (RADIs) was also investigated as comparative studies. As a result, structure-activity relationship studies by substitution of these isosteres provided useful information for design of non-peptide antagonists based on FC131 [81].

Conformationally constrained Arg mimetics, *trans*-4-guanidino-Pro and *cis*-4-guanidino-Pro, were incorporated in FC131 with the idea of fixing the backbone and side chain of Arg^1. [*trans*-4-Guanidino-Pro^1]-FC131, FC382, and [*cis*-4-guanidino-Pro^1]-FC131, FC383, showed high CXCR4-antagonistic activities that were twice as strong as that of [g-Dab^1]-FC131 (g-Dab = γ-*N*-amidino-L-2,4-diaminobutyric acid), having the same length of the linear-type side chain of the amino acid at the position 1, while [Pro^1]-FC131 did not show so high activity (Fig. (4)) [82]. In consideration of the fact that the introduction of a pyrrolidinyl ring caused a significant reduction of potency, it is thought that fixing the side chain effectively increased potency. NMR analysis of FC382 and FC383 showed similar dispositions of guanidino groups of *trans*/*cis*-4-guanidino-Pro residues in space [82], which might be the reason for essentially no difference in potency between FC382 and FC383. As the other strategies for development of refined compounds, structural tuning of FC131 was attempted based on several cyclic tetrapeptide-scaffolds. The cyclic pentapeptide, FC131, has Gly^4 as a spacer for cyclization. To reduce the ring size, the Nal^3-Gly^4 sequence of FC131 was replaced by γ-amino acids such as 4-amino-5-naphthalen-2-yl-pentanoic acid (γ-Nal) and 4-amino-5-naphthalen-2-yl-pent-2-enoic acid (γ-(*E*)-Nal). Among these γ-amino acid-containing cyclic tetrapeptides, an analog with substitution of γ-Nal for Nal^3-Gly^4, FC151, showed high CXCR4-antagonistic activity (Fig. (4)) [82]. This result suggests that the Gly residue and the amide bond of the Nal-Gly sequence are not necessary for high potency. On the other hand, to optimize the ring structures, templates different from those of peptides cyclized in peptide backbones were investigated. Since the four pharmacophore residues of T140 are located in close vicinity each other due to its disulfide bridge and cyclic peptides having the Arg-Arg-Nal sequence, such as FC131, possess high CXCR4-antagonistic activity, we have designed and prepared disulfide-bridged cyclic peptide libraries having the *N*-3-guanidinopropanoyl-L/D-Cys(S-)-L/D-Arg-L/D-Nal-L/D-Cys(S-)-NH_2 (or –tyramine) sequence (total 32 compounds). Among these libraries, 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] were found as significant CXCR4 antagonists (Fig. (4)) [82]. Since FC205 and FC225 have a common combination of chiralities of composed amino acids, both compounds might form similar conformations. Furthermore, cyclic analogs, where olefin bridges were substituted for disulfide bridges in FC205 and FC225, were synthesized using ring-closing metathesis (RCM). These olefin-bridged peptides, FC341 and FC351, showed moderate CXCR4-antagonistic activity (Fig. (4)) [82]. Further downsizing and structural tuning are now in progress.

OTHER LOW MOLECULAR WEIGHT CXCR4 ANTAGONISTS

In addition to our T22/T140-related compounds, several low molecular weight CXCR4 antagonists have been reported to date [83,84]: *i.e.* AMD series (AnorMED, Inc.) including a bicyclam AMD3100 [85], an *N*-pyridinylmethylene cyclam (monocyclam) AMD3465 [86], a non-cyclam AMD8665 [87] and AMD070 [88], ALX40-4C (Ac-[D-Arg]$_9$-NH_2; NPS Allelix) [89], CGP64222, R3G, NeoR [90-92], a distamycin analog, NSC651016 [93], a flavonoid compound, ampelopsin [94] etc. Double-functional drugs based on AMD3100 and galactosylceramide (GalCer) analog conjugates and orally bioavailable agents, KRH-1636 and KRH-2731 (Kureha Chemical & Sankyo), were also developed [95,96]. A review referring to the above compounds is reported elsewhere in detail [97]. Furthermore, a dual CCR5/CXCR4 antagonist AMD3451, which has antiviral activity against R5, R5/X4(dual-tropic) and X4-HIV-1 strains, was also developed [98].

CONCLUSIONS AND PERSPECTIVES

GPCRs such as chemokine receptors are principal targets in recent proteomics studies, and their selective ligands become useful probes for chemical biology studies [99]. The authors have developed selective antagonists against CXCR4, which is an important chemokine receptor in terms of physiology as well as pathology. HIV-entry inhibitors, T22 and T140, which inhibit an X4-HIV-1 entry to T-cells through their specific binding to the coreceptor CXCR4,

have been developed. These peptides were also identified as multiple agents possessing anti-cancer-metastasis, anti-leukemia and anti-RA activities. Downsizing of T140 was achieved to develop a cyclic pentapeptide FC131. Structural tuning and reduction of peptide character based on FC131 were explored to develop new useful leads. We must recognize that CXCR4 plays physiological roles in embryogenesis, homeostasis and inflammation in fetus, particularly in embryonic development of hemopoietic, cardiovascular and central nervous systems, and that risky reactions derived from blocking the CXCL12-CXCR4 axis can be occurred toward living human bodies. Under the above careful consideration, these CXCR4 antagonists might be used as promising agents for clinical chemotherapy of AIDS/HIV infection, cancer metastasis, leukemia progression and RA. In addition, a combined use of CXCR4 antagonists with other entry inhibitors such as CCR5 antagonists and fusion inhibitors might improve clinical chemotherapy of HIV infection and AIDS. Furthermore, recent papers suggest that CXCL12 and CXCR4 have parallel effects in the immune and nervous systems [100]. Thus, the development of CXCR4 antagonists might provide useful therapeutic strategies for the treatment of immunological and neurological disorders associated with CXCR4. In future, more investigational researches on development of optimized CXCR4 antagonists and efficient administration routes might bring us great perspective in the chemotherapy of multiple CXCR4-relevant diseases.

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Inhibitors of the Chemokine Receptor CXCR4: Chemotherapy of AIDS, Metastatic Cancer, Leukemia and Rheumatoid Arthritis

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Abstract: The interaction between the chemokine, CXCL12 and its receptor, CXCR4 is known to be involved in several intractable disease processes, including HIV infection, cancer cell metastasis, leukemia cell progression and rheumatoid arthritis. It is conjectured that this interaction may be an important therapeutic target in all of these diseases, and various CXCR4 antagonists have been proposed as potential drugs. This article describes the development in our laboratory of a number of specific CXCR4 antagonists.

Keywords: Cancer metastasis, Chemokine receptor, HIV infection, Leukemia cell progression, Low molecular weight CXCR4 antagonist, Rheumatoid arthritis.

INTRODUCTION

Transmembrane proteins are attractive targets for drug development. The interaction between CXCR4, a G-protein-coupled receptor and CXCL12/stromal cell-derived factor-1 (SDF-1), a unique ligand for CXCR4 [1-4] is essential to the migration of progenitor cells during embryonic development of the cardiovascular, hemopoietic, and central nervous systems. Differences in the expression level of CXCR4 among central memory, effector memory and $CD28^-$ $CD4^+$ T cells have significant affect on the homing of T cells in peripheral lymphoid organs [5]. Up-regulation of CXCR4 expression by common-gamma chain-dependent interleukins enhances this homing [6]. It is known that the CXCR4-CXCL12 pair is involved in various disease processes such as HIV infection [7], cancer cell metastasis [8-25], leukemia cell progression [26-28] and rheumatoid arthritis [29].

CXCR4 was initially identified as a second cellular receptor (co-receptor) of T cell line-tropic (X4-) HIV-1 entry through its association with CD4 [7]. In the late stages of HIV infection, X4 HIV-1 strains become dominant, while macrophage-tropic (R5-) HIV-1 strains, which use the chemokine receptor CCR5 as another co-receptor, are major in early stages [30-34]. Recently, it has also been reported that CXCR4 is expressed on the surfaces of several types of cancer cell, and that CXCL12 is highly expressed in internal organs that are the primary goals of cancer cell metastasis [8-14]. It has been shown that the CXCL12-CXCR4 axis is associated with metastasis of several types of cancer including cancer of the breast, pancreas, prostate, kidney, and lung and melanoma, neuroblastoma, non-Hodgkin's lymphoma, ovarian cancer, multiple myeloma and malignant brain tumors [8-25]. This axis is also correlated with the progression of chronic lymphocytic leukemia (CLL) B-cells and precursor-B (pre-B) acute lymphoblastic leukemia (ALL) cells [26-28]. Rheumatoid arthritis (RA) is caused mainly by $CD4^+$ memory T cell accumulation in the inflamed synovium. Nanki *et al.* reported that CXCR4 is highly expressed on the surface of memory T cells and CXCL12 concentration is extremely elevated in the synovium of RA patients. Further, CXCL12 stimulates migration of the memory T cells and thereby inhibits T cell apoptosis. This indicates that the CXCR4-CXCL12 interaction plays a crucial role in the accumulation of T cells in the RA synovium [29]. In the light of these observations, CXCR4 appears to be an attractive therapeutic target for these diseases and in this review article, our recent research concerning the development of CXCR4 antagonists is discussed.

SELECTIVE INHIBITION OF X4-HIV-1 ENTRY USING CXCR4 ANTAGONISTS DERIVED FROM T140

Highly active anti-retroviral therapy (HAART) is frequently used as a clinical treatment for HIV-1-infected patients. In HAART, a combination is used of two or three different agents comprising reverse transcriptase inhibitors and protease inhibitors. HAART shows significant positive effects, but fails to resolve some serious problems. These include the emergence of the viral strains with multiple drug resistance (MDR), considerable adverse effects and the high cost [35, 36]. The molecular mechanism of HIV-1 replication has been elucidated in detail, especially for a dynamic supramolecular mechanism relevant to HIV entry/fusion process, suggesting that blocking of HIV entry/fusion is a more effective strategy for HIV infection/AIDS therapy. At the first stage, an envelope protein gp120 on the viral surface binds to a cell surface protein CD4. This induces a conformational change of gp120 and its subsequent binding to a co-receptor, CCR5 [30-34] or CXCR4 [5]. The interaction between gp120 and

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each of the co-receptors leads to penetration of another envelope subunit, gp41, which anchors the HIV envelope from the *N*-terminus end on the membrane. Finally, the formation of a "trimer-of-hairpins" structure of gp41 causes membrane fusion of HIV to the cells resulting in completion of the infection [37]. Elucidation of the above dynamic molecular mechanism of the fusion process has encouraged many researchers to develop inhibitors aimed at blocking HIV-entry or fusion. These generally target the co-receptor, CCR5 or CXCR4, and the dynamic process involving formation of the gp41 trimer-of-hairpins structure. Enfuvirtide (DP-178, T-20, Fuzeon, Trimeris & Roche), a 36-mer peptide derived from the *C*-terminal helical region of gp41, has been used clinically as a fusion inhibitor targeting MDR HIV-1 strains [38]. Our research has focused on a search for drugs that target CXCR4 (Fig. 1). Antagonists of another co-receptor, CCR5, and fusion inhibitors targeting gp41, have been reviewed elsewhere [39].

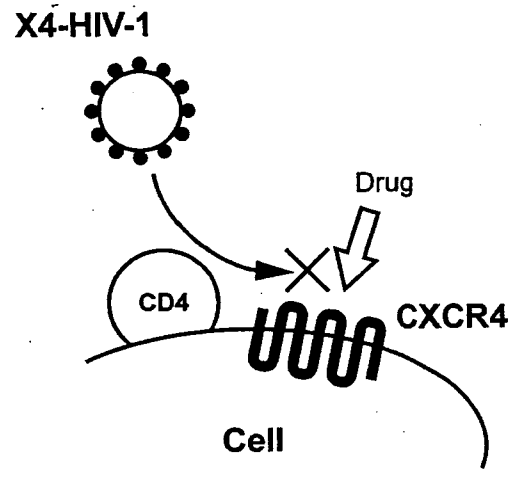


Fig. (1). Identification of the chemokine receptor CXCR4 as a co-receptor of X4 HIV entry and inhibitors targeting CXCR4.

The tachyplesins and polyphemusins, antibacterial and antiviral peptides, were isolated from the hemocyte debris of the Japanese horseshoe crab (*Tachypleus tridentatus*) and the American horseshoe crab (*Limulus polyphemus*) respectively, which are 17-mer and 18-mer peptides, used in self-defense (Fig. 2) [40, 41]. Our earlier structure-activity relationship studies of these peptides led to the development of T22 ([$Tyr^{5, 12}$, Lys^{7}]-polyphemusin II) [42, 43] and its shorter 14-mer peptide, T140, which possesses anti-HIV activity (Fig. 2) [44]. T22 and T140 block X4-HIV-1 entry to the cell effectively by binding specifically to CXCR4, and inhibit Ca^{2+} mobilization induced by CXCL12 stimulation through CXCR4 [45-47]. The T140 analogue exhibited a remarkable delaying of the appearance of drug-resistant strains in *in vitro* passage experiments using cell cultures [48], and it was presumed that the development of T140 analogues 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 Cys^{4} and Cys^{13} and connected by a type II' β-turn [49]. Four amino acid residues in T140, Arg^{2}, Nal^{3} (L-3-(2-naphthyl)alanine), Tyr^{5} and Arg^{14}, were identified as residues indispensable for significant activity [50].

T140 is unstable in mouse/feline serum or in rat liver homogenate [51, 52]. When deletion of indispensable residues (Arg^{14} in serum; Arg^{2}, Nal^{3} and Arg^{14} in liver homogenate) from the *N*- or the *C*-terminus occurs, the efficacy of degraded peptides T140 is drastically diminished. Modification of T140 analogues at each terminus suppresses the biodegradations and leads to development of novel and effective compounds, which shows highly CXCR4-antagonistic activity and increased biological stability. Furthermore, the *N*-terminal modification studies 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 analogues, 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011, which contain an *N*-terminal 4-fluorobenzoyl moiety, have anti-HIV activity two orders of magnitude higher than that of T140 and enhanced stability in serum/liver homogenates (Fig. 2) [53].

Anti-metastasis activity of T140 analogues against breast cancer, melanoma, pancreatic cancer and small cell lung cancer.

Müller *et al.* reported that 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, the common metastatic destinations of breast cancer. This suggests that the CXCL12-CXCR4/CCR7-CXCL12 axis might determine the metastatic destination of tumor cells and cause organ-preferential metastasis [8]. Practically, metastasis of breast cancer cells to the lung in mice can be inhibited by neutralizing CXCR4 with anti-CXCR4 antibodies. To evaluate the potency of CXCR4 antagonists as anti-metastatic agents, we investigated the inhibitory activity of our T140 analogues against the migration of breast cancer cells *in vitro* and metastasis of breast cancer cells *in vivo* [24]. In cell migration assays using cell culture chambers, the T140 analogues dose dependently inhibit the migration of a CXCR4-positive human breast carcinoma cell line MDA-MB-231 induced by CXCL12. The inhibitory effect of the bio-stable CXCR4 antagonist, 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. Mice subcutaneously injected with an Alzet osmotic pump (DURECT Corp., Cupertino, CA, USA) with 4F-benzoyl-TN14003 show an effective suppression of tumor accumulation on the lung surface as a result of MDA-MB-231 metastasis. This suggests that small molecule CXCR4 antagonists, such as T140 analogues, could replace anti-CXCR4 antibodies as neutralizers of metastasis of breast cancer.

Murakami *et al.* reported that an excessive expression of CXCR4 drastically enhances the metastatic accumulation of B16 melanoma cells in mouse lung, and that the CXCR4 antagonist T22 blocks pulmonary metastasis in mice injected with CXCR4-transduced B16 cells Åm54Ån. We investigated whether T140 analogues inhibit pulmonary metastasis in mice injected with B16 cells, which are not transduced with CXCR4 [10]. Poly D,L-lactic acid (PLA) microcapsules containing 4F-benzoyl-TE14011 was used in

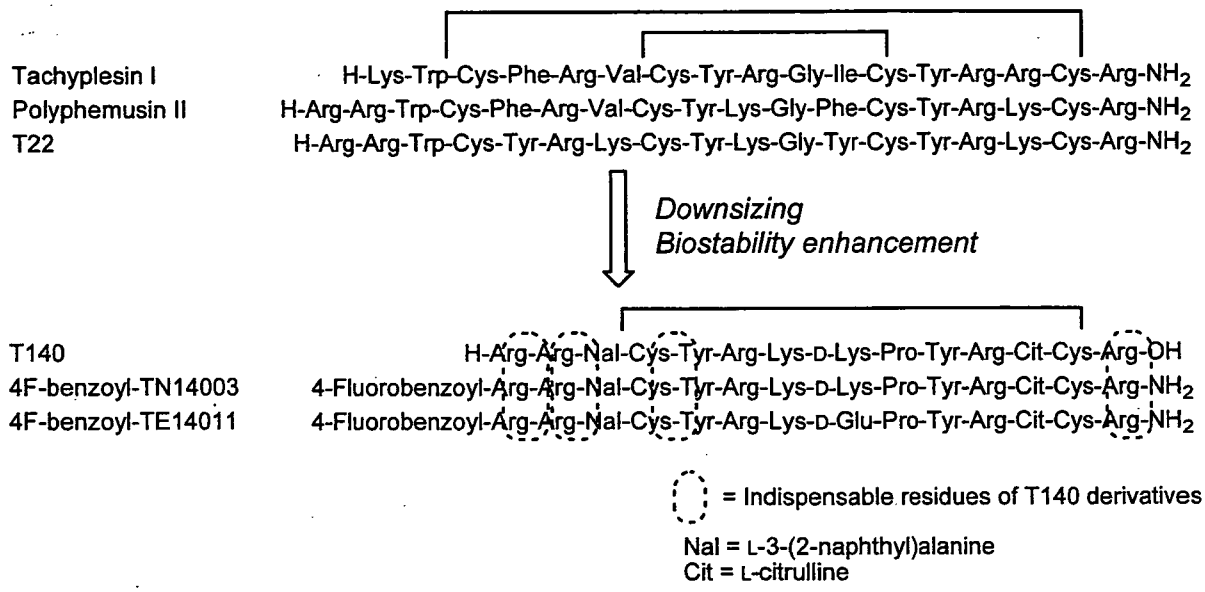


Fig. (2). Development of the CXCR4 antagonist T140 and its biologically stable analogues, 4F-benzoyl-TN14003 and 4F-benzoyl-TE14011, based on horseshoe crab peptides. Disulfide bridges are shown by solid lines.

experimental metastatic models of CXCR4-positive B16-BL6 melanoma cells [10]. This T140 analogue is released in a controlled fashion from the PLA microcapsules for a long period *in vivo*, maintaining 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, suggesting that a controlled release of CXCR4 antagonists might lead to effective suppression of cancer metastasis.

CXCL12 could play a potential role in the recruitment and activation of osteoclast precursors to the bone marrow because the CXCL12 level is correlated to the expression of multiple radiological bone lesions in individuals with multiple myeloma. It was observed that 4F-benzoyl-TE14011 significantly inhibits CXCL12-mediated osteoclast activity in addition to the osteoclast activity stimulated by the medium conditioned by myeloma plasma cell line (RPMI-8226) *in vitro* [17]. This suggests that blockade of the CXCL12-CXCR4 axis might be an effective remedy 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) [11]. Stimulation by CXCL12 induces both migration and invasion of pancreatic cancer cells, AsPC-1, PANC-1 and SUIT-2, in dose-dependent manners *in vitro*, suggesting that the interaction between CXCL12 and CXCR4 is related to pancreatic cancer cell progression and metastasis. The migration and invasion of these cells caused by CXCL12 stimulation are dose-dependently suppressed by T140 analogues [12]. Treatment of PANC-1 cells with CXCL12 induces a drastic increase in actin polymerization (cytoskeleton), leading to the invasion and subsequent metastasis of malignant cells into tissues. The T140 analogue inhibits this phenomenon effectively.

Small cell lung cancer (SCLC) which comprises 20-25% of lung cancers is the leading cause of death in Western countries [55]. CXCL12 is constitutively secreted by marrow stromal cells and plays an important role in homing of hematopoietic cells to the marrow. CXCR4 is highly expressed on the surface of primary tumor cells isolated from SCLC patients. Burger *et al.* showed that CXCL12 stimulated both SCLC cell invasion into the extracellular matrix, and firm adhesion to marrow stromal cells. Such invasion and adhesion are effectively suppressed by T140 *in vitro*, confirming the involvement of the CXCL12-CXCR4 interaction in SCLC metastasis [19]. Adhesion of SCLC cells to the extracellular matrix or to accessory cells within the tumor microenvironment causes cell adhesion-mediated drug resistance (CAM-DR) *via* integrin signaling. CXCL12 was found to induce activation of α_2, α_4, α_5 and β_1 integrins through CXCR4, which in turn is inhibited by the T140 analogue. It was shown that stromal cells protect SCLC cells from apoptosis induced by an anti-cancer drug (etoposide), and that this protection is inhibited by the T140 analogue [56]. Thus the use of T140 analogues in combination with anti-cancer drugs might overcome CXCL12-mediated CAM-DR in SCLC.

CXCR4 is expressed in malignant cells in at least 23 different types of cancers [57], including those discussed above. Antagonists of CXCR4 such as the T140 analogues might be useful lead compounds for the development of anti-metastatic agents in several types of cancer.

EFFECT OF T140 ANALOGUES AGAINST CLL AND ALL

B cell CLL, the most common leukemia in adults in Western countries, is caused by the accumulation of long-lived, monoclonal B malignant cells in blood, secondary lymphoid organs and bone marrow. CLL B cells that express CXCR4 highly are activated by CXCL12 released

from marrow stromal cells or nurselike cells. CXCL12 rescues the CLL B cells from apoptosis and contributes to their accumulation. Consequently, the CXCL12-CXCR4 system should also be a therapeutic target in B cell CLL [26]. In fact, T140 analogues inhibit chemotaxis of CLL cells induced by CXCL12, their migration beneath marrow stromal cells and actin polymerization in dose-dependent manners, *in vitro* [27]. In addition, T140 analogues reduce the anti-apoptotic effect of CXCL12 and prevent stromal cells from protecting against spontaneous apoptosis of CLL cells. Co-cultures of CLL cells and marrow stromal cells protect CLL cells from apoptosis induced by a drug (fludarabine) followed by causing stromal CAM-DR. Treatment with T140 analogues re-sensitizes these CLL cells to fludarabine-induced apoptosis. Thus, T140 analogues might be able to overcome CAM-DR which is a serious problem in the clinical chemotherapy of CLL.

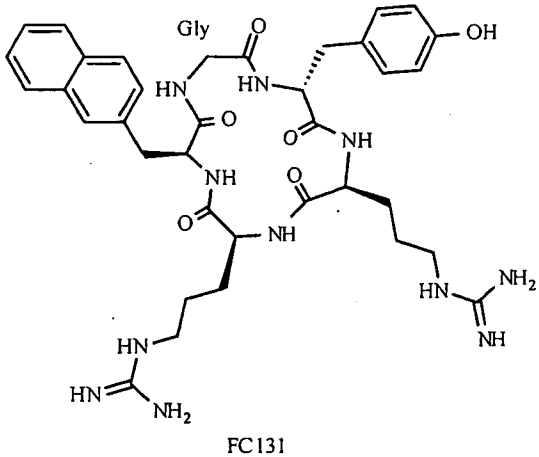
On the other hand, growth and survival of pre-B cell ALL might be caused by intimate contact with bone marrow stromal layers using the β_1 integrins. Migration of these pre-B cells into stromal layers is regulated by CXCL12; CXCR4 has been uniformly and highly expressed in the cells. T140 blocks CXCL12-induced chemotaxis and attenuates the migration of pre-B ALL cells into bone marrow stromal layers. In addition, T140 analogues enhance the cytotoxic and anti-proliferative effects of other anti-cancer agents such as vincristine and dexamethasone, suggesting that T140 analogues might overcome CAM-DR in ALL chemotherapy [28].

ANTI-RA ACTIVITY OF A BIO-STABLE T140 ANALOGUE

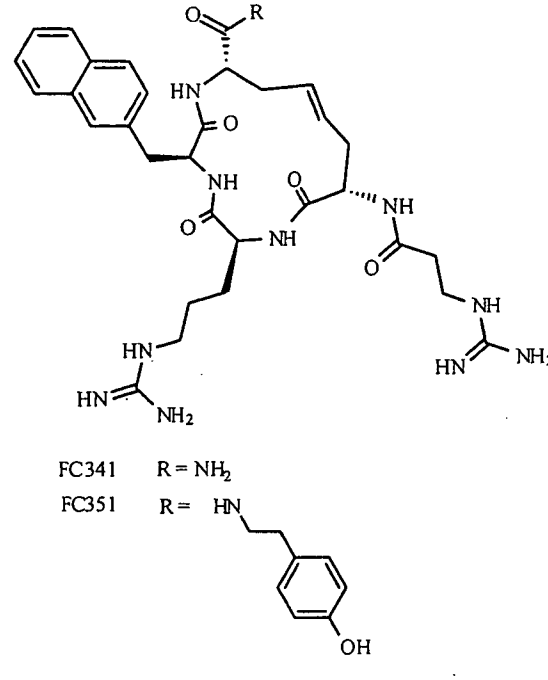
Inflammatory cytokines, such as tumor necrosis factor, (TNF)-α, interferon, IFN-γ, and the interleukins, IL-1 and IL-6, play an important role in RA [29]. The development of biological drugs such as monoclonal antibodies, that target these cytokines, has produced useful results in clinical RA therapy, but complete curative effects have not yet been achieved. To improve chemotherapy for RA, other drugs independent of the functions of these cytokines are required. As an *in vivo* experimental model of the cellular immune response, evaluation of the 4F-benzoyl-TN14003 activity, delayed-type hypersensitivity (DTH) reaction induced by sheep red blood cells (SRBC) was performed [58]. Subcutaneous injection of 4F-benzoyl-TN14003 using an Alzet osmotic pump significantly suppressed the footpad swelling (the DTH response) in a dose-dependent manner. Collagen-induced arthritis (CIA) in mice was adopted as the second *in vivo* experimental RA model. It was observed that several symptoms of arthritis (score increase, body weight loss, ankle swelling and limb weight gain) were markedly suppressed. At the same time, 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 shows a blockade effect to the humoral immune response to CII. These results suggest that CXCR4 antagonists such as T140 analogues might also be useful leads for anti-RA agents.

CYCLIC PEPTIDES WITH CXCR4 ANTAGONISTIC ACTIVITY DERIVED FROM T140

As described in the previous section, Arg^2, Nal^3, Tyr^5 and Arg^{14} of T140 are indispensable to high antagonistic activity toward CXCR4 [50]. These residues are located in close proximity to each other in an antiparallel β-sheet structure. Downsizing of T140 analogues was attempted with a pharmacophore-guided approach using cyclic pentapeptide libraries with two L/D-Arg, L/D-Nal and L/D-Tyr in addition to Gly as a spacer. This led to FC131 [*cyclo*(-Arg^1-Arg^2-Nal^3-Gly^4-D-Tyr^5-)], which showed strong



FC131



FC341 R = NH_2

FC351 R =

Fig. (3). Structures of a cyclic pentapeptides FC131, FC341 and FC351.

CXCR4-antagonistic activity comparable to that of T140 (Fig. 3) [59]. Structural analysis of FC131 by NMR and simulated annealing molecular dynamics (SA-MD) revealed the near-symmetrical pentagonal backbone structure shown in Fig. 3.

In the interests of bioavailability, reduction of the peptide character of FC131 was pursued by introduction of (*E*)-alkene dipeptide isosteres (EADIs) [60-63]. Several FC131 analogues, in which such isosteres were substituted for the amide bond backbones, were developed on the basis of the synthetic strategy reported previously [64-66]. Structure-activity relationship studies of the isostere-substituted compounds provided useful information concerning the reduction of peptide character [67].

Several cyclic tetrapeptide-scaffolds have been prepared and investigated with a view to tuning the spatial arrangement of pharmacophores. FC151, a γ-amino acid-containing peptide containing 4-amino-5-naphthalen-2-yl-pentanoic acid in the place of the Nal-Gly sequence, various 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], all showed significant CXCR4-antagonistic activity. The cyclic compounds FC341 and FC351, which contain an olefin bridge formed by ring-closing metathesis (RCM) exhibited moderate CXCR4-antagonistic activity [68]. Attempts at further downsizing and structural tuning are currently in progress.

The 4-fluorophenyl moiety identified as a pharmacophoric element through the *N*-terminal modification study of T140 analogues [53] was introduced into cyclic pentapeptides. Since the phenol group of D-Tyr^5 could not be replaced by the 4-fluorophenyl group, the 4-fluorophenyl group was incorporated into position 1 and the resulting compound, FC401 ([Phe(4-F)^1]-FC131) shows significant CXCR4-binding activity [69]. Since a second Arg residue is thought to be indispensable for high activity and an aromatic residue [L/D-Phe(4-F)] has been incorporated into position 1, D-Tyr^5 was replaced by L/D-Arg^5. Among four analogues [L/D-Phe(4-F)^1, L/D-Arg^5]-FC131, 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 [69]. Thus, FC602 may be useful as a novel lead different from FC131, but involving the pharmacophore.

Several non-T140-related CXCR4 antagonists have been reported to date [70, 71]. These include AMD3100 with two cyclam groups (AnorMED, Inc.) [72], an *N*-pyridinylmethylene cyclam (monocyclam) AMD3465 (AnorMED, Inc.) [73], AMD8665 without a cyclam group (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], and a flavonoid compound, ampelopsin [81]. AMD3100 and galactosylceramide (GalCer) analogue conjugates have also been reported as doubly-functionalized drugs [82]. KRH-1636 (Kureha Chemical & Sankyo) is an orally bioavailable agent containing *N*-pyridinylmethylene, Arg and Naphthalene moieties [83]. A review of these CXCR4 antagonists but excluding T140-related compounds has been published [84].

CONCLUDING REMARKS

T22 and its downsized analogue T140, developed as HIV-entry inhibitors, binds specifically to the co-receptor CXCR4 and thus inhibit entry of X4-HIV-1 to T-cells. T140 and its analogues also show effective activity against cancer metastasis, leukemia and rheumatoid arthritis. T140 was modified by structural tuning, downsizing and reduction of peptide character in attempt to develop new low-molecular weight CXCR4 antagonists. These antagonists might be promising agents for clinical chemotherapy of HIV infection, cancer metastasis, leukemia progression and RA. Blocking of the CXCL12-CXCR4 axis however is a risky procedure in human subjects 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 an encouraging candidate for clinical chemotherapy of HIV infection and AIDS. Further development of CXCR4 antagonists involving investigation of different administration routes could become important in the chemotherapy of multiple diseases relevant to CXCR4.

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Impact of glycosylation on antigenicity of simian immunodeficiency virus SIV239: induction of rapid V1/V2-specific non-neutralizing antibody and delayed neutralizing antibody following infection with an attenuated deglycosylated mutant

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Infection of rhesus macaques with a deglycosylation mutant, Δ5G, derived from SIV239, a pathogenic clone of simian immunodeficiency virus (SIV), led to robust acute-phase viral replication followed by a chronic phase with undetectable viral load. This study examined whether humoral responses in Δ5G-infected animals played any role in the control of infection. Neutralizing antibodies (nAbs) were elicited more efficiently in Δ5G-infected animals than in SIV239-infected animals. However, functional nAb measured by 90% neutralization was prominent in only two of the five Δ5G-infected animals, and only at 8 weeks post-infection (p.i.), when viral loads were already below 10^4 copies ml^{-1}. These results suggest a minimal role for nAbs in the control of the primary infection. In contrast, whilst Ab responses to epitopes localized to the variable loops V1/V2 were detected in all Δ5G-infected animals at 3 weeks p.i., this response was associated with a concomitant reduction in Ab responses to epitopes in gp41 compared with those in SIV239-infected animals. These results suggest that the altered surface glycosylation and/or conformation of viral spikes induce a humoral response against SIV that is distinct from the response induced by SIV239. More interestingly, whereas V1/V2-specific Abs were induced in all animals, these Abs were associated with vigorous Δ5G-specific virion capture ability in only two Δ5G-infected animals that exhibited a functional nAb response. Thus, whereas the deglycosylation mutant infection elicited early virion capture and subsequent nAbs, the responses differed among animals, suggesting the existence of host factors that may influence the functional humoral responses against human immunodeficiency virus/SIV.

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INTRODUCTION

The precise role of antibody (Ab) responses in the containment of human immunodeficiency virus (HIV) remains a subject of intense study and debate. Besides the classical direct virus neutralization properties, antibodies are also capable of blocking infection via other pathways such as antibody-dependent complement-mediated inactivation of virus (Aasa-Chapman *et al.*, 2005) and antibody-dependent cellular lysis (Ahmad & Menezes, 1996; Forthal *et al.*, 2001). Acquiring an understanding of these various mechanisms for their exploitation in the

development of candidate vaccines has been a major challenge.

The envelope protein (Env) of HIV/simian immunodeficiency virus (SIV) comprises an exterior protein (gp120) and a transmembrane (TM) protein (gp41), and trimers of the gp120/gp41 complexes form viral spikes that promote binding to receptors and co-receptors on the cell membrane for entry into the target cells (Wyatt & Sodroski, 1998). The major viral receptors of HIV/SIV include CD4 and a variety of co-receptors such as CCR5 or CXCR4. One desirable target epitope for neutralizing antibody (nAb) that shows relative conservation across clades is the binding site for the co-receptor (Burton *et al.*, 2004; Zolla-Pazner, 2004); however, this site is conformationally cryptic within the viral spike up until immediately after binding of the viral spike to CD4, providing an effective shielding mechanism to the virus. Another distinct feature of HIV/SIV Env is the extensive glycosylation that also effectively prevents access to antibodies directed at the epitopes (Chen *et al.*, 2005; Wyatt & Sodroski, 1998; Wyatt *et al.*, 1998). The gp120 protein possesses 18–33 Asn–X–Ser/Thr sequences, signals for the attachment of *N*-linked carbohydrate side chains (Leonard *et al.*, 1990; Ohgimoto *et al.*, 1998; Regier & Desrosiers, 1990; Zhang *et al.*, 2004). As the carbohydrate moiety is generally weakly immunogenic and is recognized to a large extent as self by the host immune system, the massive glycans on the surface of viral spikes constitute an immunologically silent facade (Wyatt & Sodroski, 1998; Wyatt *et al.*, 1998). As a result, mature viral spikes are protected from nAb and other host immune responses by a massive carbohydrate 'glycan shield' (Chen *et al.*, 2005; Wyatt & Sodroski, 1998; Wyatt *et al.*, 1998). In fact, a prominent role of carbohydrates of HIV/SIV in evasion from immune surveillance has been reported previously as follows. Variants of SIV that have evolved to acquire additional glycans in the variable regions of Env have increased neutralization resistance compared with the parental virus (Chackerian *et al.*, 1997; Cheng-Mayer *et al.*, 1999). Similarly, the appearance of neutralization escape mutants has been associated with altered glycosylation in HIV-1 evolved during the course of infection (Wei *et al.*, 2003). Conversely, infection with SIV239 mutants with deglycosylated Env (lacking *N*-linked glycosylation sites) in the variable loops V1/V2 of gp120 elicited markedly increased titres of nAb (Reitter *et al.*, 1998). We have reported that a deglycosylation mutant, Δ5G, lacking *N*-linked glycosylation sites at aa 79, 146, 171, 460 and 479 in gp120 of SIV239 displayed an attenuated phenotype when used to infect rhesus macaques (Mori *et al.*, 2001; Ohgimoto *et al.*, 1998). In addition, animals infected with Δ5G exhibited almost sterile protection against rechallenge with SIV239 (Mori *et al.*, 2001).

Thus, we suggest that studies aimed at identifying the mechanisms underlying the early and potent immune control of deglycosylated SIV may provide knowledge for the formulation of effective HIV/SIV vaccines. Studies performed herein were therefore directed at attempts to define more precisely the early humoral responses (both virus-specific nAb and non-nAb) generated after infection with Δ5G in rhesus macaques and to compare these responses with those observed in macaques inoculated with wild-type SIV239, with the rationale that results from such studies may help to identify their potential contribution towards viral control of primary infection.

METHODS

Viruses. The molecular pathogenic clone of SIV239 (Regier & Desrosiers, 1990) and its derived deglycosylated mutant, Δ5G, were used in this study. Δ5G was derived by mutagenesis of an SIV239 infectious DNA clone so that the asparagine residues for *N*-glycosylation at aa 79, 146, 171, 460 and 479 in gp120 were converted to glutamine residues (Fig. 1a) (Ohgimoto *et al.*, 1998). Viral stocks of SIV239 and Δ5G were prepared as reported previously (Mori *et al.*, 2001).

Peptides. A series of 72 consecutive 25 mer peptides overlapping by 13 aa were synthesized based on the entire SIV239 Env sequence (Env-1–72). These peptides were synthesized by the Microchemical Facility, Emory University School of Medicine, Atlanta, USA. Another set of 15 mer peptides overlapping by 11 aa around the V1/V2 region in gp120 (V1V2-1–12) was synthesized by Sigma-Aldrich Japan based on the Δ5G sequence (see Fig. 5b). All peptides were dissolved in DMSO diluted in PBS.

Animal infection. Juvenile rhesus macaques originating from Myanmar (Burma) (Mm12, Mm13, Mm20, Mm23 and Mm26) or from Laos (Mm07, Mm22 and Mm25) were used following the results of screening for SIV, simian T-cell lymphotropic virus, B virus and type D retrovirus infection, which were all negative prior to inception of the study. All animals were housed in individual cages and maintained according to the rules and guidelines for experimental animal welfare as outlined by the National Institute of Infectious Diseases and National Institute of Biomedical Innovation. Animals were infected intravenously with Δ5G or SIV239 as described previously (Mori *et al.*, 2001).

Plasma viral load measurements. SIV infection was monitored by measuring the plasma viral RNA load using a highly sensitive quantitative real-time RT-PCR. Viral RNA was isolated from plasma samples from infected animals using a commercial viral RNA isolation kit (Roche Diagnostics). SIV *gag* RNA was amplified and quantified using a method originally developed by Hofmann-Lehmann *et al.* (2000) using a TaqMan EZ RT-PCR kit (Applied Biosystems). The detection sensitivity of plasma viral RNA by this method was 100 viral RNA copies per ml plasma (given as copies ml^{-1}).

Neutralization assay. SIV neutralization was tested according to a protocol using CEMx174/SIVLTR-SEAP cells, originally described by Means *et al.* (1997). To measure low levels of nAb, IgG was purified from plasma as described below and concentrated virus stocks were used.

Anti-gp120 Ab ELISA and anti-Env peptide ELISA. Recombinant SIV239 gp120 and Δ5G gp120 were expressed utilizing a Sendai virus vector as described previously (Mori *et al.*, 2005; Yu *et al.*, 1997). Culture supernatant containing approximately 2 μg secreted SIV gp120 ml^{-1} was diluted with an equal amount of PBS, dispensed into each well of an ELISA plate and allowed to incubate at 4 °C overnight.

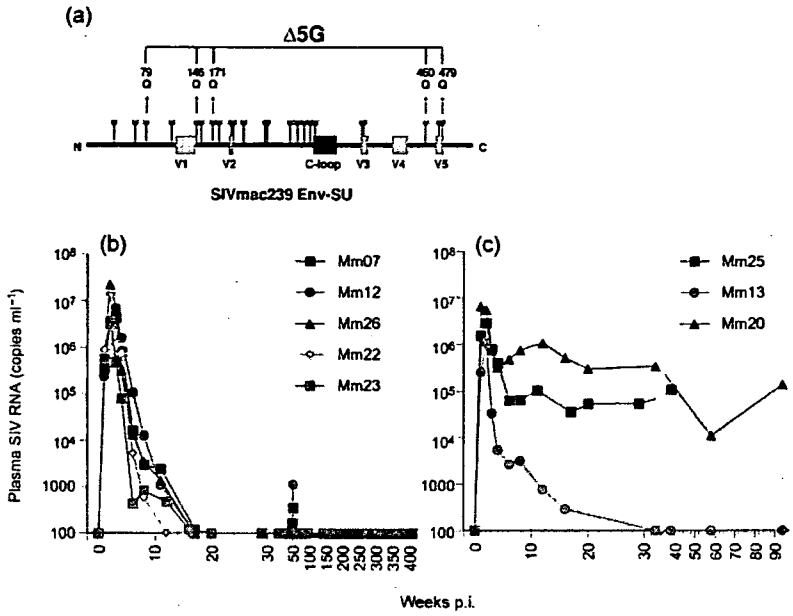

Fig. 1. Plasma SIV RNA loads in animals infected with Δ5G or SIV239. (a) *N*-Glycosylation sites in SIV239 gp120 and deglycosylation sites in Δ5G gp120. The locations of 23 *N*-glycosylation sites in SIV239 gp120, variable regions (V1–V5) and cysteine loops (C-loop) are shown. Δ5G was deglycosylated by N→Q substitutions at aa 79, 146, 171, 460 and 479 in Env (Ohgimoto *et al.*, 1998). (b, c) Plasma viral load in Δ5G-infected (b) and SIV239-infected (c) animals was measured in plasma samples using sensitive real-time RT-PCR to indicate when viral loads declined below 100 copies ml^{-1}. Three Δ5G-infected animals (Mm07, Mm12 and Mm26) were challenged with SIV239 at 48 weeks p.i.; thus, slightly increased viral loads were detected in those animals during weeks 49–51 p.i. (Mori *et al.*, 2001).

For the peptide ELISA, each peptide was diluted to 0.5 μM with 50 mM carbonate buffer (pH 9.5) and captured on Nunc Immobilizer amino plates (Nalge Nunc) at 4 °C overnight. A 1 : 100 dilution (150 μl) of the plasma sample to be tested was dispensed into antigen-immobilized plates and incubated at 37 °C for 1 h. Ab responses were detected using peroxidase-conjugated goat anti-monkey IgG and *o*-phenylenediamine. Absorbance was measured at 490 nm.

Removal of linear V1/V2 epitope-specific Abs from IgG fractions. A mixture of the peptides (V1V2-9, -10 and -11; see Fig. 5b) was conjugated to a HiTrap NHS-activated HP column (GE Healthcare). IgGs from plasma samples were fractionated using a mAb trap kit (GE Healthcare) and applied to the peptide-conjugated column. The flow-through fractions devoid of anti-V1V2-9, -10 and -11 peptide-specific Abs were collected. The concentration of IgG was determined using a protein assay kit (Bio-Rad).

Virion capture assay. The virion capture assay was modified using a method reported by Nyambi *et al.* (1998). ELISA plates were coated with the IgG samples described above at a concentration of 20 μg ml^{-1} in 50 mM carbonate buffer (pH 9.5) and incubated at 4 °C for 48 h. The plates were washed three times with PBS and blocked with 3 % BSA in PBS at 37 °C for 1 h. The plates were then washed three times with serum-free RPMI 1640. Δ5G or SIV239 virion solutions with a $p27^{gag}$ concentration of 15, 7.5 and 3.75 ng in 10 % fetal bovine serum/RPMI 1640 were added to each well of the IgG-coated plate and incubated at 37 °C for 3 h. The wells were washed five times with serum-free RPMI 1640 to remove unbound virus. The virus bound to IgG was lysed using MagNA Pure LC Lysis/Binding Buffer (Roche Diagnostics). The viral lysates were subjected to viral RNA purification using a MagNA Pure Compact nucleic acid purification kit (Roche Diagnostics). The copy number of the isolated SIV RNA was determined by real-time RT-PCR for SIV239 as described above.

Statistical analysis. Correlation analysis was done using Spearman's non-parametric rank test and the Mann–Whitney test using GraphPad Prism 4.0 software. Correlations were considered to be statistically significant for values of $P<0.05$.

RESULTS

Plasma viral loads of a quintuple deglycosylated SIV239 mutant in rhesus macaques

Eight rhesus macaques were infected intravenously with Δ5G ($n=5$) or SIV239 ($n=3$) (Mori *et al.*, 2001). Plasma viral RNA loads were assayed for up to 400 weeks p.i. and the data obtained in the Δ5G-infected (Fig. 1b) or SIV239-infected (Fig. 1c) animals were plotted. Both Δ5G and SIV239 replicated with similar kinetics during the early phase of primary infection for up to 4 weeks p.i. However, subsequent to this acute infection phase, virus replication was markedly different in the two groups of monkeys: SIV239-infected animals exhibited viral load set points around 10^5 copies ml^{-1} in two of three animals, with one animal (Mm13) having an undetectable viral load (<100 copies ml^{-1}) by 30 weeks p.i. (Fig. 1c). In contrast, the Δ5G-infected animals showed uniformly controlled viraemia reaching undetectable levels by 12–16 weeks p.i. and maintained this control for more than 6 years p.i. (Fig. 1b).

nAb response in Δ5G-infected animals

Although failure to detect a nAb response is characteristic of SIV239-infected rhesus macaques (Johnson *et al.*, 2003; Means *et al.*, 1997), the rapid control of viraemia in Δ5G-infected animals prompted us to determine whether nAb played a role in this control of viraemia. We hypothesized that the deglycosylation might lead to the elicitation of a markedly more vigorous nAb response than infection with SIV239. To maximize the detection sensitivity of weak nAb responses at early time points p.i., an assay that measures neutralization titres based on 50 % inhibition of virus replication (IC_{50}) in $CD4^+$ T-cell lines was initially used.

Consistent with the reported results in SIV239-infected animals, no appreciable nAb titre was detected in two animals (Mm13 and Mm25), despite the fact that viral load in Mm13 was distinctively decreased by 30 weeks p.i. However, we observed a rare animal (Mm20) that elicited a robust nAb response against SIV239 and a relatively delayed nAb response against Δ5G, despite the maintenance of a high viral load (Fig. 2a). These results indicated the lack of correlation of nAb response with viral load in SIV239-infected animals. In contrast, nAb was detected in two Δ5G-infected animals (Mm07 and Mm22) starting at 8 weeks p.i. and in two additional animals (Mm12 and Mm23) at 12 weeks p.i. (Fig. 2b, left panel). These titres peaked at either 12 or 18 weeks p.i., and the peak was followed by a decrease in titre that varied among animals. Mm12 and Mm23, which exhibited nAb induction at 12 weeks p.i., had essentially low titres, whilst Mm07 and Mm22, which exhibited nAb induction at an earlier time point, maintained vigorous nAb titres of >1 : 100. Of note, plasma from Mm26 did not contain detectable levels of nAb at any time p.i. In contrast, nAb against SIV239 was not induced in any of the Δ5G-infected animals (Fig. 2b, right panel). As low-level nAb may play a role in control of virus replication, purified IgG from the plasma samples was used to measure neutralizing activity. However, the results from the purified IgG corresponding to the plasma at a 1 : 3 dilution did not change the kinetics of nAb response in Δ5G-infected animals (data not shown).

In experiments where the passive administration of monoclonal HIV nAb successfully prevented the infection of macaques with simian–human immunodeficiency virus, the results unequivocally indicated that high titres of nAb were needed to achieve such protection (Nishimura *et al.*, 2002). In consideration of these results, data were recalculated based on a cut-off value of 90 % inhibition of virus replication (IC_{90}) in $CD4^+$ T-cell lines. As a result, nAb responses were detected in only two of the animals, Mm07 and Mm22, but with titres of 1 : 100 and 1 : 500, respectively (Fig. 2b, middle panel). Next, we examined the correlation between viral load and nAb titre at 8 and 12 weeks p.i. and found that the correlation was not statistically significant (Fig. 2c).

Anti-gp120 Ab response in Δ5G-infected animals

Next, we measured binding Ab responses against gp120. When the plasma samples were assayed for levels of Ab that bound to SIV239 gp120 or Δ5G gp120, essentially identical values were obtained. Fig. 3 shows the data obtained using SIV239 gp120. Remarkably, anti-gp120 responses during the early period p.i. between the two groups of monkeys were distinct. Whereas anti-gp120-specific Ab responses

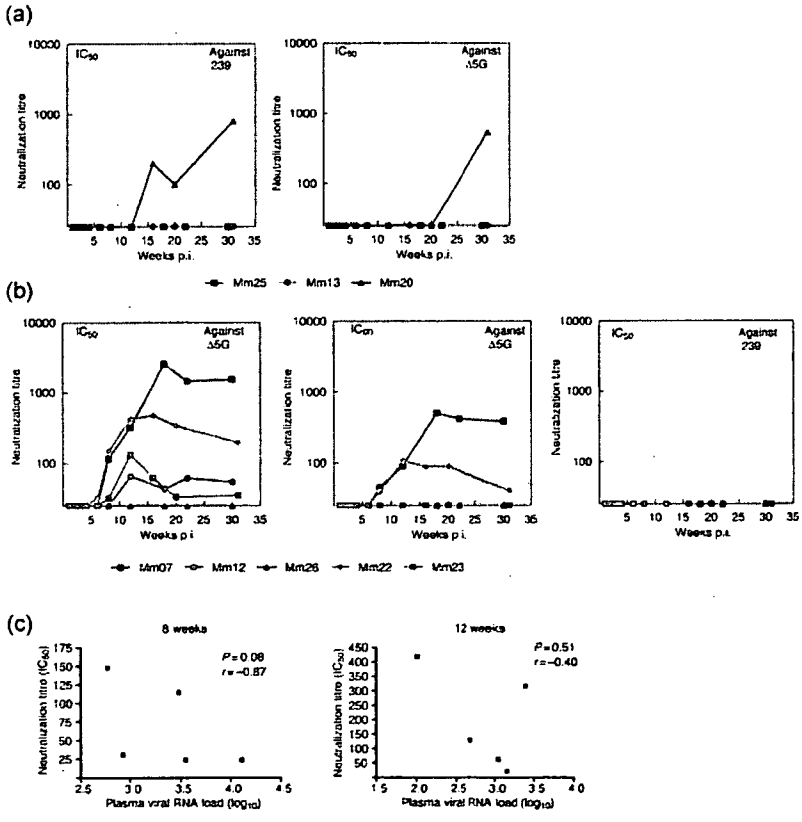

Fig. 2. nAb titres in SIV-infected animals. (a) nAb titres in SIV239-infected animals are indicated as the plasma dilution yielding 50 % inhibition (IC_{50}) of SIV239 infection (left) or Δ5G infection (right) in CEMx174/SIVLTR-SEAP cells. (b) nAb titres in Δ5G-infected animals are indicated as the plasma dilution that yielded 50 % inhibition (IC_{50}, left) and 90 % inhibition (IC_{90}, middle) of Δ5G infection or 50 % inhibition of SIV239 infection (right) in CEMx174/SIVLTR-SEAP cells. (c) Correlation between IC_{50} nAb titres and plasma viral RNA load at 8 and 12 weeks p.i. in Δ5G-infected animals.