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Development of cell-expressed and virion-incorporated CCR5-targeted vaccine ☆

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ABSTRACT

Our previous study demonstrated that the immunization with a cycloimmunogen derived from extracellular loop-2 (ECL-2) of CCR5 (cDDR5) attenuated acute phase of CCR5-tropic simian-human immunodeficiency virus $(SHIV)_{SF162P3}$ replication *in vivo*. Although the study showed that the antisera raised against cDDR5 reacted with cell-expressed CCR5, we have not yet demonstrated whether the antisera can react with virion-incorporated CCR5. Here, we show that rhesus cDDR5 (rcDDR5)-specific antibodies react with not only cell-expressed but also virion-incorporated simian CCR5s (siCCR5s), but may predominantly exert their inhibitory effects on simian immunodeficiency virus (SIV) infection by the binding of cell-expressed rather than virion-incorporated CCR5s. These results suggest that the virion-incorporated CCR5 may contribute to the reactivation of the anti-rcDDR5 antibody-producing B-cells by SIV particles after rcDDR5 immunization, although the binding of anti-rcDDR5 antibody to virion-incorporated CCR5 results in a partial inhibitory effect on SIV infection.

The human immunodeficiency virus (HIV) uses cell-surface receptors to gain entry into target cells. CD4 is the main receptor, and a chemokine receptor CCR5 is the coreceptor utilized by CCR5-tropic (R5) viruses. CXCR4, an alternative chemokine receptor, is used by CXCR4-tropic viruses. The distribution of these receptors is critical for HIV transmission and infection. In fact, the most striking resistance to HIV infection is in the naturally occurring delta32 CCR5 mutation in approximately 1% of Caucasians [1,2]. These individuals lack the cell-surface expression of CCR5. Furthermore, some studies showed that mucosal antibody responses may contribute to the apparent resistance to HIV-1 infection. The studies, in which humoral responses against HIV-1 in the vaginal secretions of women who remain uninfected despite frequent unprotected sex with HIV-1-infected partners were analyzed, indicated the presence of CCR5-specific mucosal autoantibodies [3].

As attempts to reproduce some of the functional aspects of the natural resistance to HIV infection, some vaccination strategies of inducing CCR5-specific autoantibodies have been reported. Our previous attempts were to induce CCR5-specific autoantibodies with anti-R5 HIV-1 activity by the inoculation of cDDR5 from the UPA (from Arg_{168} to Cys_{178}) of ECL-2 in CCR5 into Balb/c mice [4] and cynomolgus macaques [5]. Other attempts include the induction of CCR5-specific autoantibodies with anti-R5 HIV-1 activity by the inoculation of recombinant papillomavirus-like particles, which represent an extracellular loop of CCR5, into C57BL/6 mice and pig-tail macaques [6,7], and by the immunization of rhesus macaques with synthetic linear peptides (*N*-terminal peptide $_{1-20}$, first-loop $peptide_{89-102}$, and second-loop $peptide_{178-197}$) derived from the *N*-terminus, first loop, and second loop in CCR5 [8]. Results of these studies indicate that vaccines aimed at inducing CCR5-specific autoantibodies can be developed to reproduce some of the functional aspects of this natural resistance by blocking and down-modulating the CCR5 on the surface of HIV-targeted cells, as well as conventional viral-protein-based vaccines.

A recent intriguing study by Yusa and coworkers [9] has suggested that CCR5 is incorporated into HIV virions. Depending on the surface of the host cell, HIV-1 incorporates cell-derived molecules into its envelope [10,11]. Previous studies showed that HLA-class I, CD54, and other cellular surface proteins were incorporated into a budding virion [12], whereas CD4, CXCR4, and CCR5 were not detectable [13]. However, Yusa and coworkers found that CCR5 is incorporated into budding virions using the various types of monoclonal antibodies against the cell-surface molecules. The

Abbreviations: SHIV, simian-human immunodeficiency virus ECL-2, extracellular loop-2 siCCR5, simian CCR5 SIV, simian immunodeficiency virus UPA, undecapeptidyl arch HIV, human immunodeficiency virus R5, CCR5-tropic

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result suggests that CCR5-specific autoantibodies induced by vaccines may be capable of not only blocking and down-modulating the CCR5 on the surface of HIV-targeted cells but also directly neutralizing HIV infection.

In this study, we suggest that siCCR5 is incorporated into SIV_{mac239} particles, and cDDR5 derived from ECL-2 of siCCR5 is an attractive mimotope for inducing anti-siCCR5 antibodies against cell-expressed and virion-incorporated siCCR5s.

Materials and methods

Preparation of SIV_{mac239} lysate. The supernatants from the culture media of SIV_{mac239}-infected HSC-F cells [14] and rhesus peripheral blood mononuclear cells (PBMCs) were filtered through a 0.45 μm disposable filter and then centrifuged at 43,000g for 3 h at 4 °C. The pellet was resuspended in PBS(−) and then centrifuged at 100,000g for 1 h at 4 °C. The resulting pellet and SIV_{mac239}-infected HSC-F cells were lysed in 200 μl of lysis buffer (125 mM Tris–HCl (pH 6.8) containing 4% SDS and 20% glycerol).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and western immunoblot analysis. The diluted lysate was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [15] (PAG Mini "DAIICHI" 4–20%, Daiichi Pure Chemicals, Tokyo, Japan) and the separated proteins were subsequently electroblotted onto a polyvinylidene difluoride membrane (Immobilon, Millipore Corporation, Bedford, MA, USA). Antigens were probed with anti-siCCR5 antibody, 3A9 (BD Biosciences), and murine anti-SIV_{mac251} gp130 monoclonal antibody (Immuno Diagnostic Inc.). The bands were visualized by chemiluminescence detection (NEN Life Science Products, Boston, MA, USA).

Preparation of rhesus CCR5-derived cDDR5-keyhole limpet hemocyanin (KLH). A siCCR5-derived linear dodecapeptide (linear rhesus DDR5, H_2N-DRSQREGLHYTG-COOH), in which all side-chain groups are protected, was synthesized using an automatic peptide synthesizer and cyclized, as previously described [4]. The molecular masses of rhesus macaque CCR5-derived cDDR5 (rcDDR5) were determined by MALDI-TOF-mass spectrometry (Burker Franzen Analytik). The gamma-carboxyl group of Glu_1 in the protected rcDDR5 was conjugated to ethylenediamine and then coupled to KLH through Bis(sulfosuccinimidyl)suberate (Thermo Fisher Scientific Inc.).

Immunization and screening. Ten BALB/c mice were immunized intraperitoneally with 200 μg of rcDDR5-KLH in Freund's adjuvant at 1-week intervals and administered an intravenous boost of 40 μg of rcDDR5-KLH 3 days prior to splenectomy. Eleven hybridomas were generated by a standard method, by which splenocytes were fused with P3U1 cells and selected in hypoxanthine-, aminopterin-, and thymidine-supplemented media. In the screening, supernatants were tested for reactivity to rcDDR5-Multi-Pin Block in accordance with the method described in Ref. [4]. Hybridomas that produced high titers of anti-rcDDR5 antibodies (MARS4 and MARS8) were then cloned. MARS4 and MARS8 were found to be monoclonal and immunoglobulin G_3 and G_{2a} isotypes, respectively.

Flow cytometry. HSC-F cells were preincubated with or without MIP-1beta (1 ng) at 37 °C for 30 min. These cells were washed with a washing buffer (phosphate-buffered saline (PBS) containing 2% fetal calf serum and 0.02% NaN_3), and then stained with MARS4 at 4 °C for 30 min. These cells were washed with a washing buffer, and FITC-conjugated anti-mouse IgG was used for antibody staining. After 30 min of incubation at 4 °C, the cells were washed and then analyzed using an EPICS XL flow cytometer (Beckman Coulter).

Chemotaxis assay. A chemotaxis assay was performed using the protocol of Gosling et al. [16] with HSC-F cells (5×10^5 cells) treated with or without MARS8. The assay was conducted in the presence of 10 ng/ml MIP-1beta placed in the lower chamber. Transwells (pore size, 5 μm; Corning Inc., Corning, NY) were incubated for 5 h at 37 °C. The cells that migrated from the upper chamber to the lower chamber were quantified by trypan blue dye exclusion.

Virus-binding ELISA. The pellet of the purified SIV_{mac239} (50 ng of p27 antigen) was suspended in anti-rcDDR5 serum, normal mouse serum, 1 μg of antibodies to SIV ENV protein (murine anti-SIV_{mac251} gp130 monoclonal antibody (Immuno Diagnostic Inc.)), or to siCCR5 (3A9, BD Biosciences), then incubated on ice for 30 min, and washed with PBS(−). The resulting viral pellet was resuspended with 50 μl of protein G microBeads (Miltenyi Biotec), incubated on ice for 30 min, and washed with 200 μl of PBS(−). The immune complex of antibody–virus–protein G microBeads was purified in accordance with the manufacturer's instructions, and lysed with 20 μl of 95 °C-prewarmed lysing buffer in Retro-Tek SIV p27 Antigen ELISA kit (ZeptoMetrox Corporation) for 5 min. The lysate was eluted with 50 μl of elution buffer (lysing buffer:PBS(−) = 1:9) before quantification of p27 by ELISA.

Total viral DNA detection assay. To evaluate the inhibitory effect of MARS4, the HSC-F cells (1×10^6 cells) or SIV_{mac239} was pretreated in the following three different ways with MARS4. (Cell block) HSC-F cells were preincubated with MARS4 for 30 min and then washed. The cells were then incubated with SIV_{mac239} (50 ng of p27 antigen) in the presence of DEAE dextran (20 μg/ml) for 4 h, washed twice with the culture medium, and cultured in fresh medium (200 μl) for 40 h. (Virus block) SIV_{mac239} was preincubated with MARS4 for 30 min and then washed by centrifugation (100,000g). The virus was incubated with HSC-F cells in the presence of DEAE dextran for 4 h. Finally, the HSC-F cells were washed twice with the culture medium and cultured in fresh medium (200 μl) for 40 h. (Cell/virus block) HSC-F cells were preincubated with MARS4 for 30 min. Without washing, the cells were then incubated with SIV_{mac239} (50 ng of p27 antigen) in the presence of MARS4 and DEAE dextran (20 μg/ml) for 4 h, washed twice with the culture medium, and cultured in fresh medium (200 μl) for 40 h. After this, the HSC-F cells were harvested. The nucleic acid obtained after the purification procedure [17] was used for the PCR amplification. cDNA duplicates were amplified by SYBR green real-time PCR assay as previously described [18] with some modifications. Briefly, primers that recognize specific and highly conserved sequences on the *gag* region of SIV described by Ui et al. [19] were selected. The sequences of SIV *gag* primers were 5′-GGAAATTACCCAGTACAACAAATAGG-3′ and 5′-TCTATCAATTTTACCCAGGCATTTA-3′. The SIV *gag* gene was amplified in 20 μl of a PCR mixture consisting of 10 μl of 2× master mix containing modified DyNAmo hot start DNA polymerase, SYBR green I, optimized PCR buffer, 5 mM $MgCl_2$, a dNTP mix including dUTP (Finnzymes), 2 μl of each primer, and 8 μl of viral DNA. PCR was conducted as follows: initial activation of hot start DNA polymerase at 95 °C for 15 min; 40 cycles of four steps of 95 °C for 10 s, 57 °C for 20 s, 72 °C for 20 s, and 76 °C for 2 s. At the end of the amplification cycle, melting temperature analysis was conducted by gradually increasing the temperature (0.5 °C/s) to 95 °C. Amplification, data acquisition, and analysis were conducted with the DNA Engine Opticon 2 System (Bio-Rad Laboratories Inc.) using Opticon Monitor version 2.02 software (Bio-Rad Laboratories Inc.).

Results

Incorporation of siCCR5 into SIV_{mac239}

To examine the incorporation of siCCR5 into the envelope of SIV_{mac239}, the lysates from viruses grown in either HSC-F or rhesus PBMC were subjected to western immunoblot analysis with anti-siCCR5 antibody, 3A9 (Fig. 1A and B), and murine anti-SIV_{mac251} gp130 monoclonal antibody that cross-reacted with SIV_{mac239} Env protein (Fig. 1C and D). Consistent with other reports [20,21], a band corresponding to siCCR5 monomer was detected in both

cases, which migrated with an apparent 48 kD mass (Fig. 1A and B). Furthermore, higher-molecular-weight species as shown in Refs. [20,21] were also detected in SIV_{mac239} from rhesus PBMC (Fig. 1B) but not from HSC-F cells that predominantly expressed CCR5 having a molecular weight of approximately 48 kDa (Fig. 1A). As a positive control, anti-SIV_{mac251} gp130 monoclonal antibody was used, which detected SIV_{mac239} Env protein in both cases (Fig. 1C and D). Although supernatants from uninfected cells as a negative control were also subjected to western immunoblot analysis, a band corresponding to siCCR5 was not detected (data not shown).

rcDDR5 synthesis and peptide analysis

It is generally considered that the conformational B-cell epitopes involved in the induction of a conformation-specific antibody would be difficult to mimic using a simple synthetic linear peptide. To mimic the UPA of ECL2 of rhesus CCR5, a linear side-chain group-blocked oligopeptide (linear rhesus DDR5 (rDDR5): H_2N-ERSQREGLHYTG-COOH) with a free-amino-terminal head and a carboxyl-terminal tail was first synthesized and then cyclized by peptidyl bond formation between the amino group of Glu_1 and the carboxyl group of Gly_{12} (Fig. 2A). After the removal of the side-chain-blocking group, rcDDR5 (cyclized at the head and tail of linear rDDR5) was purified, and its molecular masses was determined by MALDI-TOF-MS using alpha-cyano-4-hydroxy-cinnamic acid as a matrix. The spectrum of purified rcDDR5 exhibited major peaks at m/z 1414.66 (Fig. 2B), suggesting that the structure of rcDDR5 is cyclo($ER_{168}S_{169}Q_{170}R_{171}E_{172}G_{173}L_{174}H_{175}Y_{176}T_{177}G$).

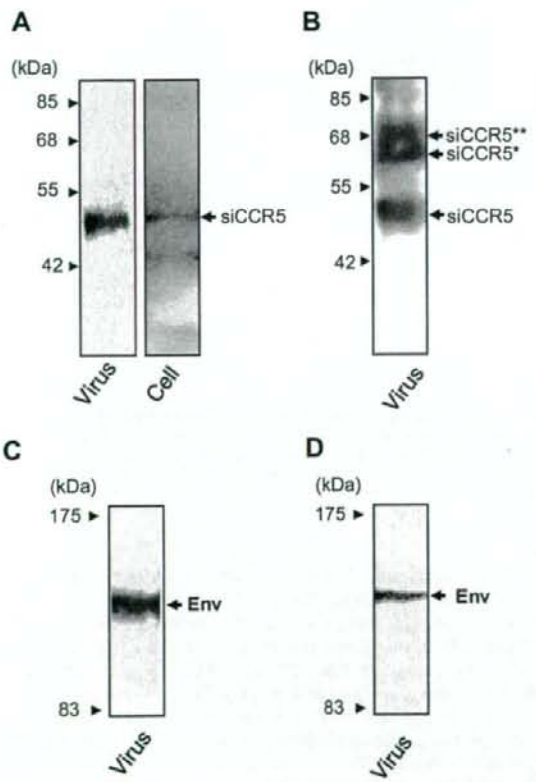

Fig. 1. Incorporation of siCCR5 into SIV_{mac239}. Viral lysates (2 μg) produced from HSC-F or rhesus PBMCs were resolved by 4–20% SDS–PAGE. Western immunoblot analysis was performed using anti-siCCR5 antibody, 3A9 (A and B), and murine anti-SIV_{mac251} gp130 monoclonal antibody (C and D). Furthermore, the cell lysate of HSC-F cells was also subjected to western immunoblot analysis using 3A9 for comparison (A).

Immunochemical specificity of the anti-rcDDR5-MAP antibodies, MARS4 and MARS8

Among the many antibody-producing clones, two clones producing the antibody to rcDDR5-KLH was effectively selected using the rcDDR5-Multi-Pin Block. The novel monoclonal antibodies, MARS4 and MARS8 (IgG_3 and IgG_{2a} isotypes, respectively) were selected (Fig. 2C). The immunochemical specificities of MARS4 and MARS8 were determined using flow cytometry (Fig. 2D) and chemotaxis assay (Fig. 2E). MARS4 was bound to CCR5-expressing HSC-F cells (Fig. 2D) but the binding was significantly competed with MIP-1beta (Fig. 2D). Furthermore, MARS8 significantly interfered with the chemotaxis induced by MIP-1beta (Fig. 2E). These results suggest that rcDDR5 immunization can induce the anti-siCCR5 antibody.

Binding of anti-rcDDR5 serum to virion-incorporated siCCR5

To examine whether anti-rcDDR5 serum from mice immunized with rcDDR5-KLH can recognize the virion-incorporated siCCR5, we measured the amount of intact SIV_{mac239} particles captured by anti-rcDDR5 serum using a virus-binding ELISA as shown in "Materials and Methods". SIV_{mac239} particles were treated with anti-rcDDR5 serum or normal mouse sera. Alternatively, both the commercially available anti-siCCR5 antibody, 3A9 and the anti-SIV ENV protein antibody were also used as controls. As expected, the anti-rcDDR5 serum captured the SIV_{mac239} grown in HSC-F cells (Fig. 3A). As a positive control, the anti-SIV ENV protein antibody effectively captured the HSC-F cell-derived virions, and 3A9 also captured the virions although the binding efficiency of 3A9 to siCCR5 was lower than that of the anti-rcDDR5 serum. These results suggest that the UPA is an attractive target for immune strategies aimed at generating anti-CCR5 antibodies.

Antiviral activity

Because siCCR5 is the main coreceptor for SIV_{mac239} and is incorporated into the SIV_{mac239} envelope, we investigated whether MARS4 could inhibit SIV_{mac239} entry via cell-expressed or virion-incorporated CCR5. The anti-SIV_{mac239} activities of MARS4 were determined using HSC-F cells that express CCR5 and SIV_{mac239} grown in HSC-F cells. The pretreatment of cells with MARS4 resulted in a significant inhibitory effect on SIV_{mac239} infection (Fig. 3B). On the other hand, the pretreatment of the virus with MARS4 resulted in a partial inhibitory effect on SIV_{mac239} infection (Fig. 3B). Furthermore, when MARS4-pretreated cells were inoculated with SIV_{mac239} in the presence of MARS4, it resulted in the most effective inhibitory effect (Fig. 3B). These results suggested that the rcDDR5-induced anti-CCR5 antibody may effectively show inhibitory effects through the binding to the cell-expressed rather than virion-incorporated CCR5s.

Discussion

Could CCR5 be an attractive target for the development of HIV vaccines? Persons with the homozygous delta32 CCR5 mutation, a 32-base-pair deletion of the CCR5 gene that results in a lack of cell-surface expression of CCR5, have strongly reduced susceptibility to CCR5-dependent HIV-1 infection [1,22,23]. Furthermore, Pastori et al. found that long-lasting CCR5 internalization by anti-CCR5 antibodies in a subset of long-term nonprogressors is associated

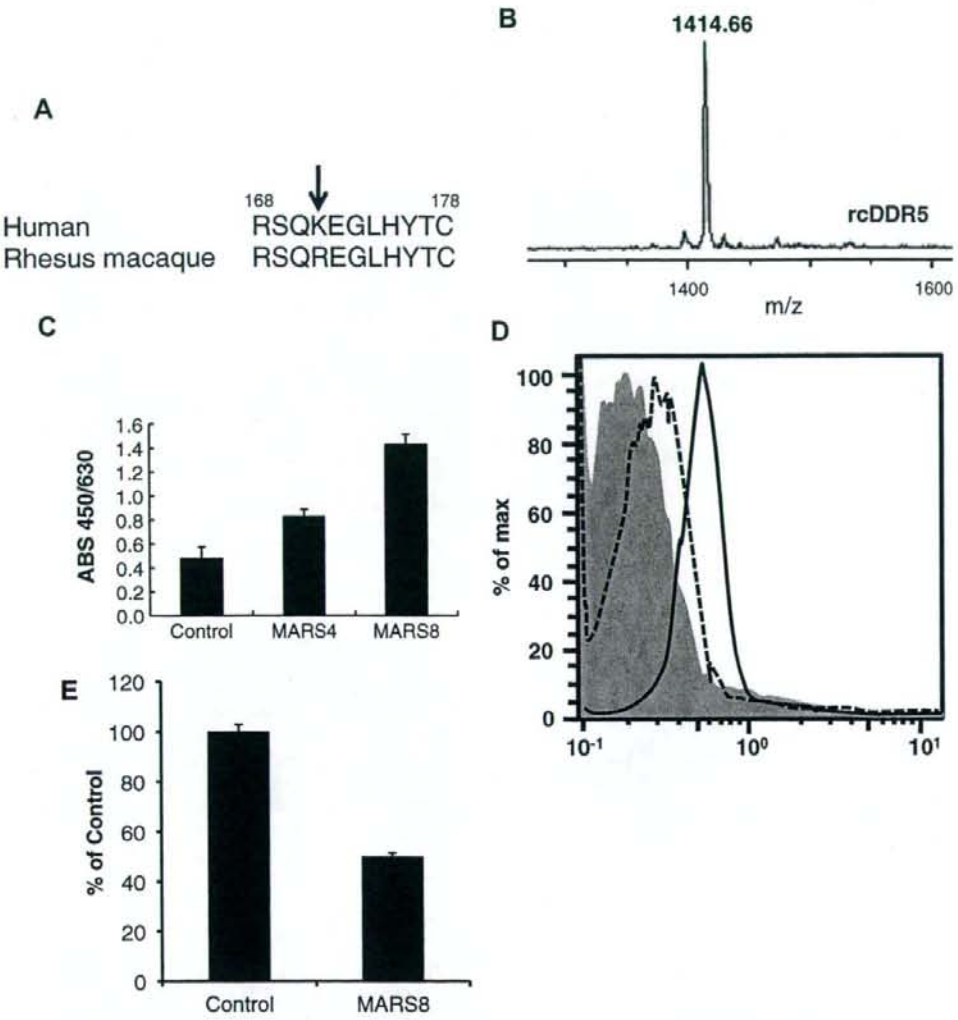

Fig. 2. Cloning of anti-rcDDR5 antibodies, MARS4 and MARS8, and their specificities. (A) Difference in UPA sequence between human and rhesus macaque. (B) MALDI-TOF-MS spectrum of rcDDR5. The spectrum exhibited a peak at *m/z* 1414.66. (C) Screening of anti-rcDDR5 antibodies, MARS4 and MARS8, using rcDDR5-Multi-Pin ELISA as described in "Materials and methods." (D) MARS4 binds specifically to siCCR5 on the cell surface. HSC-F cells were exposed to MARS4 in the absence (dotted line) or presence (bold line) of MIP-1beta or in the presence of an isotype-matched control antibody (gray shadow) at 4 °C. (E) MARS8 interferes with MIP-1beta-induced THP-1 chemotaxis. Results are expressed in terms of % of control, which represents the number of cells migrating in response to MIP-1beta over the number of cells migrating spontaneously in the control medium multiplied by 100.

with a possible protective effect against disease progression [24], suggesting that the induction of anti-CCR5 antibodies by a vaccine could reproduce the immune status in long-term nonprogressors. Thus, these data make CCR5 an attractive potential target for the development of HIV vaccines.

In general, it is considered that antibodies neutralize enveloped viruses by diverse mechanisms, such as disruption of receptor binding, interference with conformational changes required for virus entry, steric hindrance, or virus aggregation [25,26]. We previously showed that the antisera raised against cDDR5 mimicking the UPA of human CCR5 reacted with human CCR5, and potently suppressed infection by the R5 HIV-1 isolates [4,5], suggesting that HIV infection can be neutralized by the anti-CCR5 antibody-mediated interference with receptor binding of the envelope glycoproteins. Recently, Yusa and coworkers found that CCR5 is incorporated into budding virions [9]. The result suggests that CCR5-specific antibodies induced by vaccines may be capable of not only blocking the CCR5 on the surface of HIV-targeted cells but also directly neutralizing HIV infection. In this study, the immunization with rcDDR5-conjugated KLH induces anti-rcDDR5-specific monoclonal IgGs and anti-rcDDR5 serum that specifically bind to both the cell-expressed and virion-incorporated siCCR5s. Although the antibody binding to virion-incorporated CCR5 had a potential to inhibit SIV_{mac239} infection, only partial inhibition was observed. These results suggest that the rcDDR5-induced antibody binding to cell-expressed CCR5 may predominantly establish a steric block to a step in the virus entry process rather than that to virion-incorporated CCR5.

The incorporation of CCR5 in virions raises another hypothesis that the anti-CCR5 antibody can be simultaneously induced

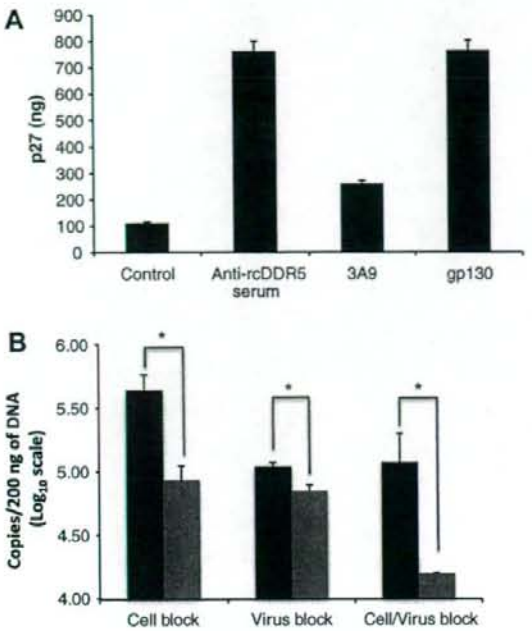

Fig. 3. Reactivity of anti-rcDDR5 serum to siCCR5 on the SIV_{mac239} envelope and the antiviral activity of MARS4. (A) The capture of SIV_{mac239} by anti-rcDDR5 serum was determined by a virus-binding ELISA as described in "Materials and methods". (B) The antiviral activity was measured as described in "Materials and methods". Each control experiment was carried out without MARS4 pretreatment. Results represent the amount of viral DNA (copies/200 ng of DNA) in each sample pretreated with MARS4 (gray column) or in each control sample (black column). $^*P<0.05$ by Mann–Whitney *U*-test.

when HIV invades the human body after cDDR5 immunization. In cDDR5-based vaccine development, it is very important to determine whether anti-cDDR antibody-producing B-cells could be reactivated when the CCR5-incorporated viruses invade the body. As shown in Fig. 3A, the epitopes of anti-rcDDR5 antibodies are likely to be effectively exposed on the surface of SIV_{mac239}. This result may support our hypothesis. Thus, these results suggest that the UPA in CCR5 is an attractive target for immune strategies aimed at reproducing the immune response in a subset of long-term non-progressors with anti-CCR5 antibodies.

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Expert Opinion

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A future perspective on the development of chemokine receptor CXCR4 antagonists

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

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

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1. Introduction

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

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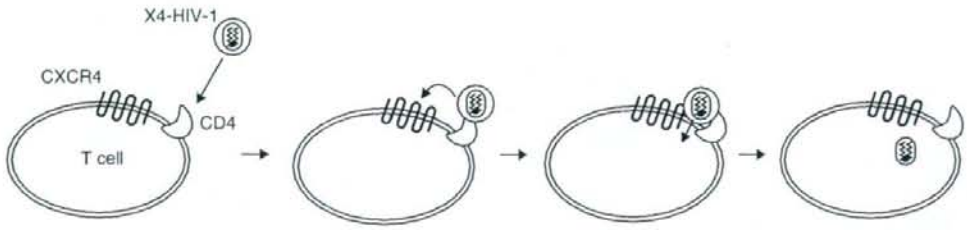

Figure 1. Correlation of CXCR4 to X4-HIV-1 infection. X4-HIV-1 strains enter T cells through association with the first receptor, CD4, and the second receptor, CXCR4.

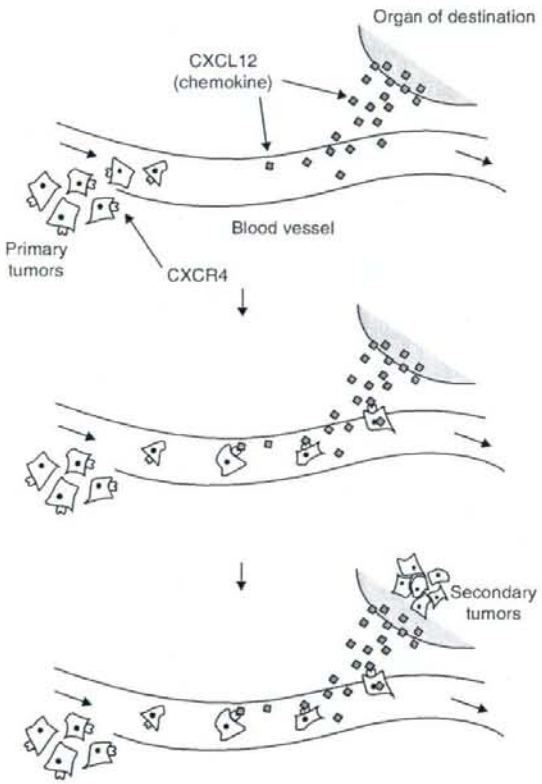

Figure 2. Correlation of the CXCL12–CXCR4 axis to cancer cell metastasis. A metastatic destination of tumor cells might be determined by the CXCL12–CXCR4 axis.

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

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

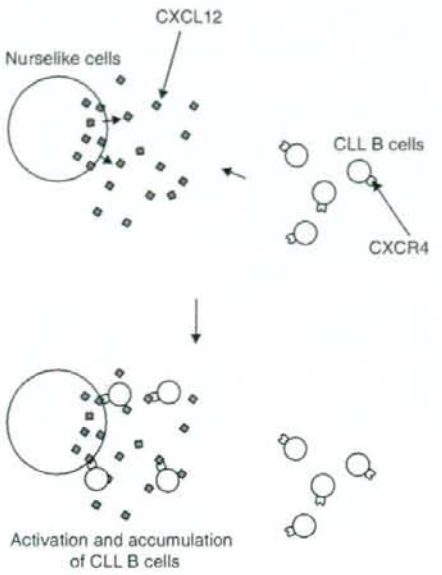

Figure 3. Correlation of the CXCL12–CXCR4 axis to CLL.
CLL B-cells are rescued from apoptosis through their activation by CXCL12 and thereby are accumulated.
CLL: Chronic lymphocytic leukemia.

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

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

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

3. Anticancer metastatic activity of CXCR4 antagonists

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

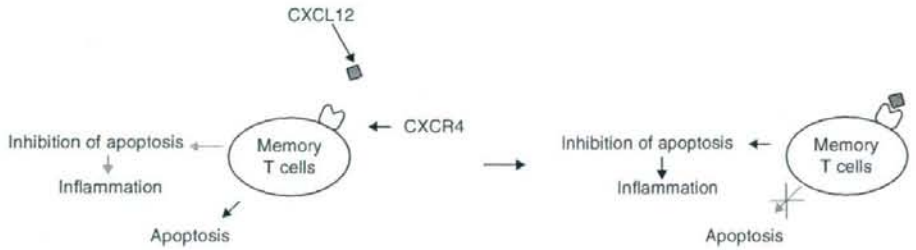

Figure 4. Correlation of the CXCL12–CXCR4 axis to rheumatoid arthritis. CXCL12 stimulates migration of the memory T cells and thereby inhibits T-cell apoptosis.

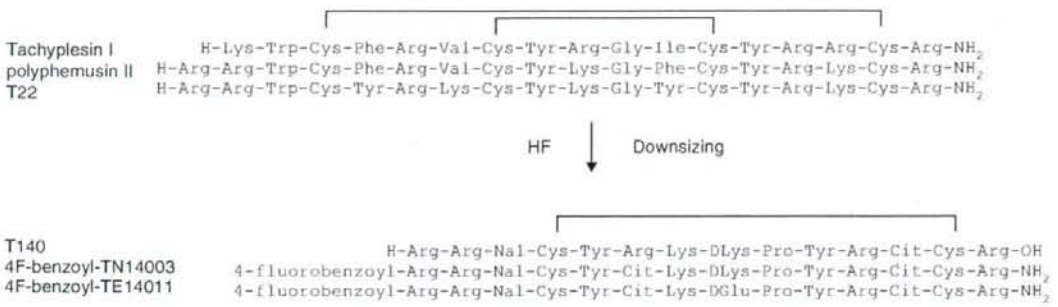

Figure 5. Structures of tachyplesin I, polyphemusin II and their analogues. Disulfide bridges of these peptides are shown by solid lines.
Cit: L-Citrulline; DGlu: D-Glutamic acid; DLys: D-Lysine; Nal: L-3-(2-Naphthyl)alanine.

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

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

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

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

4. Antileukemia activity of CXCR4 antagonists

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

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

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

5. Anti-RA activity of CXCR4 antagonists

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

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

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

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

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

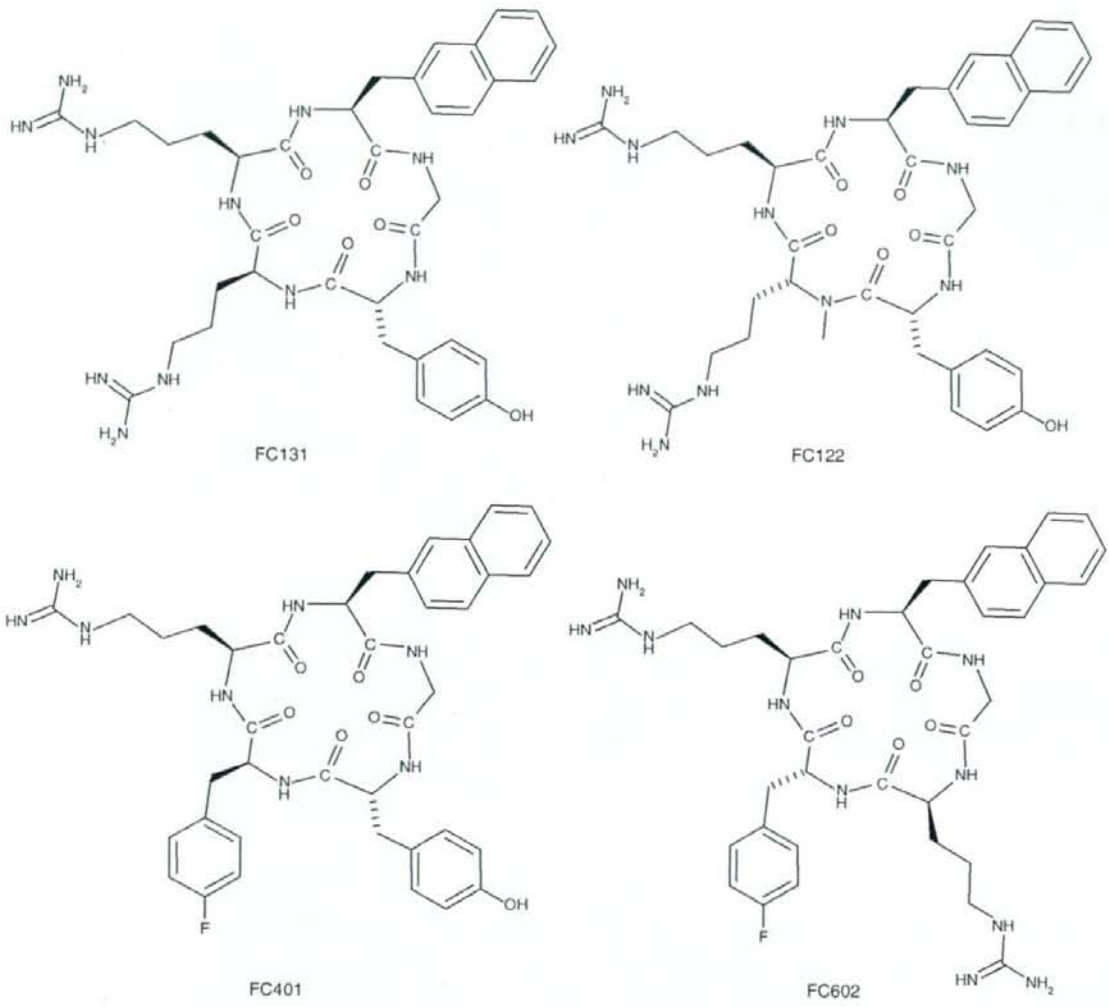

Figure 6. Structures of cyclic pentapeptides FC131, FC122, FC401 and FC602.

8. Development of linear small molecules with CXCR4 antagonistic activity

Development of small linear molecules with CXCR4 antagonistic activity was investigated based on identification of the T140 pharmacophore groups involving Arg, Nal and Tyr together with a novel pharmacophore moiety for CXCR4 antagonism, such as a 4-fluorophenyl moiety. Combination of substructure units of the pharmacophore moieties led to the design and synthesis of several compounds using combinatorial chemistry. As a result, compounds 1 – 4 shown in **Figure 7**, linear-type moderate CXCR4 antagonists, were found [57]. These compounds are generally less potent than the cyclic pentapeptide FC131, suggesting that conformational restriction implicit in the cyclic pentapeptide template is essential for potency. Furthermore, introduction of pharmacophores involving guanidine and aromatic groups into constrained and drug-like scaffolds, such as benzodiazepine, indole and quinoxaline, has provided a new type of nonpeptide CXCR4 antagonist such as **5** [58].

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

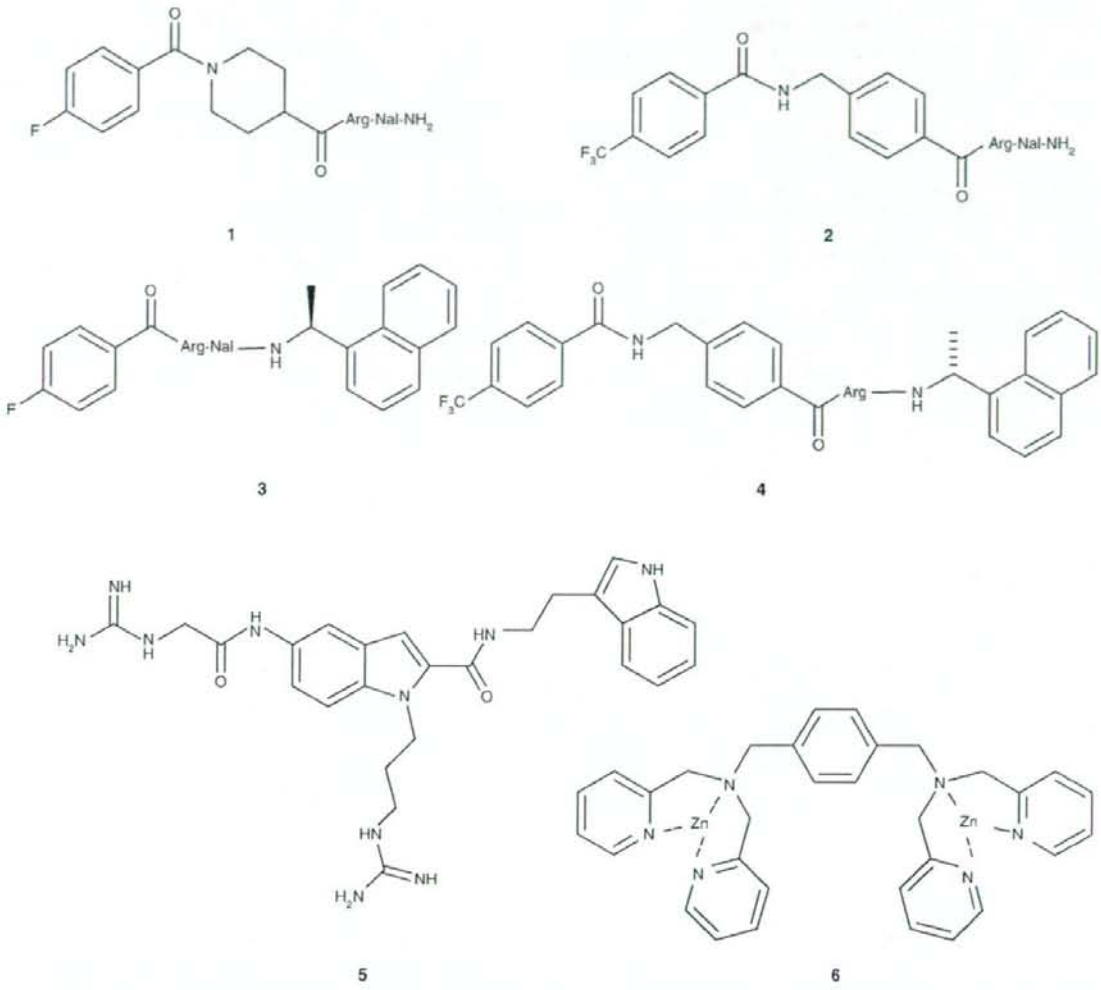

Figure 7. Structures of a linear type of low molecular weight CXCR4 antagonists.

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

9. Other CXCR4 antagonists

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

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

10. Conclusion

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

11. Expert opinion

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

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

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

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

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

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Declaration of interest

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

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

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

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

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

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

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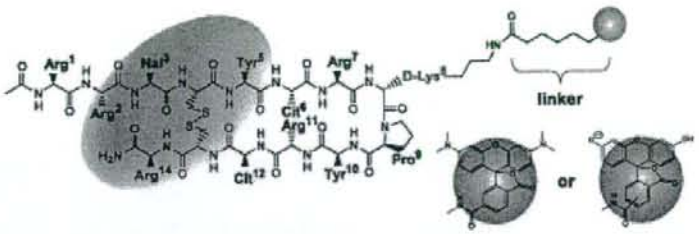


Figure 1. Design of fluorophore-labeled Ac-TZ14011. The amino acid residues in the red area are critical to CXCR4 binding activity. Fluorophores are shown as blue spheres.

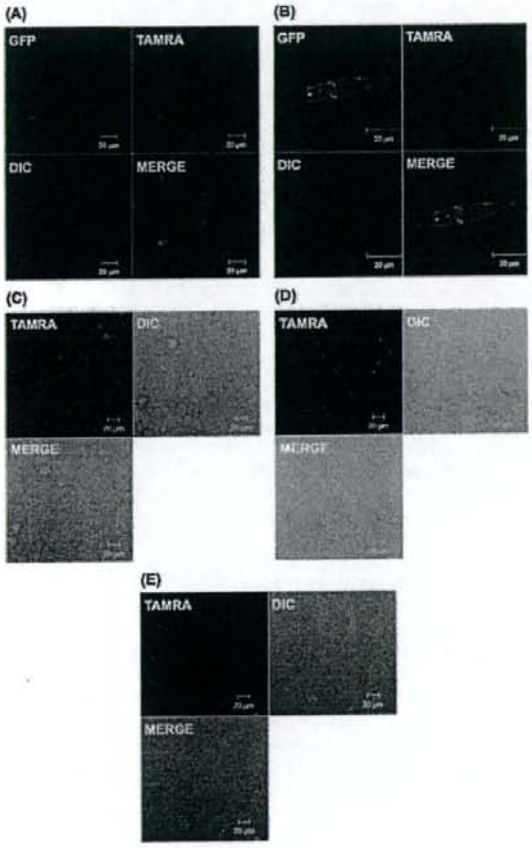


Figure 2. Confocal microscopy assays of TAMRA-Ac-TZ14011 binding to CXCR4. The signals of GFP and TAMRA are displayed in green and red, respectively. (A) Binding to NP2-GFP-CXCR4 cells. (B) Competitive binding to NP2 cells with excess amount of Ac-TZ14011. (C) Binding to HeLa-CD4-CCR5 cells. (D) Competitive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

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

To investigate the utility of fluorescein-labeled Ac-TZ14011, cell-based binding assays were performed. In this binding assay, fluorescein-Ac-TZ14011 was utilized as a competitor to derivatives of FC131 (*8*) and the dipicolylamine-*p*-xylene Zn(II)

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

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

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

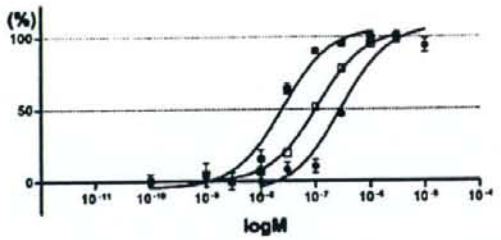
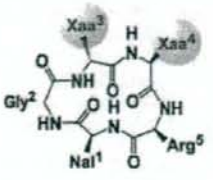


Figure 3. Curve fitting for CXCR4 binding of T140 (■), Zn^{2+}-(Dpa)-*p*-Xyl (●), and FC131 (□) in competitive assays by fluorescein-Ac-TZ14011. The *x* and *y* axes show concentrations and inhibition percentages of the binding of test compounds, respectively.

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

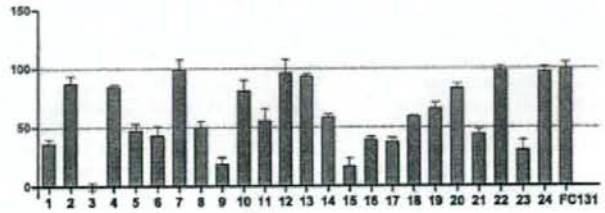
In the application of high-throughput screening for pharmacophores of CXCR4 ligands, it is important to be able to rapidly determine IC_{50} values. To test whether fluorescein-Ac-TZ14011 could be useful as a ligand in high-throughput screening, binding inhibition analyses at constant compound concentrations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

(A)

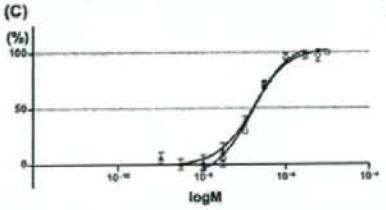


compound	Xaa^3	Xaa^4	compound	Xaa^3	Xaa^4
1	Arg	Nal	13	Arg	D-Nal
2	Nal	Arg	14	Nal	D-Arg
3	Arg	Trp	15	Arg	D-Trp
4	Trp	Arg	16	Trp	D-Arg
5	Arg	Nal(1)	17	Arg	D-Nal(1)
6	Nal(1)	Arg	18	Nal(1)	D-Arg
7	D-Arg	Nal(1)	19	D-Arg	D-Nal
8	D-Nal	Arg	20	D-Nal	D-Arg
9	D-Arg	Trp	21	D-Arg	D-Trp
10	D-Trp	Arg	22	D-Trp	D-Arg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

(B)



(C)



(D)

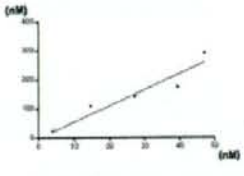


Figure 4. (A) Design of cyclic pentapeptides. Xaa^3 and Xaa^4 (red area) were manually randomized. (B) Results of single concentration point assays for determination of the binding activity of library compounds at one time. The *x* and *y* axes show concentrations and inhibition percentages of binding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

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

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

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

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

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