

A Small Molecule CXCR4 Inhibitor that Blocks T Cell Line-tropic HIV-1 Infection

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Summary

Several members of the chemokine receptor family have been shown to function in association with CD4 to permit human immunodeficiency virus type 1 (HIV-1) entry and infection. The CXC chemokine receptor CXCR4/fusin is a receptor for pre-B cell growth stimulating factor (PBSF)/stromal cell-derived factor 1 (SDF-1) and serves as a coreceptor for the entry of T cell line-tropic HIV-1 strains. Thus, the development of CXCR4 antagonists or agonists may be useful in the treatment of HIV-1 infection. T22 ([Tyr^{5,12},Lys⁷]-polyphemusin II) is a synthesized peptide that consists of 18 amino acid residues and an analogue of polyphemusin II isolated from the hemocyte debris of American horseshoe crabs (*Limulus polyphemus*). T22 was found to specifically inhibit the ability of T cell line-tropic HIV-1 to induce cell fusion and infect the cell lines transfected with CXCR4 and CD4 or peripheral blood mononuclear cells. In addition, T22 inhibited Ca²⁺ mobilization induced by pre-B cell growth stimulating factor (PBSF)/SDF-1 stimulation through CXCR4. Thus, T22 is a small molecule CXCR4 inhibitor that blocks T cell line-tropic HIV-1 entry into target cells.

In addition to the primary receptor for HIV-1, CD4, other cellular factors (coreceptors) are required for HIV-1 entry into target cells (1–3). It was recently found that the CXC chemokine receptor CXCR4/fusin is a coreceptor for the entry of T cell line-tropic (T-tropic) strains of HIV-1 (4), and the CC chemokine receptor CCR5 serves as a coreceptor for macrophage tropic (M-tropic) strains of HIV-1 (5–9). In addition, the CXC chemokine pre-B cell growth stimulating factor (PBSF)/stromal cell-derived factor 1 (SDF-1), recently shown to be a ligand for CXCR4, blocks infection of lymphocytes by T-tropic HIV-1 isolates (10, 11), and the CC chemokines regulated on activation, normal T expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1 α , and MIP-1 β , which are ligands for CCR5, inhibit infection by M-tropic HIV-1 isolates (12). These observations suggest that chemokines,

chemokine derivatives, or small molecule chemokine receptor antagonists or agonists may be useful agents for the treatment of HIV-1 infection. It was reported that an analogue of RANTES that consists of 60 amino acid residues and acts as a CCR5 antagonist could inhibit infection by M-tropic HIV-1 (13). However, no antagonist of CXCR4 has been reported so far. We previously found a derivative of horseshoe crab blood cell-derived peptide, T22 ([Tyr^{5,12}, Lys⁷]-polyphemusin II), which consists of 18 amino acid residues showed strong anti-HIV-1 activity in vitro (14). Here, we show that T22 is a small molecule CXCR4 antagonist, and specifically inhibits the entry of T cell line-tropic HIV-1 into target cells.

Materials and Methods

Cell Lines. Human osteosarcoma-derived cells (HOS) were grown in Eagle's MEM supplemented with 1% nonessential amino acid (GIBCO BRL, Gaithersburg, MD) and 10% FCS.

T. Nakajima and T. Murakami contributed equally to this work.

Human HeLaS3 cells were grown in RPMI 1640 supplemented with 10% FCS. Mouse NIH3T3 cells and human glioma cell lines U87MG were grown in DMEM supplemented with 10% FCS.

PBMCs. PBMCs were obtained from healthy donors by Ficoll-Hypaque separation and grown in RPMI 1640 with 20% FCS and 1 μ g/ml PHA for 72 h. PBMCs (5×10^5 cells) were incubated with virus in 2 ml for 2 h at 37°C at a multiplicity of infection of 0.001. After washing residual virions, cells were resuspended in fresh medium containing rIL-2 (100 U/ml; Shionogi Co., Osaka, Japan) with 20% FCS and the peptides, and the concentration of HIV-1 gag p24 in the culture supernatant was determined by ELISA (Cellular Products, Buffalo, NY).

Viruses. T. Shioda (University of Tokyo, Tokyo, Japan) provided us with the following panel of recombinant vaccinia viruses: Vac.Env (NL432 Env), Vac.Env JRCSF (JRCSF Env), and Vac T4 (CD4). M. Kohara (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) provided us with recombinant vaccinia virus LO-T7 encoding the T7 polymerase. A. Adachi (University of Tokushima, Tokushima, Japan) provided us with HIV-1 strain NL432 (15). J.A. Levy (University of California, San Francisco, CA) provided us with HIV-1 strain SF162 (16). HIV-1 chimeric clone NL432_{env162} was generated using standard techniques by Y. Isaka (Shionogi Institute for Medical Science, Osaka, Japan).

Peptides. T22 and 4Ala-T-I were synthesized by Fmoc-based solid-phase or solution-phase peptide synthesis as described previously. Murine PBSF/SDF-1 was synthesized at the Peptide Institute, Inc. (Minoo, Japan). Murine PBSF/SDF-1 has a single conservative Val to Ile substitution compared to the human PBSF/SDF-1.

Transfection of Cell Lines. NIH3T3 cells were transfected with coreceptor in pBluescript by lipofectamine and used for fusion assay. U87MG and HOS cells, stably expressing human CD4 and long terminal repeat (LTR)- β galactosidase (β -gal) were transiently transfected with 15 μ g of coreceptor by modified calcium phosphate method. The cells were detached with 0.5 mM EDTA/PBS(-) 18 h later and passaged to 12-well plate followed by incubation at 37°C overnight. The next day, cells were tested for infection assay.

Cell Fusion Assays. To quantitate cell-cell fusion events, we used a modified version of the fusion assay using α complementation of β -gal as reported previously. In brief, effector HeLaS3 cells (10^5 cells/well in 24-well plates) were infected with recombinant vaccinia virus vectors containing the α subunit of β -gal and env proteins. Target NIH3T3 cells (5×10^4 cells/well in 24-well plates) were infected with the recombinant vaccinia virus vectors containing human CD4, the ω subunit of β -gal and T7 RNA polymerase, and subsequently transfected with plasmids containing coreceptors under control of the T7 promoter. 16 h later, effector and target cells were washed with PBS containing 0.5 mM CaCl₂ and suspended in Hanks' balanced salt solution containing 3 mM CaCl₂, pH 7.6, followed by treatment with anti-vaccinia virus antibody 2D5 in the presence or absence of T22 peptide for 1 h. Cells were plated in 24-well plates, centrifuged at 1,300 rpm for 5 min, and incubated for 12 h at 37°C in 5% CO₂. If fusion occurs, mixing of the cytoplasmic contents of the cells lead to α complementation between the α and ω subunits of β -gal resulting in active enzyme. After coincubation, the cells were lysed in 200 μ l/well of a solution containing 8 mM of chlorophenolred- β -d-galactopyranoside (Boehringer Mannheim, Indianapolis, IN), 45 mM of 2-mercaptoethanol, 1 mM of MgCl₂, 100 mM of Hepes, pH 8.0, 0.5% NP-40, and 0.1 mg/ml of DNase I, and incubated for 30 min at 37°C. Reactions were

stopped by adding 200 μ l/well of 2% SDS and each aliquot was assayed for β -gal activity at OD₅₉₀.

Infection Assay. U87MG and HOS cells were transfected with human CD4 and an integrated HIV-1 LTR-driven reporter gene, *lacZ*, which is induced by the HIV-1-encoded transactivating protein Tat. Cells stably expressing human CD4 and LTR- β -gal were then transiently transfected with 15 μ g of CXCR4 or CCR5 by a modified calcium phosphate method. The cells were plated in 12-well tissue culture plates. The next day, cells were incubated with T22 or 4Ala-T-I peptide at 37°C for 1 h and infected with cell-free virus stock (reverse transcriptase [RT] activity, 2×10^6 RT/ml for SF162, and NL432_{env162}, 5×10^6 RT/ml for IIIB, and 3×10^6 RT/ml for NL432, 200 μ l/well) for 2 h at 37°C in 5% CO₂. After infection, 2.5 ml of culture medium was added to each well. After 4 d of infection, cells were lysed in 400 μ l of Reporter lysis buffer (Promega Corp., Madison, WI), followed by freezing to -80°C and thawing. Samples were then centrifuged at 12,000 rpm for 5 min at 4°C, and the supernatant was assayed for β -gal activity using Luminescent β -gal Detection Kit (Clontech, Palo Alto, CA).

Calcium Fluorimetry. The stable CXCR4- or CCR2B-transfected Chinese hamster ovary (CHO) cell lines (5×10^6 cells) were incubated in 1 ml of Hanks' balanced salt solution containing 1% FCS, 10 mM Hepes, pH 7.2, and 2 μ M of Fura-PE3 AM for 30 min at 37°C. The loaded cells were washed twice with Hanks' balanced salt solution containing 1% FCS and 10 mM Hepes, pH 7.2, resuspended in Hanks' balanced salt solution containing 1 mg/ml (wt/vol) of BSA and 10 mM Hepes, pH 7.2, added to a stirred cuvette (5×10^6 cells in 500 μ l) and inserted into a spectrofluorometer (LS 50B; Perkin-Elmer Corp., Norwalk, CT). Cells were incubated in the presence or absence of T22 or 4Ala-T-I peptide for 2 min, and chemokines were added in a volume of 5 μ l at the indicated time points. Real time recording of [Ca²⁺]_i changes in the stable CXCR4- or CCR2B-transfected CHO cell lines loaded with Fura-PE3 AM were performed as described (17).

Results

First, to investigate whether T22 (Fig. 1) specifically inhibits HIV-1 infection mediated by CXCR4, we examined the effect of T22 on HIV-1 infection using target cells expressing CD4 and CXCR4 or CCR5. U87MG and HOS cells were used as target cells since these cell lines are non-permissive for HIV-1 infection unless transfected with plasmid expressing human CD4 and chemokine receptors specific for particular viral strains. Target cells were infected with T-tropic HIV-1 strains (NL432, IIIB) or M-tropic HIV-1 strains (SF162, NL432_{env162}). T-tropic HIV-1 infection to the cells expressing CXCR4 and CD4 was inhibited by 0.3 μ M T22, but not by 3 μ M 4Ala-T-I (Fig. 1), a control peptide that has almost the same hydrophobicity and basic properties as T22 (Fig. 2, A and B). In contrast,

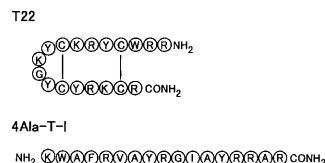


Figure 1. Amino acid sequences of T22 and 4Ala-T-I peptides.

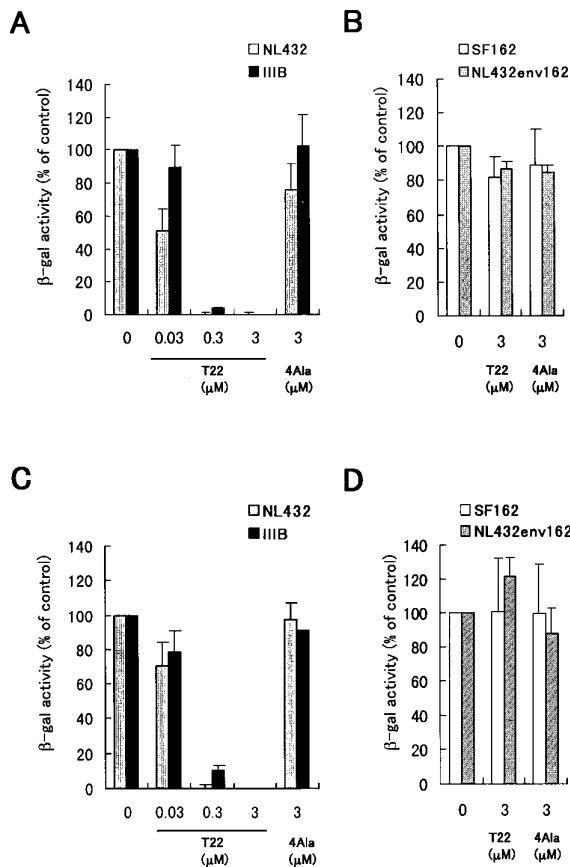


Figure 2. Inhibition of CXCR4-mediated HIV-1 infection by T22. (A and B) U87MG.CD4 transfected with either CXCR4 (A) or CCR5 (B) were incubated with the designated HIV-1 isolates in the presence of the indicated concentrations of T22 or 4Ala-T-I. Cells were then assayed for β -gal activity. Results are indicated as percentage of β -gal activity observed in the absence of peptide. Error bars show standard deviations from triplicate experiments. (C and D) HOS.CD4 cells expressing either CXCR4 (C) or CCR5 (D) were infected with the indicated HIV-1 isolates in the presence of T22 or 4Ala-T-I, and then assayed for β -gal activity. Results are indicated as percentage of β -gal activity compared with that of the no peptide control samples. Error bars show standard deviations from triplicate experiments.

T22 did not inhibit infection to cells expressing CCR5 and CD4 by either M-tropic HIV-1 or a recombinant T-tropic HIV-1 clone that has the M-tropic HIV-1 Env region. Similar results were obtained with HOS.CD4 cells transfected with either CXCR4 or CCR5 (Fig. 2, C and D). These data indicate that T22 inhibits the T-tropic HIV-1 infection mediated by CXCR4, but not the M-tropic HIV-1 infection mediated by CCR5.

Second, to examine whether T22 inhibits the infection of T-tropic HIV-1 at the stage of HIV-1 Env-mediated membrane fusion, we used a modified fusion assay as reported previously (18). As shown in Fig. 3 A, membrane fusion mediated by CXCR4 and NL432 Env was strongly inhibited in a dose-dependent manner by 0.03–3 μ M T22, but not by the control peptide 4Ala-T-I. In contrast, T22 had no effect on membrane fusion mediated by CCR5 and

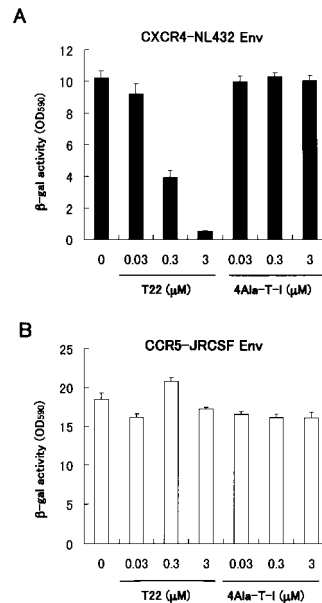


Figure 3. Effects of T22 on CXCR4-mediated cell fusion. NIH3T3 target cells were transfected with plasmids containing the CXCR4 (A) or CCR5 (B) gene driven by the T7 promoter and coinfecting with recombinant vaccinia viruses expressing CD4, T7 RNA polymerase, and the ω subunit of β -gal. HeLaS3 effector cells were coinfecting with recombinant vaccinia viruses expressing the α subunit of β -gal, and either the NL432 Env (A) or JRCSF Env (B) proteins. Cells were allowed to fuse in the presence of either T22 or 4Ala-T-I, and then assayed for β -gal activity as an indicator of cell fusion. Each value represents absorbance per 30 min at 590 nm. Error bars indicate standard deviations from triplicate assays.

JRCSF Env (Fig. 3 B). These results indicate that T22 inhibits membrane fusion mediated by CXCR4, CD4, and T-tropic HIV-1 Env proteins.

Third, to examine whether T22 interacts with CXCR4, we tested the effect of T22 on Ca^{2+} mobilization induced by PBSF/SDF-1, the ligand for CXCR4. CXCR4-expressing CHO cells were stimulated with PBSF/SDF-1 or monocyte chemoattractant protein 1 (MCP-1) in the presence of T22 or 4Ala-T-I, and intracellular Ca^{2+} levels were measured. As shown in Fig. 4 A, addition of 3 μ M T22 to CXCR4 transfectants abrogated responsiveness to a subsequent addition of PBSF/SDF-1, whereas the same concentration of T22 had no effect on Ca^{2+} mobilization induced by MCP-1 (Fig. 4 B). In contrast, 4Ala-T-I had no inhibitory effect on Ca^{2+} mobilization induced by either SDF-1 or MCP-1 (Fig. 4, A and B). These results indicate that T22 is a CXCR4 inhibitor, and suggest that the interaction of T22 with CXCR4 is involved in the suppressive effect of T22 on T cell line-tropic HIV-1 infection.

Finally, to examine the possibility of adapting the T22 or its counterpart as an agent for the treatment of HIV-1 infection, PBMCs were used as target cells in infection assays. As shown in Fig. 5, 0.008–0.2 μ M T22 inhibited infection by T cell line-tropic HIV-1 in a dose-dependent manner, but not by M-tropic HIV-1 strains, indicating that T22 specifically inhibits the infection of PBMCs by T-tropic HIV-1 isolates as well as cell lines expressing CD4 and CXCR4.

Discussion

In this report, we demonstrate that T22 is a small molecule CXCR4 antagonist and a potent inhibitor of infection by T-tropic HIV-1 strains which uses CXCR4 as a coreceptor, but not by M-tropic HIV-1 strains, which uses CCR5.

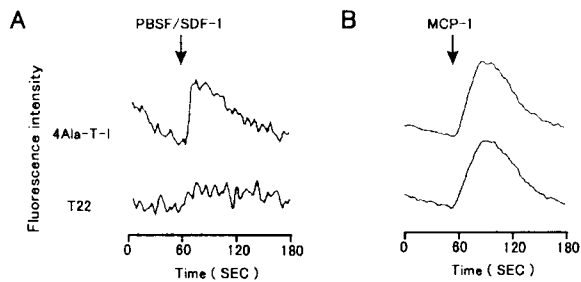


Figure 4. Inhibitory activities of T22 peptide on PBSF/SDF-1 induced $[Ca^{2+}]_i$ mobilization. Stable CXCR4- (A) or CCR2B- (B) transfected CHO cell lines were loaded with Fura-PE3 AM and treated sequentially with either T22 or 4Ala-T-I, followed with the indicated chemokines. Transient increases in $[Ca^{2+}]_i$ were measured using a spectrofluorometer. PBSF/SDF-1 and MCP-1 were added at a final concentration of 100 nM, and T22 and 4Ala-T-I were used at a final concentration of 3 μ M.

Our results suggest the hypothesis that binding of T22 to CXCR4 inhibits T-tropic HIV-1 entry into target cells, although we can not rule out the possibility that T22 also binds to regions of the T-tropic HIV-1 Env protein V3 region or CD4 critical for viral entry. It has been shown that a ligand of CXCR4 inhibits viral entry (10, 11) and that the regions of CCR5 important for chemokine receptor function partially overlap the regions used for viral entry by HIV-1 (19, 20). These results support our hypothesis.

We demonstrated that T22 specifically inhibited infection by T-tropic virus strains. M-tropic virus strains are isolated from individuals shortly after seroconversion and during the asymptomatic period of the disease, appear to be responsible for the sexual and parenteral transmission, and represent the most prevalent phenotype. In contrast, T-tropic HIV-1 isolates tend to appear in infected individuals at the later stages of the infection during the transition from the asymptomatic to the symptomatic state, and may be involved in the rapid decline of CD4⁺ T lymphocytes and progression to AIDS. It is therefore important to find means to inhibit CXCR4-mediated infection by T-tropic HIV-1 in order to block progression to AIDS.

Our results showed that T22 is a CXCR4 inhibitor. Previous studies have demonstrated that agonists for CCR5, MIP-1 α , MIP-1 β , and RANTES inhibit entry by M-tropic HIV-1 isolates that use CCR5 for entry. Infection by the

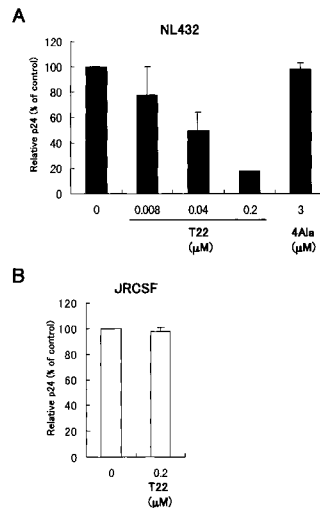


Figure 5. Inhibition of HIV-1 infection in PBMCs by T22. PHA-stimulated PBMCs from healthy seronegative donors were infected with either NL432 (A) or JRCSF (B) HIV-1 isolates in the presence or absence of different concentrations of T22. Aliquots of the culture supernatant were assayed at day 4 for HIV-1 gag p24 content by enzyme-linked immunosorbent assay. Results are indicated as the percentage of p24 content observed in the absence of peptide. Error bars indicate standard deviations from triplicate assays.

subset of M-tropic isolates that use CCR3 as a coreceptor is inhibited by eotaxin, the main CCR3 ligand. However, high doses of such chemokines were shown to produce harmful results by stimulating HIV-1 replication in macrophages and/or intensifying virus-induced inflammation (21). It therefore might be preferable to use antagonists rather than agonists to avoid such side effects that may be crucial by the latter. We recently found that a ligand for CXCR4, PBSF/SDF-1, is essential for perinatal viability, B lymphopoiesis, bone marrow myelopoiesis, and cardiac ventricular septal formation (22). However, it is not clear whether blocking of the function of CXCR4 is detrimental to the adult host.

Our findings that T22 inhibited Ca^{2+} mobilization induced by PBSF/SDF-1 suggest that the structure of T22 mimics the region in PBSF/SDF-1 that is involved in the binding to CXCR4. In fact, the CXC chemokines have a core structure consisting of three antiparallel β sheets and T22 also has an antiparallel β sheet structure maintained by two disulfide bonds (23). T22 is 18 amino acids in length and much smaller than PBSF/SDF-1. Thus, the three-dimensional structure of T22 would assist in the rational design of agents capable of inhibiting viral entry mediated by CXCR4 and the CD4-gp 120 complex.

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