

compounds were dissolved in dimethyl sulfoxide (DMSO) at a final concentration of 0.1%.

Cell culture. Jurkat and its subline Jurkat E6-1 were used in this study. The cells were cultured in a complete medium (CM) composed of RPMI-1640 (Sigma, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.1% 2-Mercaptoethanol at 37°C in a humidified environment with a 5% CO₂ atmosphere.

Fluorescence-activated cell sorter (FACS) analysis. Expression of CXCR4 and CD4 on Jurkat cells was measured by flow cytometry. The cells were suspended at 1×10^5 cells/mL in PBS containing 1% FCS. The cells were reacted with phycoerythrin (PE)-labeled mouse monoclonal antibodies (mAbs) to human CXCR4 (12G5; eBioscience, San Diego, USA) and CD4 (Leu3a; Becton Dickinson, Tokyo, Japan) as a positive control at 4°C for 1 h. The treated cells were washed and fixed with 1% formalin in PBS. Fluorescence of the stained cells was detected by a FACSCalibur (Becton Dickinson), followed by the analysis of fluorescence intensity by CellQuest software (Becton Dickinson).

Cytotoxic assay. Jurkat cells were treated with CXCR4 antagonists at 37°C for 1 h. The cells were harvested and resuspended in a 96-well plate. The viability of the treated cells was measured using a Cell Counting Kit-8 (Dojindo, Tokyo, Japan).

Chemotaxis assay. Cellular chemotaxis was investigated using a 24-well culture plate with 8-µm-pore filters (Transwell; Corning, Tokyo, Japan). Jurkat cells were washed three times in a FCS-free medium and suspended at 3×10^6 cells/mL in RPMI-1640 containing 0.1% bovine serum albumin (control medium). The control medium (0.2 mL) containing 3×10^5 cells was added to the upper well; the control medium (0.6 mL) with or without SDF-1α (100 ng/mL) or CXCR4 antagonists (10 µM) was added to the lower well. The culture plate was incubated for 3 h at 37°C; thereafter, the cells in the upper or lower well were then harvested and resuspended in a 96-well plate. The number of cells in each well was measured using a Cell Counting Kit-8. Optical density (OD) (455 nm/650 nm) values were measured on a microplate reader. The chemotaxis index was calculated as follows: [(OD of treated cells in the lower well - OD of control medium in the lower well)/(OD in sum of the lower and upper wells - OD of control medium in the lower well)] × 100.

For evaluating the inhibitory effect of the CXCR4 antagonists on chemotaxis, cells were pretreated with CXCR4 antagonists at 37°C for 1 h, followed by the chemotaxis assay as stated above.

Anti-HIV-1 assay. Human peripheral blood mononuclear cells, which were activated with immobilized anti-CD3 mouse mAb in RPMI-1640 medium supplemented with 10% FCS for 3 days, were infected with NL4-3 at a multiplicity of infection of 0.001. After 3 h of adsorption, the cells were washed, and cultured in CM supplemented with recombinant human interleukin-2 (50 U/mL), in the presence or absence of the test compounds. Amounts of HIV-1 capsid (p24) antigen produced in the culture supernatants were measured by an enzyme-linked immunosorbent assay kit (ZeptoMatrix Corp., Buffalo, NY, USA) 7–10 days after infection.

Results

The initial purpose of this study was to evaluate whether a series of CXCR4 antagonists could inhibit cancer metastasis, which is promoted by the interaction between SDF-1α and CXCR4. In order to evaluate the antagonistic effect of the compounds, we sought to develop an assay system for quantitatively detecting SDF-1α-mediated chemotaxis induced by the interaction. In this experiment, we employed CD4⁺ leukemic cell line Jurkat as a CXCR4⁺ indicator.⁽²⁰⁾ Since Jurkat sublines have different characteristics, we compared CXCR4 expression in the original Jurkat cells and its subline E6-1 by using flow cytometry. As expected, CXCR4 expression was comparable in both cell lines, while CD4 expression was greater in the Jurkat cells (Fig. 1a).

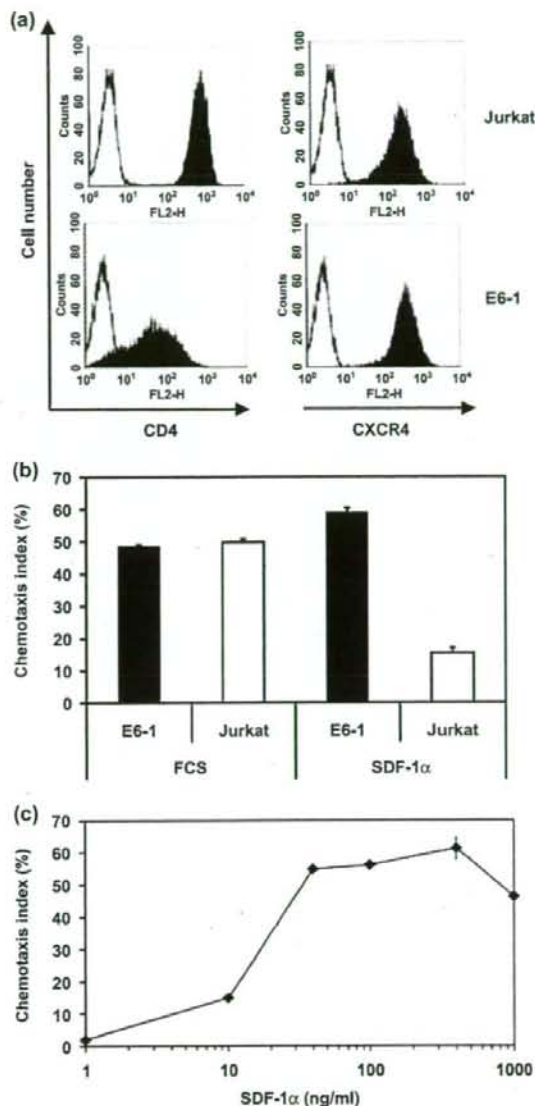


Fig. 1. A quantitative assay system for stromal cell-derived factor-1α (SDF-1α)-mediated chemotaxis. (a) Evaluation of CD4 and CXCR4 expression on Jurkat and its subline E6-1. The cells were stained with phycoerythrin-labeled anti-CXCR4 or anti-CD4 mouse monoclonal antibodies. Open and closed lines indicate fluorescence of the control and stained cells, respectively. (b) Effect of SDF-1α on chemotaxis of Jurkat and its subline E6-1. The cell lines were incubated with the control medium including 400 ng/mL of SDF-1α or 10% fetal calf serum (FCS) for 24 h at 37°C. The results are shown as a chemotaxis index and standard deviation. The calculation of the chemotaxis index is described in 'Materials and Methods'. (c) Dose-dependent effect of SDF-1α on the chemotaxis of E6-1 cells. Increasing amounts of SDF-1α were treated with E6-1 cells for 3 h and the levels of migration to the lower well are indicated as a chemotaxis index.

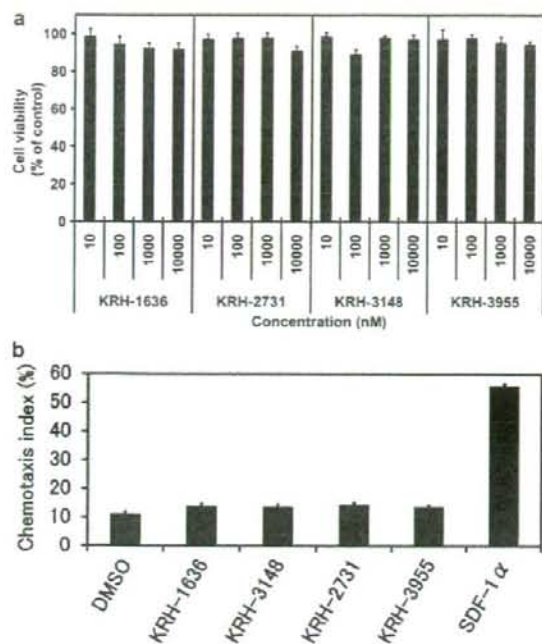


Fig. 2. CXC chemokine receptor-4 (CXCR4) antagonists exhibited neither cytotoxic nor agonistic effects. (a) Increasing amounts of CXCR4 antagonists were examined for their cytotoxic effect on E6-1 cells. (b) CXCR4 antagonists (10 μ M) or stromal cell-derived factor-1 α (SDF-1 α) (100 ng/mL) were added to the lower wells in a chemotaxis assay and were incubated at 37°C for 3 h. The treated E6-1 cells were evaluated for the chemotaxis index. DMSO, dimethyl sulfoxide.

Next, the two cell lines were analyzed for SDF-1 α -mediated chemotaxis activity; after 24 h of incubation, about 30% of both Jurkat and E6-1 migrated to the lower wells in the presence of the control medium. Since the value was the background for this chemotaxis assay, we subtracted this value from the subsequent experiments. We decided to use 400 ng/mL of SDF-1 α for the chemotaxis assay as previously described by Liang *et al.*⁽²¹⁾ It was found that SDF-1 α induced a four-fold increase in the migration efficiency of E6-1 cells compared to the original Jurkat cells (Fig. 1b). Therefore, we decided to use E6-1 cells for the subsequent experiments.

Next, we attempted to optimize the experimental conditions for the SDF-1 α -mediated chemotaxis assay. The chemotaxis index plateaued at approximately 60% after 3 h incubation of E6-1 cells with 400 ng/mL of SDF-1 α (data not shown). We then examined the effect of increasing concentration of SDF-1 α on the chemotaxis index and found that the level of chemotaxis was augmented in a dose-dependent manner and plateaued when more than 40 ng/mL of SDF-1 α was used (Fig. 1c). Accordingly, the optimal condition for the chemotaxis assay in subsequent experiments was 100 ng/mL of SDF-1 α for a 3 h incubation period.

Next, we analyzed the cytotoxicity of CXCR4 antagonists to E6-1 cells. As indicated in Figure 2(a), the CXCR4 antagonists were not cytotoxic for E6-1 cells at a 10 μ M concentration. To ascertain the possibility of these antagonists also exhibiting agonistic activities, we examined the chemotaxis activity of the antagonists. We observed that 100 ng/mL SDF-1 α efficiently induced migration of E6-1; however, none of antagonists induced migration even at 10 μ M (Fig. 2b). This indicated that the CXCR4 antagonists did not possess agonistic properties.

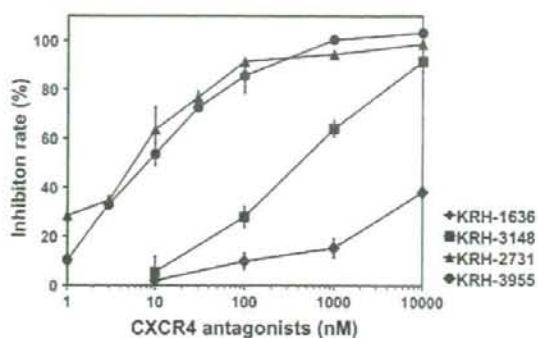


Fig. 3. Dose-dependent effect of CXC chemokine receptor-4 (CXCR4) antagonists on inhibition of stromal cell-derived factor-1 α (SDF-1 α)-mediated chemotaxis. E6-1 cells were pretreated with each concentration of CXCR4 antagonists at 37°C for 1 h, followed by incubation with 100 ng/mL of SDF-1 α for 3 h. The cells were evaluated for the chemotaxis index. The inhibition rate was calculated as the percentage inhibition of chemotaxis by the antagonists.

Table 1. Inhibitory effects of CXCR4 antagonists on SDF-1 α -mediated chemotaxis and HIV-1 infection

CXCR4 antagonists	CXCR4 (EC ₅₀ , nM)	
	Chemotaxis	HIV-1
KRH-1636	>10 000	42
KRH-3148	396.7	4
KRH-2731	9.2	0.9
KRH-3955	5.3	1

The effect of CXCR4 antagonists on the chemotaxis was investigated under the same conditions as described above. The prototype antagonist KRH-1636 inhibited the SDF-1 α -mediated chemotaxis up to approximately 40% at a maximal concentration (10 μ M). By contrast, KRH-3148 almost completely inhibited the chemotaxis at the maximal concentration; moreover, KRH-2731 and KRH-3955 showed the maximum inhibition rate even at 1 μ M (Fig. 3). In order to quantitatively compare these efficacies, 50% effective concentration (EC₅₀) was calculated (Table 1). The results from this study clearly showed that KRH-2731 and KRH-3955 were effective at >1000-fold as compared with KRH-1636.

We further evaluated the effect of the compounds on HIV-1 infection. Anti-HIV-1 activities in nM of KRH-1636, KRH-3148, KRH-2731, and KRH-3955, which were shown as EC₅₀, were 42, 4, 0.9, and 1, respectively (Table 1). The efficacy of the antagonists was highly correlated with their inhibitory effects on HIV-1 infection by interrupting the association of the Env with CXCR4. Interestingly, inhibition of chemotaxis by KRH-1636 and KRH-3148 was relatively lower than that of HIV-1 infection compared with KRH-2731 and KRH-3955. The difference may be because action sites of KRH-2731 or KRH-3955 against CXCR4 are somewhat different from those of KRH-3148 (Sei Kumakura, unpublished data). In summary, these results demonstrate that both KRH-2731 and KRH-3955 are capable of efficiently inhibiting SDF-1 α -mediated chemotaxis as well as infection of T cell-tropic HIV-1.

Discussion

The present study demonstrated that the novel CXCR4 antagonists efficiently inhibited SDF-1 α -mediated chemotaxis as well as

infection of T cell-tropic HIV-1. Two compounds KRH-2731 and KRH-3955 were found to be highly potent inhibitors for both efficacies without any cytotoxicity or agonistic activity, indicating that they may be promising as anti-cancer metastasis and anti-HIV-1 drugs. In particular, both KRH-2731 and KRH-3955 efficiently inhibited calcium signaling induced by SDF-1 α at a concentration of 10 nM, while KRH-3148 and KRH-1636 inhibited at 100 nM and at greater than 10 μ M, respectively (Sei Kumakura *et al.*, unpublished results). This indicated that their antagonistic effects were highly correlated with their abilities to inhibit chemotaxis and HIV-1 infection.

While the Jurkat cell line expressed a smaller but almost comparable level of CXCR4 compared with E6-1 cells (Fig. 1a), their migration levels in the presence of SDF-1 α were quite different (Fig. 1b). It is possible that the original Jurkat cells express non-functional CXCR4 with regard to signal transduction that is required for chemotaxis.

Tumor cells from various types of human cancers of epithelial, mesenchymal, and hematopoietic origins express high levels of CXCR4.^(14,16) The interaction of SDF-1 α with its receptor CXCR4 contributes to metastasis of breast cancer as well as a number of other malignancies in the lung, brain, and prostate. Furthermore, patients with cancers expressing high levels of CXCR4 have more extensive metastasis at lymph nodes compared with low CXCR4-expressing ones.⁽²²⁾ On this basis, the efficient CXCR4 antagonists demonstrated in this study may be highly valuable for the regulation of cancer metastasis. In fact, a synthetic peptide against CXCR4 efficiently inhibited metastasis of breast cancer in a mouse model,⁽²¹⁾ thus providing support to our notion. However, a hurdle remains for the delivery of the

peptide inhibitor to the primary focus of cancer in patients, thus impeding the clinical application of the inhibitor. In this regard, our low molecular weight CXCR4 antagonists are promising because they are non-cytotoxic and can be administered orally. In fact, KRH-3955 showed oral bioavailability of 25.6% in rats and its oral administration blocked X4 HIV-1 replication in the human peripheral blood lymphocytes and in severe combined immunodeficiency mouse system (Tsutomu Murakami *et al.*, manuscript in preparation). It is notable that AMD3100, another small non-peptide CXCR4 antagonist, has been shown to inhibit metastasis of cancer cells *in vitro* and *in vivo*.^(23,24) Moreover, our preliminary data suggested that injection of the breast cancer cell line MDA-231 produced a huge tumor at the inoculated site as well as aggressive metastasis in the lungs of mice, and that our compounds partially inhibited both the primary tumor growth and the metastasis (data not shown).

In conclusion, CXCR4 antagonists, which can be orally administered, are promising agents for SDF-1 α -mediated metastasis of cancer cells and also for the treatment and prophylaxis of a number of diseases related to the interaction between CXCR4 and SDF-1 α , the best example of which would be an anti-HIV-1 drug.

Acknowledgments

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References

- 1 Ward SG, Westwick J. Chemokines: understanding their role in T-lymphocyte biology. *Biochem J* 1998; **333**: 457-70.
- 2 Le Y, Zhou Y, Iribarren P *et al.* Chemokines and chemokine receptors: their manifold roles in homeostasis and disease. *Cell Mol Immunol* 2004; **1**: 95-104.
- 3 Burger JA, Kipps TJ. CXCR4: a key receptor in the cross-talk between tumor cells and their microenvironment. *Blood* 2006; **107**: 1761-7.
- 4 Horuk R. Chemokine receptors. *Cytokine Growth Factor Rev* 2001; **12**: 313-15.
- 5 Nagasawa T, Hirota S, Tachibana K *et al.* Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* 1996; **382**: 635-8.
- 6 Tachibana K, Hirota S, Iizasa H *et al.* The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature* 1998; **393**: 591-4.
- 7 Zou YR, Kottmann AH, Kuroda M *et al.* Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature* 1998; **393**: 595-9.
- 8 Burns JM, Summers BC, Wang Y *et al.* A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *J Exp Med* 2006; **203**: 2201-13.
- 9 Berger EA, Murphy PM, Farber JM. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. *Annu Rev Immunol* 1999; **17**: 657-700.
- 10 Su L, Kaneshima H, Bonyhadi M *et al.* HIV-1-induced thymocyte depletion is associated with indirect cytopathogenicity and infection of progenitor cells *in vivo*. *Immunity* 1995; **2**: 25-36.
- 11 Bleul CC, Farzan M, Choe H *et al.* The lymphocyte chemoattractant SDF-1 is a ligand for LESTR/fusin and blocks HIV-1 entry. *Nature* 1996; **382**: 829-33.
- 12 Alkhatib G, Combadiere C, Broder CC *et al.* CC CKR5: a RANTES, MIP-1 α , MIP-1 β receptor as a fusion cofactor for macrophage-tropic HIV-1. *Science* 1996; **272**: 1955-8.
- 13 Cocchi F, DeVico AL, Garzino-Demo A *et al.* Identification of RANTES, MIP-1 α , and MIP-1 β as the major HIV-suppressive factors produced by CD8⁺ T cells. *Science* 1995; **270**: 1811-15.
- 14 Balkwill F. Cancer and the chemokine network. *Nature Rev Cancer* 2004; **4**: 540-50.
- 15 Luker KE, Luker GD. Functions of CXCL12 and CXCR4 in breast cancer. *Cancer Lett* 2006; **238**: 30-41.
- 16 Muller A, Homey B, Soto H *et al.* Involvement of chemokine receptors in breast cancer metastasis. *Nature* 2001; **410**: 50-6.
- 17 Liotta LA. An attractive force in metastasis. *Nature* 2001; **410**: 24-5.
- 18 Santiago B, Balex F, Palao G *et al.* CXCL12 is displayed by rheumatoid endothelial cells through its base amino-terminal motif on heparan sulfate proteoglycans. *Arthritis Res Ther* 2006; **8**: R43.
- 19 Ichiyama K, Yokoyama-Kumakura S, Tanaka Y *et al.* A duodenally absorbable CXC chemokine receptor 4 antagonist, KRH-1636, exhibits a potent and selective anti-HIV-1 activity. *Proc Natl Acad Sci USA* 2003; **100**: 4185-90.
- 20 Tamamura H, Hori A, Kanzaki N *et al.* T140 analogs as CXCR4 antagonists identified as anti-metastatic agents in the treatment of breast cancer. *FEBS Lett* 2003; **550**: 79-83.
- 21 Liang Z, Wu T, Lou H *et al.* Inhibition of breast cancer metastasis by selective synthetic polypeptide against CXCR4. *Cancer Res* 2004; **64**: 4302-8.
- 22 Dewan MZ, Ahmed S, Iwasaki Y *et al.* Stromal cell-derived factor-1 and CXCR4 receptor interaction in tumor growth and metastasis of breast cancer. *Biomed Pharmacother* 2006; **60**: 273-6.
- 23 Yoon Y, Liang Z, Zhang X *et al.* CXC chemokine receptor-4 antagonist blocks both growth of primary tumor and metastasis of head and neck cancer in xenograft mouse models. *Cancer Res* 2007; **67**: 7518-24.
- 24 Li JK, Yu L, Shen Y *et al.* Inhibition of CXCR4 activity with AMD3100 decreases invasion of human colorectal cancer cells *in vitro*. *World J Gastroenterol* 2008; **14**: 2308-13.