TABLE 3. KRH-3955 susceptibilities of drug-resistant viruses[a]

Virus[b]	IC_{50} (nM)[c]	
	KRH-3955	AMD3100
NL4-3	0.50	4.6
HXB2	0.60	6.2
NRTI-Res (HXB2-env)	0.60	9.0
NNRTI-Res (HXB2-env)	0.80	7.0
PI-Res (HXB2-env)	0.70	9.2
MDR (HXB2-env)	0.70	5.3
T20-Res (NL4-3-env)	0.40	2.3

[a] Susceptibility of drug-resistant HIV-1 was measured by using a single-cycle recombinant virus assay (see Materials and Methods).
[b] The pseudoviruses containing X4-tropic envelope (HXB2 or NL4-3) and patient-derived PR and RT sequences containing mutations associated with resistance to PR (PI-Res), RT (NRTI-Res or NNRTI-Res), or both (MDR) (the mutations are not shown). T20-Res contains a site-directed mutation (Q40H) in the NL4-3 envelope.
[c] IC50, 50% inhibitory concentration of CXCR4 antagonists.

of ^{125}I-labeled SDF-1α, ^{125}I-labeled RANTES, ^{125}I-labeled MCP-1, ^{125}I-labeled TARC, ^{125}I-labeled RANTES, or ^{125}I-labeled IL-8 to CXCR4, CCR1, CCR2b, CCR4, CCR5, or CXCR1, respectively (Fig. 2). Thus, KRH-3955 selectively blocks the binding of SDF-1α to CXCR4.

KRH-3955 exhibits inhibition of Ca^{2+} signaling through CXCR4. We next examined whether KRH-3955 acts as an agonist or antagonist of CXCR4 by using CXCR4-expressing CHO cells. The addition of KRH-3955 inhibited the SDF-1α-induced increase in the intracellular Ca^{2+} concentration in a dose-dependent manner, whereas 100 nM AMD3100 did not affect Ca^{2+} mobilization (Fig. 4). KRH-3955 itself did not affect Ca^{2+} mobilization at up to 1 μM (data not shown). We performed the Ca^{2+} mobilization assay with human PBMCs but could not detect an SDF-1α-induced Ca^{2+} signal mainly due to low expression of CXCR4 (data not shown). Thus, KRH-3955 inhibits Ca^{2+} signaling through CXCR4.

Effect of KRH-3955 on anti-CXCR4 antibody binding to CXCR4-expressing cells. To localize the binding site(s) of KRH-3955, the effects of KRH-3955 and AMD3100 on the binding of four types of anti-CXCR4 MABs were first examined. We used MAbs A145, 12G5, 44717, and A80, which are specific for the N terminus, extracellular loop 1 (ECL1) and ECL2, ECL2, and ECL3, respectively. Neither KRH-3955 nor AMD3100 inhibited A145 binding to CXCR4-expressing Molt-4 cells (Fig. 5). Both compounds inhibited the binding of MAbs 12G5, 44717, and A80 to Molt-4 cells in a dose-depen-

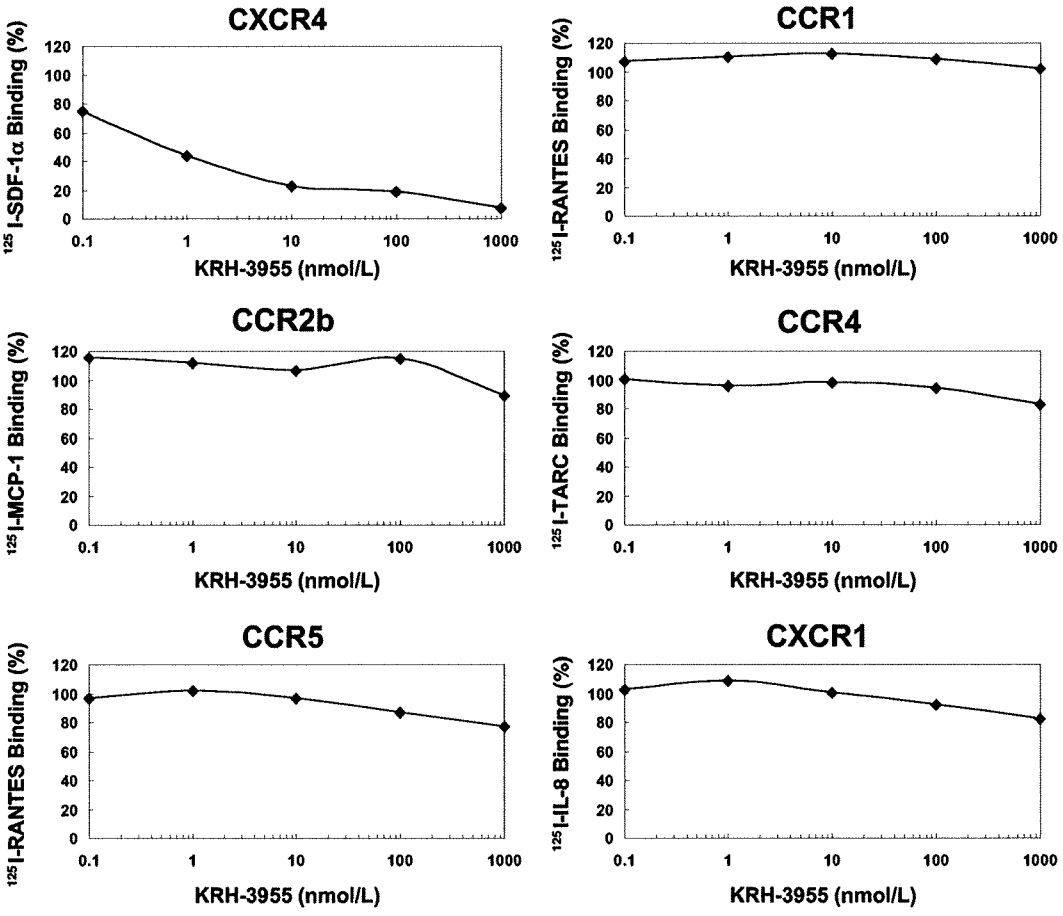

FIG. 2. Inhibitory effects of KRH-3955 on chemokine binding to CXCR4-, CCR1-, CCR2b-, CCR4-, CCR5-, or CXCR1-expressing CHO cells. Chemokine receptor-expressing CHO cells were incubated with various concentrations of KRH-3955 in binding buffer containing ^{125}I-labeled chemokine. Binding reactions were performed on ice and were terminated by washing out the unbound ligand. Cell-associated radioactivity was measured with a scintillation counter. Percent binding was calculated as 100 × [(binding with inhibitor − nonspecific binding)/(binding without inhibitor − nonspecific binding)]. The data represent the means in duplicate wells in a single experiment.

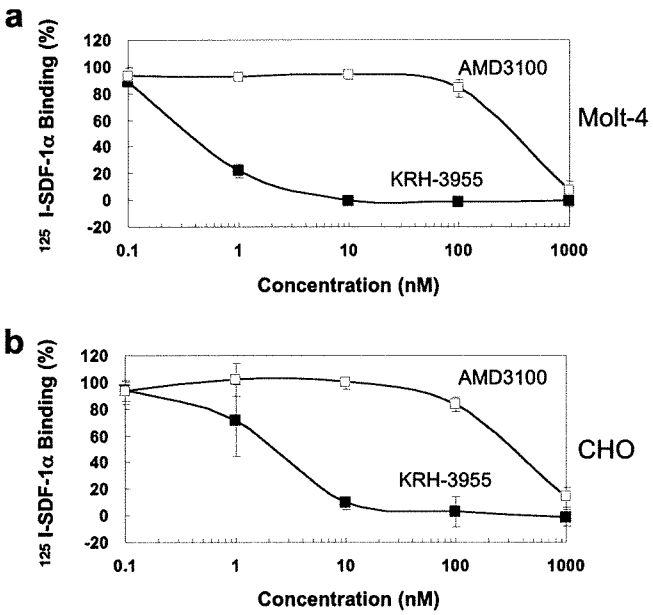

FIG. 3. Concentration-dependent inhibition by KRH-3955 of SDF-1α binding to (a) Molt-4 and (b) CXCR4-expressing CHO cells. CXCR4-expressing CHO cells were incubated with various concentrations of KRH-3955 (■) or AMD3100 (□) in binding buffer containing ^{125}I-labeled SDF-1α. Binding reactions were performed, and percent binding was calculated as described in the legend to Fig. 2. The data represent the means ± standard deviations of three independent experiments.

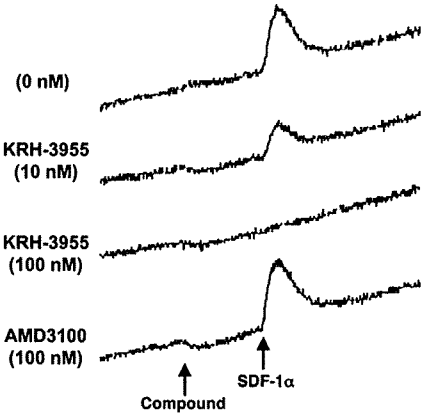

FIG. 4. Inhibitory effects of KRH-3955 on SDF-1α-induced Ca^{2+} mobilization in CXCR4-expressing CHO cells. Fura-2-acetoxymethyl ester-loaded CXCR4-expressing CHO cells were incubated in the presence or absence of various concentrations of KRH-3955 or AMD3100. Changes in intracellular Ca^{2+} levels in response to SDF-1α (1 μg/ml) were determined with a fluorescence spectrophotometer. The data show representative data for two independent experiments.

dent manner. The inhibitory activity of KRH-3955 is similar to its anti-HIV-1 activity, whereas the inhibitory activity of AMD3100 is much weaker than its anti-HIV-1 activity. Similar data were obtained when activated human PBMCs were used as target cells (data not shown). KRH-3955 itself did not induce internalization of CXCR4 at concentrations of up to 1 μM (data not shown), as KRH-1636 did (23). These results suggest that the binding sites of KRH-3955 are located in a region composed of all three ECLs of CXCR4.

Long-lasting inhibitory effects of KRH-3955 on the binding of MAb 12G5. The inhibitory effect of KRH-3955 on the binding of MAb 12G5 was examined with or without washing of the compound from the cells. Molt-4 cells were treated with 10 nM KRH-3955 or 1,000 nM AMD3100 for 15 min. With or without washing, the cells were stained with MAb 12G5-PE and the amount of bound antibody was analyzed by flow cytometry. KRH-3955 strongly inhibited MAb 12G5 binding to Molt-4 cells irrespectively of washing (Fig. 6a). In contrast, AMD3100 efficiently inhibited MAb 12G5 binding without washing away of the compound but lost its inhibitory activity after washing away of the compound (Fig. 6a). The long-lasting inhibitory effect of KRH-3955 on the binding of MAb 12G5 was further tested. Molt-4 cells were preincubated with or without KRH-3955 at 10 nM. The compound was washed away, and the cells were further incubated at 37°C in compound-free growth medium. At 0, 3, and 6 h after compound removal, the cells were stained with MAb 12G5-PE and analyzed by flow cytometry. Even at 6 h after washing away of the compound, KRH-3955 inhibited MAb 12G5 binding by approximately 40% (Fig. 6b). These results suggest that KRH-3955 has a strong binding affinity for CXCR4 and a slow dissociation rate, although competition assays with the two molecules (KRH-3955 versus MAb 12G5 with radioactive, nonradioactive, or different labeling) are necessary to provide definitive conclusions.

Inhibition of MAb 12G5 binding to CXCR4 mutants by KRH-3955. The effects of different CXCR4 mutations on the inhibitory activity of KRH-3955 against MAb 12G5 binding to CXCR4 were examined. HEK293-CXCR4 transfectants were preincubated with various concentrations of KRH-3955 and AMD3100, after which the compound was washed away. The binding of PE-conjugated MAb 12G5 was measured by flow cytometry. As reported previously, AMD3100 substantially lost its blocking activity against MAb 12G5 binding for D171A (TM4), D262A (TM6), and E288A/L290A (TM7) mutants, as shown by previous reports (Table 4) (20, 37, 38). In contrast, the blocking activity of KRH-3955 against MAb 12G5 binding was not affected by the above mutations. In contrast, the H281A (ECL3) mutant displayed decreased inhibition of MAb 12G5 binding by KRH-3955 (Table 4). These data further support the hypothesis that the CXCR4 interaction sites of KRH-3955 are different from those of AMD3100.

Pharmacokinetic studies of KRH-3955 in rats. In pharmacokinetics studies, KRH-3955 was orally or intravenously administrated to Sprague-Dawley rats at a dose of 10 mg/kg. The plasma concentration of R-176211, the free form of KRH-3955, was monitored by liquid chromatography-tandem mass spectrometry. In these studies, KRH-3955 was found to be well absorbed and the absolute oral bioavailability in rats was calculated to be 25.6% based on the area under the plasma concentration-time curve (Table 5). However, KRH-3955 also showed a long elimination half-life after single-dose administration to rats, suggesting long-term accumulation of the compound in tissues (Table 5). KRH-3955 was found to be stable in human hepatic microsomes, and no significant inhibition of CYP450 liver enzymes by this compound was observed (data

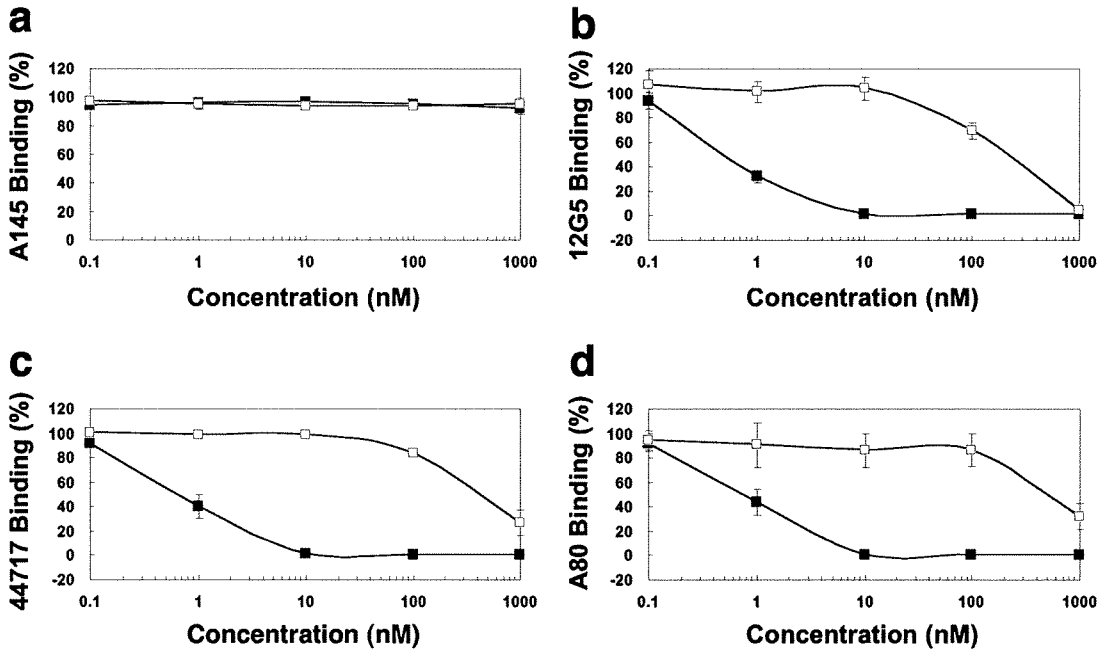

FIG. 5. Effect of KRH-3955 on the binding of four different MAbs to the CXCR4 receptor. Molt-4 cells were incubated with various concentrations of KRH-3955 (■) or AMD3100 (□). The cells were stained directly with MAbs 12G5 (recognizes ECL1 and ECL2 of CXCR4)-PE, 44717 (recognizes ECL2 of CXCR4)-PE, and A145 (recognizes the N terminus of CXCR4)-FITC or indirectly with MAb A80 (recognizes ECL3 of CXCR4). The mean fluorescence of the stained cells was analyzed with a FACScalibur flow cytometer. Percent binding was calculated with the equation described in the legend to Fig. 2. The data represent the means ± standard deviations of three independent experiments.

not shown). Thus, orally administered KRH-3955 is bioavailable in rats.

KRH-3955 efficiently suppresses X4 HIV-1 infection in hu-PBL–SCID mice. We then examined whether KRH-3955 can interfere with X4 HIV-1 infection in vivo by using hu-PBL–SCID mice. Mice were administrated a single dose (10 mg/kg) of either KRH-3955 or tartrate (as a control) p.o. and fed for 2 weeks. These mice were then engrafted with human PBMCs, and after 1 day, these "humanized" mice were infected with infectious X4 HIV-1 (NL4-3). After 7 days, human lymphocytes harvested from the peritoneal cavities and spleens of the infected mice were cultured for 4 days in vitro in the presence of rhIL-2 in order to determine the level of HIV-1 infection by the p24 enzyme-linked immunosorbent assay. The maximum concentration of KRH-3955 in blood after p.o. administration was estimated to be 100 nM (data now shown). Under these conditions, four of five mock-treated mice were infected whereas only one of five mice treated with KRH-3955 was infected (Table 6). The one infected mouse in the KRH-3955-treated group (no. 5)

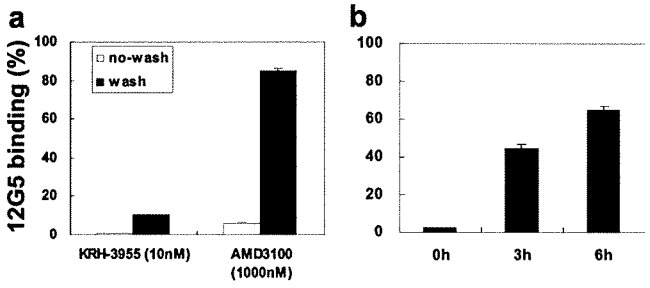

FIG. 6. Long-lasting inhibitory effects of KRH-3955 on the binding of MAb 12G5. (a) Molt-4 cells were treated with 10 nM KRH-3955 or 1,000 nM AMD3100 for 15 min. With (■) or without (□) washing, the cells were staining with MAb 12G5-PE and analyzed by flow cytometry. (b) Long-lasting inhibitory effect of KRH-3955 on the binding of MAb 12G5. Molt-4 cells were preincubated with or without KRH-3955 at 10 nM. The compound was washed away, and the cells were further incubated at 37°C in compound-free RPMI medium. At 0, 3, and 6 h after removal of the compound, the cells were staining with MAb 12G5-PE and analyzed by flow cytometry. The data represent the means of triplicate wells in a single experiment.

TABLE 4. Affinity of KRH-3955 and AMD3100 for wild-type CXCR4 and various mutant forms of CXCR4[a]

CXCR4 (location)	KRH-3955		AMD3100	
	IC_{50}	IC_{90}	IC_{50}	IC_{90}
Wild type	2.8 ± 0.5	8.2 ± 0.4	289.1 ± 25.5	971.1 ± 31.2
V99A (ECL1)	1.5 ± 0.2	7.4 ± 0.2	258.5 ± 25.9	>1,000
V112A (TM3)	2.2 ± 0.2	>10	196.6 ± 28.5	821.3 ± 15.4
H113A (TM3)	0.8 ± 0.3	6.3 ± 0.2	296.4 ± 112.2	>1,000
D171A (TM4)	3.2 ± 0.1	>10	>1,000	>1,000
D181A (ECL2)	0.5 ± 0.1	5.1 ± 0.3	143.7 ± 29.3	795.6 ± 79.9
H203A (TM5)	0.5 ± 0.1	5.3 ± 0.1	259.0 ± 11.5	860.6 ± 22.4
D262A (TM6)	1.6 ± 0.3	8.1 ± 0.5	>1,000	>1,000
E275A (ECL3)	1.0 ± 0.2	6.4 ± 0.1	235.6 ± 30.2	930.2 ± 26.1
E277A (ECL3)	3.1 ± 0.1	8.7 ± 0.1	469.5 ± 19.2	>1,000
V280A (ECL3)	1.0 ± 0.2	6.1 ± 0.1	175.3 ± 10.3	821.2 ± 47.3
H281A (ECL3)	14.1 ± 5.2	248.3 ± 74.9	72.7 ± 42.9	572.2 ± 118.1
W283A (ECL3)	1.3 ± 0.2	6.9 ± 0.2	300.2 ± 10.5	>1,000
I284A (TM7)	1.2 ± 0.2	6.8 ± 0.5	265.8 ± 20.8	>1,000
E288A/L290A (TM7)	1.6 ± 0.1	7.7 ± 0.3	>1,000	>1,000

[a] The data shown, which represent means ± SDs (n = 3) of nanomolar concentrations, were obtained from competition binding on HEK293 cells expressing the wild-type or mutant CXCR4 receptors with MAb 12G5.

TABLE 5. Pharmacokinetic parameters of KRH-3955 after single oral administration in rats[a]

Parameter	Value when given i.v. or p.o. at 10 mg/kg
Bioavailability (%)[b]	25.6
I.v. half-life (h)	99.0 ± 13.1
I.v. CL (liters/h/kg)[c]	3.9 ± 0.07
V_1 (ss) (liters/kg)[d]	374.0 ± 14
P.o. C_{max} (ng/ml)[e]	86.3 ± 23.6
T_{max} (h)[f]	2.3 ± 1.53
P.o. AUC_{0-336} (ng · h/ml)[g]	325.0 ± 38

[a] The data shown are means ± SDs (n = 3).
[b] Bioavailability = $(AUC_{oral}/AUC_{i.v.}) \times (dose_{i.v.}/dose_{oral}) \times 100$.
[c] CL, clearance.
[d] V_1 (ss), volume of distribution in central compartment at steady state.
[e] C_{max}, maximum concentration of drug in serum.
[f] T_{max}, time to maximum concentration of drug in serum.
[g] AUC_{0-336}, area under the plasma concentration-time curve from time zero to 336 h.

TABLE 6. Inhibition of infection of hu-PBL–SCID mice with X4 HIV-1 by KRH-3955[a]

Group and mouse no.	p24 produced (pg/ml)
Control	
1	747
2	10,263
3	<5
4	5,821
5	1,902
KRH-3955	
6	<5
7	<5
8	<5
9	<5
10	36

[a] Two groups of C.B-17 SCID mice (n = 5) were administrated a single dose of either KRH-3955 or tartrate (as a control) p.o. and fed for 2 weeks. These mice were then engrafted with human PBMCs (1×10^7 per animal i.p.), and after 1 day, these "humanized" mice were infected with 1,000 infective units of X4 HIV-1_{NL4-3}. IL-4 (2 mg per animal) was administrated i.p. on days 0 and 1 after PBMC engraftment to enhance X4 HIV-1 infection. After 7 days, human lymphocytes were harvested from the infected mice and cultured in vitro for 4 days in medium containing 20 U/ml IL-2. HIV-1 infection was monitored by measuring p24 levels. Means from duplicate determinations are shown. <5, below detection level.

showed low levels of p24 production. These results indicate that single-dose p.o. administration of KRH-3955 was very effective in protecting against X4 HIV-1 infection in an in vivo mouse model.

DISCUSSION

In this study, we clearly demonstrate that KRH-3955, a KRH-1636 derivative that is bioavailable when administered orally, is a potent inhibitor of HIV-1 infection both in vitro and in vivo. KRH-3955 selectively inhibited X4 HIV-1 strains, including clinical isolates, as we have previously shown with KRH-1636. Furthermore, KRH-3955 is approximately 40 times more potent than KRH-1636 in its anti-HIV-1 activity in activated PBMCs (Table 1). The anti-HIV-1 activity of KRH-3955 was independent of the PBMC donor (Table 2). KRH-3955 also inhibited the infectivity of recombinant viruses resistant to NRTIs, NNRTIs, PIs, and T20 (Table 3). Pharmacokinetic studies of KRH-3955 indicated that the compound is bioavailable in rats when administered orally (Table 5). In addition, oral administration of the compound efficiently inhibited the replication of X4 HIV-1 in the hu-PBL–SCID mouse model (Table 6). Although we could show that KRH-3955 is a potent inhibitor of subtype B HIV-1 isolates, we need to examine the efficacy of this compound against non-subtype B HIV-1 isolates because of the global nature of the HIV/AIDS epidemic and the regional diversity of HIV-1 subtypes.

R5 HIV-1 is isolated predominantly during the acute and asymptomatic stage (12) and is also believed to be important for virus transmission between individuals. In contrast, X4 HIV-1 strains emerge in approximately 50% of infected individuals and their emergence is associated with a rapid $CD4^+$ T-cell decline and disease progression (35, 50). One recent report also indicated that detection of X4 HIV-1 at baseline independently predicted disease progression (13), although it is still not known whether the emergence of X4 HIV-1 is a cause or outcome of disease progression. These findings strongly support the need for highly potent CXCR4 inhibitors that are bioavailable when administered orally such as KRH-3955.

Inhibition of ligand binding to chemokine receptors by KRH-3955 was specific for CXCR4 (Fig. 2), as we observed previously for KRH-1636. This specific inhibition of SDF-1α binding to CXCR4 by KRH-3955 is absolutely necessary for developing an anti-HIV agent to avoid immune dysregulation by nonspecific inhibition of binding by other chemokines. It is of note that the inhibitory activity of the compound against SDF-1α binding is similar to that against HIV-1 infection, which is different from that of control compound AMD3100. Where on the CXCR4 molecule is the binding site(s) of KRH-3955? Experiments to examine the effect of KRH-3955 on the binding of several anti-CXCR4 MAbs suggest that the binding sites of KRH-3955 are located in all three ECLs of CXCR4 (Fig. 5). To further define the binding site(s) of KRH-3955, we examined the effects of CXCR4 point mutations on the inhibitory activity of KRH-3955 against MAb 12G5 binding to the receptor. AMD3100 was used as a control. The inhibitory activity of AMD3100 against MAb 12G5 binding to the receptor was greatly reduced by the mutations D171A (TM4), D262A (TM6), and E288A/L290A (TM7), as reported previously (Table 4) (20, 37, 38). Of note, these mutations also affect SDF-1α binding and/or CXCR4 coreceptor activity (8). Unexpectedly, none of these three mutations affected the inhibition of MAb 12G5 binding by KRH-3955 (Table 4). Only the H281A (ECL3) mutant showed decreased inhibition of MAb 12G5 binding by KRH-3955 (Table 4). Interestingly, the same mutant modestly increased the blocking activity of AMD3100 against MAb 12G5 binding. In addition, the H281A mutation markedly impaired inhibition of MAb 12G5 binding by AMD3465, one of the prototype monocyclams (37). Further experiments with different CXCR4 mutants are necessary to identify the exact site(s) on CXCR4 targeted by this compound.

Pharmacological tests of KRH-3955 were performed with rats, and the compound was found to be bioavailable when administered orally (Table 5), which is favorable for anti-HIV drugs. However, the compound also indicated a long half-life after single-dose administration to rats, suggesting long-term accumulation of the compound in tissues, which can be either advantageous

in terms of inhibiting HIV-1 infection in hu-PBL–SCID mice (Table 6) or disadvantageous in terms of toxicity. Further studies are ongoing to determine the safety and pharmacokinetics of the compound in other animals such as dogs and monkeys. To evaluate the in vivo efficacy of KRH-3955, we used the hu-PBL–SCID mouse model and showed that oral administration of the compound strongly protected against X4 HIV-1 infection in this model system (Table 6). To achieve substantial replication of X4 HIV-1 in this system, recombinant IL-4 was added after human PBMC engraftment as described previously (23). Notably, KRH-3955 was administered only once 2 weeks before PBMC engraftment and was effective enough to block X4 HIV-1 infection, suggesting that the compound can be used as a preexposure prophylaxis agent to prevent HIV infection. This long-lasting antiviral effect of KRH-3955 can be partly explained by the strong affinity of the compound for CXCR4 (Fig. 6) and long-term accumulation of the compound in tissues.

In terms of safety of anti-HIV drugs, CCR5 antagonists are considered to be relatively safe because of the lack of obvious health problems in individuals homozygous for the CCR5 delta32 allele (27, 39). Indeed, maraviroc, a CCR5 antagonist, was approved by the U.S. FDA in 2007. In contrast, CXCR4 antagonists, which inhibit SDF-1α–CXCR4 interactions, may cause severe adverse effects because knocking out either the SDF-1α or the CXCR4 gene in mice causes marked defects such as abnormal hematopoiesis and cardiogenesis, in addition to vascularization of the gastrointestinal tract (32, 44, 52). However, no severe side effects have been reported for either AMD3100, a well-characterized CXCR4 antagonist, or AMD070, an oral CXCR4 antagonist, in human volunteers and/or HIV-infected patients. Milder side effects, including gastrointestinal symptoms and paresthesias, were common at higher doses of AMD3100. These results indicate the feasibility of using CXCR4 antagonists as anti-HIV-1 drugs in a clinical setting (21, 22, 41).

Besides the physiological roles mentioned above, the CXCR4–SDF-1 axis is also involved in various diseases such as cancer metastasis, leukemia cell progression, rheumatoid arthritis, and pulmonary fibrosis. CXCR4 antagonists such as AMD3100 and T140 have demonstrated activity in treating such CXCR4-mediated diseases (14, 46). In addition, AMD3100 is considered to be a stem cell mobilizer for transplantation in patients with cancers such as non-Hodgkin's lymphoma. Recently, AMD3100 has been shown to increase T-cell trafficking in the central nervous system, leading to significant improvement in the survival of West Nile virus encephalitis (29). Given its highly potent and selective inhibition of SDF-1–CXCR4 interaction and its bioavailability when administered orally, it is important to address whether KRH-3955 can also be used for such clinical applications.

One important issue to be addressed is whether HIV-1 strains resistant to other CXCR4 antagonists show cross-resistance to KRH-3955. In our preliminary studies, AMD3100-resistant HIV-1 (kindly provided by M. Baba, Kagoshima University) (4) showed ~19-fold resistance to KRH-3955 compared with parental NL4-3, whereas the resistant virus showed ~40-fold resistance to both AMD3100 and AMD070 in MT-4 cells (data not shown). Interestingly, the AMD3100-resistant HIV-1 strain was relatively sensitive to T22, another prototype CXCR4 antagonist. Thus, KRH-3955 target sites on CXCR4 seem to partially overlap those of AMD3100, although experiments with CXCR4 mutants do not support this idea. It is important to establish KRH-3955-resistant mutants and investigate whether they also show cross-resistance to other CXCR4 antagonists. Long-term culture experiments with PM1/CCR5 cells that express both CXCR4 and CCR5 infected with X4 HIV-1 in the presence of KRH-3955 are in progress.

In conclusion, KRH-3955 is a small-molecule antagonist of the CXCR4 receptor that is bioavailable when administered orally. The compound potently and selectively inhibits X4 HIV-1 infection both in vitro and in vivo. Thus, KRH-3955 is a promising antiviral agent for HIV-1 infection and should be evaluated for its clinical efficacy and safety in humans.

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Efficient inhibition of SDF-1α-mediated chemotaxis and HIV-1 infection by novel CXCR4 antagonists

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CXC chemokine receptor-4, the receptor for stromal cell-derived factor-1α as well as human immunodeficiency virus type 1, belongs to the chemokine receptor family and has been shown to play a critical role in directing the migration of cancer cells to sites of metastasis as well as human immunodeficiency virus type 1 infection. We had previously reported that a duodenally absorbable CXC chemokine receptor-4 antagonist, KRH-1636, showed a potent anti-human immunodeficiency virus type 1 activity both *in vivo* and *in vitro*. In this study, we initially examined the effect of the compound and its derivatives on stromal cell-derived factor-1α-mediated chemotaxis of cancer cells in order to evaluate if they could be applicable as a novel inhibitor of cancer metastasis. We found that both KRH-2731 and KRH-3955 were highly potent antagonists of stromal cell-derived factor-1α-mediated chemotaxis, i.e. the derivatives exhibited 50% effective concentrations of less than 10 nM, for more than 1000-fold efficacy improvement over the prototype KRH-1636. We further demonstrated the greater anti-human immunodeficiency virus type 1 efficacy of the derivatives compared with the original KRH-1636. Taken together, the KRH-1636 derivatives KRH-2731 and KRH-3955 may be promising as a novel inhibitory drug for cancer metastasis as well as for human immunodeficiency virus type 1 infection. (*Cancer Sci* 2009)

Chemokines are secretory proteins with a molecular weight of about 8–14 kDa, and are generally alkaline and heparin-bound. The small chemokine proteins are classified into four highly conserved groups, i.e. CXC, CC, C, and CX3C (X indicates the number of amino acids between the cysteine residues) on the basis of the position of the first two cysteines that are adjacent to the amino terminus.[1] An established role for several members of the CXC and CC chemokine families is to provide directional cues for the movement of leukocytes in development, homeostasis, and inflammation.[2] At the time of the movement of leukocytes, chemokine concentration gradually increases at the inflammatory site because the chemoattractants released from the luminal surface of the endothelium, the inflammatory site of the lymphocyte, are rapidly diluted and swept downstream by blood flow. Leukocytes in the mainstream of blood flow may make contact with the endothelium via a group of molecules called selectins,[3] and may then roll along the endothelial surface.

The cell surface molecule CXC chemokine receptor-4 (CXCR4) is a 7-transmembrane-spanning, G-protein-coupled receptor for the CXC chemokine stromal cell-derived factor-1α (SDF-1α)/pre-B-cell growth stimulating factor (PBSF)/CXCL12.[2] The open reading frame of the *CXCR4* gene encodes a peptide of 352 amino acids and is interrupted by one intron in the region encoding the N-terminal segment.[4]

CXCR4 is a receptor for the SDF-1α. SDF-1α interacts with CXCR4 to play a variety of physiological roles: B-cell formation in liver and bone marrow at the fetal stage, homing of bone marrow cells in the developmental process, formation of the interventricular septum, regulation of movement of the cerebellum granule cell in neurogenesis, and large vasculogenesis that nourishes the gastrointestinal tract.[2] Since both CXCR4 and SDF-1α knockout mice do not survive, the interaction between these molecules is essential in the developmental process.[5–7] It has been reported recently that CXCR7 binds with high affinity to SDF-1α and to interferon-inducible T-cell α-chemoattractant (I-TAC, also known as CXCL11).[8] However, unlike other chemokine receptors, ligand activation of CXCR7 induces neither Ca^{2+} mobilization nor cell migration.[8]

CXCR4 is also shown to be one of the coreceptors for human immunodeficiency virus type 1 (HIV-1).[9] Entry of HIV-1 into target cells involves interactions of the viral envelope protein (Env) with CD4 and a coreceptor, mainly either CXCR4 for T-cell–tropic HIV-1,[10,11] or CCR5 for macrophage–tropic HIV-1.[12,13] In acute HIV-1 infection, primarily macrophage-tropic strains are involved in transmission of the virus, whereas T-cell–tropic strains emerge later and are associated with the rapid progression to AIDS.[9]

Importantly, cancer cells originating from the pancreas, brain, breast, prostate, kidney, ovaries, thyroid, and malignant melanoma express CXCR4; however, normal tissues scarcely express CXCR4. Increasing CXCR4 promotes metastasis of these tumor cells toward SDF-1α-expressing organs including the lungs, liver, lymph nodes, bone marrow, and adrenal glands.[14–17] Further, interaction between CXCR4 and SDF-1α promotes progression of chronic and acute lymphocytic leukemia,[3] and exacerbation of chronic rheumatoid arthritis.[18]

We previously reported that a duodenally absorbable CXCR4 antagonist, KRH-1636, competitively blocked the association of the Env protein of HIV-1 with CXCR4 both *in vivo* and *in vitro* as well as the interaction of SDF-1α with CXCR4.[19] We therefore hypothesized that KRH-1636 could be a promising chemical for offering protection from both cancer metastases induced by SDF-1α and from CXCR4-tropic HIV-1 infection. In order to assess this possibility, we sought to evaluate whether the CXCR4 antagonist KRH-1636 and its derivatives could potently inhibit SDF-1α-mediated chemotaxis of cancer cells as well as HIV-1 infection.

Materials and Methods

Reagents. SDF-1α (R&D systems, Minneapolis, MN, USA) was dissolved in phosphate-buffered saline (PBS) at 1 μM. KRH-1636,[19] and its derivatives KRH-2731, -3148, and -3955 were synthesized at Kureha Chemical Industry (Tokyo, Japan). These

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Abbreviations: CXCR4, CXC chemokine receptor-4; DMSO, dimetyl sulfoxide; EC_{50}, 50% effective concentration; Env, envelope protein; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; HIV-1, human immunodeficiency virus type 1; mAb, monoclonal antibody; OD, optical density; PBS, phosphate-buffered saline; PBSF, pre-B-cell growth stimulating factor; PE, phycoerythrin; SDF-1α, stromal cell derived factor-1α.

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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% CO_2 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 (ZeptoMetrix 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).

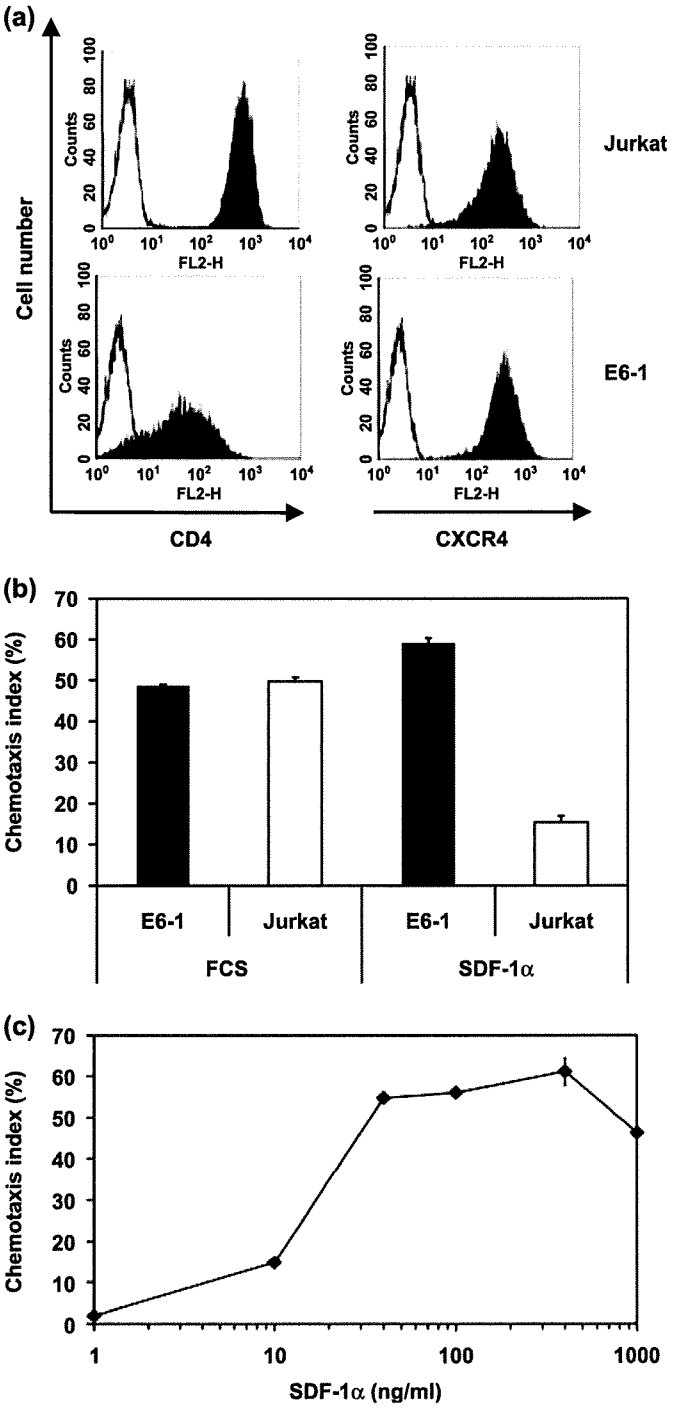

Fig. 1. A quantitative assay system for stromal cell-derived factor-1α (SDF-1α)-mediated chemotaxis. (a) Evaluation of CD4 and CXC chemokine receptor-4 (CXCR4) expression on Jurkat and its subline E6-1. The cells were stained with phycoerythrin-labeled anti-CXCR4 or anti-CD4 mouse monoclonal antibodies. 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.

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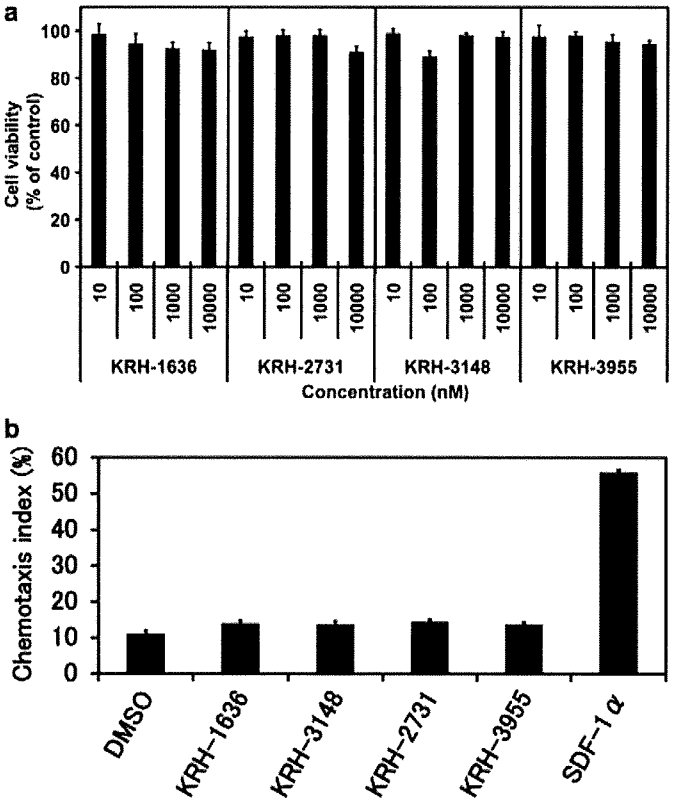

Fig. 2. CXC chemokine receptor-4 (CXCR4) antagonists exhibited neither cytotoxic nor agonistic effects. (a) Increasing amounts of CXCR4 antagonists were examined for their cytotoxic effect on E6-1 cells. (b) CXCR4 antagonists (10 μM) or stromal cell-derived factor-1α (SDF-1α) (100 ng/mL) were added to 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.

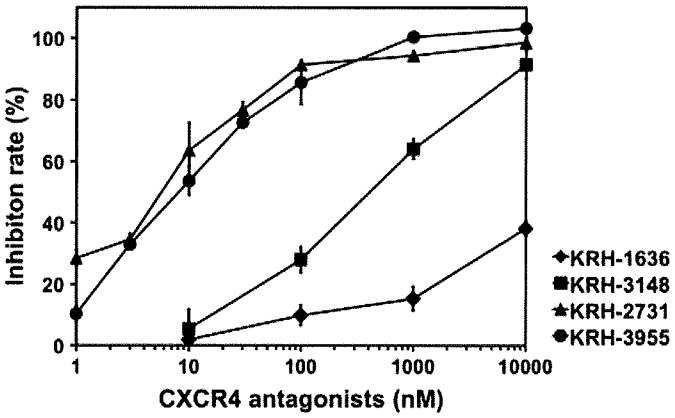

Fig. 3. Dose-dependent effect of CXC chemokine receptor-4 (CXCR4) antagonists on inhibition of stromal cell-derived factor-1α (SDF-1α)-mediated chemotaxis. E6-1 cells were pretreated with each concentration of CXCR4 antagonists at 37°C for 1 h, followed by incubation with 100 ng/mL of SDF-1α for 3 h.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

Next, the two cell lines were analyzed for SDF-1α-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-1α for the chemotaxis assay as previously described by Liang *et al.*[21] It was found that SDF-1α 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-1α-mediated chemotaxis assay. The chemotaxis index plateaued at approximately 60% after 3 h incubation of E6-1 cells with 400 ng/mL of SDF-1α (data not shown). We then examined the effect of increasing concentration of SDF-1α 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-1α was used (Fig. 1c). Accordingly, the optimal condition for the chemotaxis assay in subsequent experiments was 100 ng/mL of SDF-1α for a 3h 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 μM 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-1α efficiently induced migration of E6-1; however, none of antagonists induced migration even at 10 μM (Fig. 2b). This indicated that the CXCR4 antagonists did not possess agonistic properties.

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-1α-mediated chemotaxis up to approximately 40% at a maximal concentration (10 μM). 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 μM (Fig. 3). In order to quantitatively compare these efficacies, 50% effective concentration (EC_{50}) 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 EC_{50}, 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-1α-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-1α-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-1α at a concentration of 10 nM, while KRH-3148 and KRH-1636 inhibited at 100 nM and at greater than 10 μM, 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-1α 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-1α 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 inihibitor 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-1α-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-1α, the best example of which would be an anti-HIV-1 drug.

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