

# KRH-2731: An Orally Bioavailable CXCR4 Antagonist Is a Potent Inhibitor of HIV-1 Infection

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## Background

Chemokine receptors, CXCR4 and CCR5, which are used as coreceptors by HIV-1 are considered attractive targets for possible intervention of HIV-1 infection. We previously reported KRH-1636 as a duodenally absorbable CXCR4 antagonist and X4 HIV-1 inhibitor (Ichiyama et al., PNAS, 100, 4185-4190, 2003). Our continuous effort to find more effective CXCR4 antagonists have recently allowed us to identify **KRH-2731·5HCl**, an orally bioavailable X4 HIV-1 inhibitor.

## Methods

### Anti-HIV-1 Assays.

Human PBMCs, which were activated with immobilized anti-CD3 MAb, were infected to various HIV-1 strains including primary clinical isolates at a multiplicity of infection of 0.001. After 3 h of adsorption, the cells were washed, and cultured in RPMI 1640 medium supplemented with 10% FBS and rIL-2 (50 U/ml) in the presence or absence of the test compounds. Amounts of HIV-1 capsid (p24) antigen in the culture supernatants were measured by ELISA 5 to 7 days after infection. The cytotoxicities of the compounds were based on the viability and proliferation of the activated PBMCs, as determined with a Cell Proliferation Kit II (XTT) (Roche)(J. Immunol. Methods, 142, 257-265, 1991).

### Ligand-Binding Assays.

CHO, or chemokine receptor-expressing CHO cells were incubated with on ice in the presence of [<sup>125</sup>I] labeled chemokines and various concentrations of test compounds. After washing unbound ligand, the cell-associated radioactivities were counted using a scintillation counter.

### Chemokine Receptor-Mediated Ca<sup>2+</sup> Signalling.

CXCR4-expressing HOS cells were loaded with Fura 2AM (Sigma) and incubated in the absence or presence of various concentrations of test compounds. Changes in the intracellular Ca<sup>2+</sup> level over time in response to SDF-1 $\alpha$  (1  $\mu$ g/ml) were determined by a fluorescence spectrophotometer.

### Inhibition of MAb Binding to CXCR4 by the Receptor Antagonists.

The activated PBMCs or Molt-4 cells were treated with 1  $\mu$ M KRH-2731·5HCl or AMD3100. The cells were washed and stained with anti-CXCR4 MAb and the fluorescent dye-conjugated secondary Ab, successively. The mean fluorescence of stained cells was measured by a FACSCalibur flow cytometer.

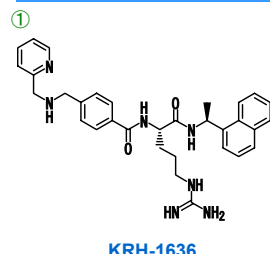
### Anti-HIV-1 Assays Using in a Hu-PBL-SCID Mice Model.

Female C.B-17 SCID mice (six to eight week old) were purchased from CLEA Japan (Tokyo). The SCID mice were depleted of NK cells by i.p. injection with TM $\beta$ -1 rat anti-mouse IL-2R $\beta$  MAb (1 mg/animal) and orally administered with KRH-2731 (10 mg/kg) two days before virus infection. After 1 day, 1x10<sup>7</sup> human PBMCs were introduced i.p. together with human rIL-4 (0.5  $\mu$ g/animal; Pepro Tech. Rocky Hill, NJ). On the next day, HIV-1<sub>NL4-3</sub> (1000 TCID<sub>50</sub> per 0.1-0.2 ml) was infected i.p. Two, 4, and 6 days after infection, additional rIL-4 (0.5  $\mu$ g/animal) was injected. KRH-2731 (10 mg/kg) had been orally administered during the course of infection. On day 7 after infection, plasma and peritoneal lavage fluid (PL) were collected and assayed for p24 antigen. A part of cells in PL were cultured for 3 days and examined for HIV-1 replication, whereas the other part of the cells were subjected to a quantitative PCR assay to determine viral DNA.

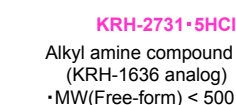
### Pharmacokinetic Studies.

After acclimation for at least 1 week, two male Wistar rats (6 weeks old, CLEA Japan) were fasted for 18 h before KRH-2731·5HCl (dissolved in 50 mM citric acid) administration. After single oral administration to the rats at a dose of 10 mg/kg, the plasma samples were prepared from blood withdrawn via the jugular vein of the rats at 0.5, 1, 2, 4, and 6 h with heparinized syringes. The obtained plasma was mixed with 0.1% formic acid/methanol. After centrifugation, the supernatant was evaporated to dryness under the stream of nitrogen, and dissolved in 25% acetonitrile/0.1% formic acid and analyzed by the liquid chromatography mass spectroscopy. For intravenous administration, two rats were dosed with KRH-2731·5HCl (dissolved in saline containing 25% DMSO) at 2.5 mg/kg. Blood samples were withdrawn at 0.08, 0.25, 0.5, 1, 2, and 4 h and the plasma samples were prepared and analyzed as described above.

## Results



- A duodenally absorbable CXCR4 antagonist
- Anti-HIV-1(NL4-3) activity EC<sub>50</sub>=42 nM (Ichiyama et al. PNAS:100 4185,2003)



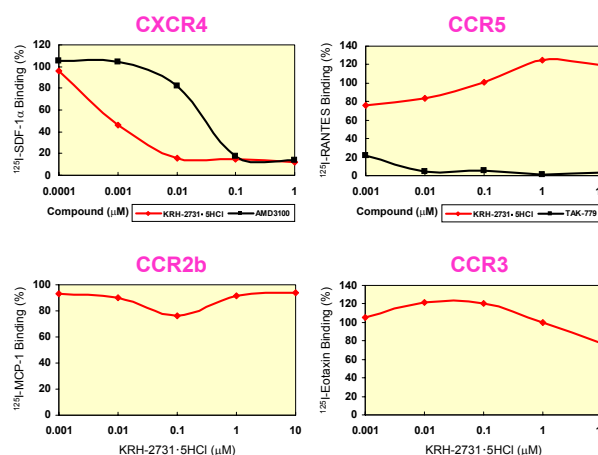
- An orally bioavailable CXCR4 antagonist
- Anti-HIV-1(NL4-3) activity EC<sub>50</sub>=1.0 nM

### Anti-HIV Activity of KRH-2731·5HCl in Activated PBMCs

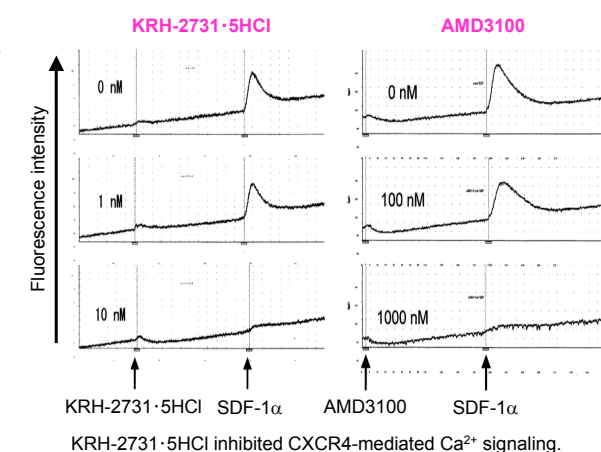
Virus	Tropism	EC <sub>50</sub> (nM)			
		KRH-2731·5HCl	AMD3100	TAK-779	AZT
NL4-3	X4	1.0	29	>1000	3.1
92HT599	X4	4.0	189	>1000	12
A018H (preAZT)	X4	1.4	38	>1000	1.9
A018G (postAZT)	X4	1.3	32	>1000	87000
89.6	R5X4	1.3	77	>1000	3.5
92HT593	R5X4	4.2	122	>1000	5.6
JR-CSF	R5	>1000	>1000	21	1.6
91US005	R5	>1000	>1000	11	5.2

KRH-2731·5HCl efficiently inhibited X4 and R5X4 HIV-1 infection in PBMCs. CC<sub>50</sub> of KRH-2731·5HCl in PBMCs was 57  $\mu$ M.

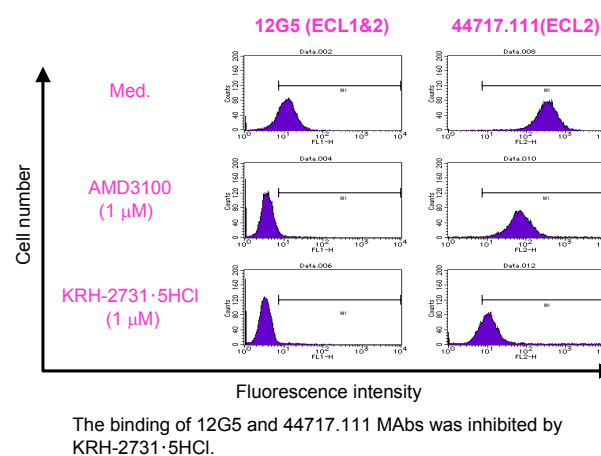
### Inhibitory effects of KRH-2731·5HCl on chemokine binding to CXCR4-, CCR5-, CCR2b, CCR3-expressing CHO cells



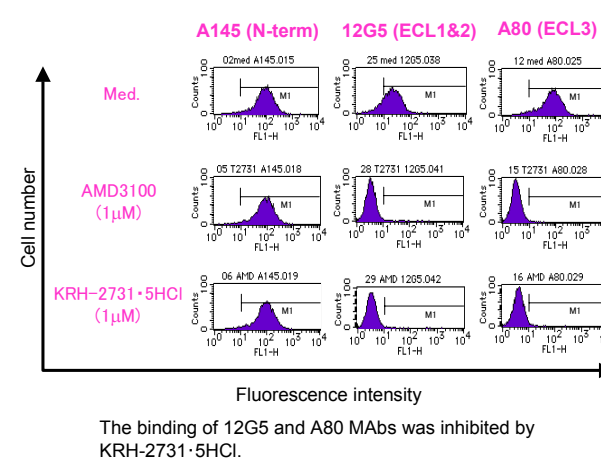
### Inhibitory Effect of KRH-2731·5HCl on SDF-1 $\alpha$ -Induced Ca<sup>2+</sup> Mobilization in CXCR4-Expressing HOS Cells



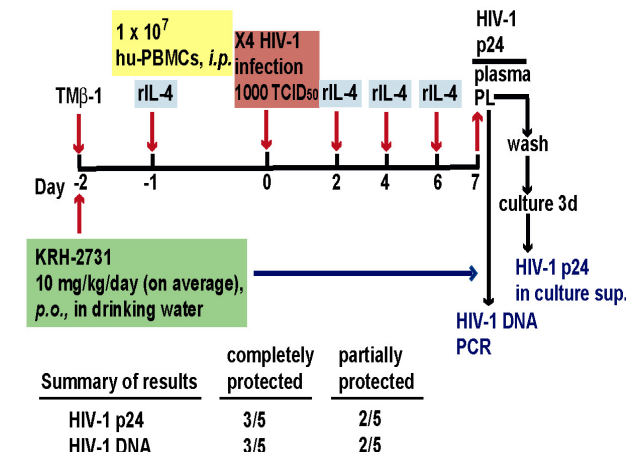
### Effect of KRH-2731·5HCl on Binding of MABs to CXCR4 in Molt-4 Cells



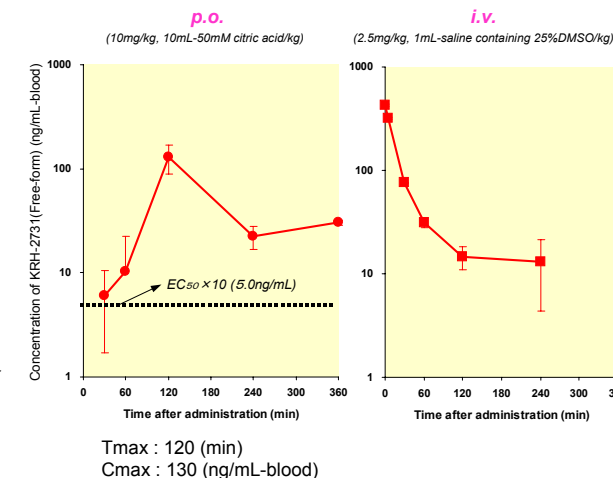
### Effect of KRH-2731·5HCl on Binding of MABs to CXCR4 in Activated PBMCs



### Anti-HIV-1 Activity of KRH-2731 in Hu-PBL-SCID Mice



### Plasma Concentration Profile after Single Dose in Rats



### Pharmacokinetic Profile of KRH-2731·5HCl

- Oral bioavailability : 37% (Rat)
- Dose-proportional increase in AUC up to 100 mg/kg, single dose in rats.
- Concentration in blood was kept at least 6 h over 10-fold of EC<sub>50</sub> (*in vitro*) after single dosing.
- IC<sub>50</sub> on CYP3A4 activity was more than 50  $\mu$ M.
- Stable in hepatic microsomes.
- 102.8% (Human), 99.7% (Rat)

## Summary

1. KRH-2731·5HCl efficiently inhibits replication of X4 and R5X4 HIV-1 in activated PBMCs (EC<sub>50</sub>=1.0-4.2 nM).
2. KRH-2731·5HCl is a CXCR4 antagonist.
3. The binding sites of KRH-2731·5HCl are located in ECL2 and ECL3 of CXCR4.
4. KRH-2731 suppresses X4 HIV-1 replication in hu-PBL-SCID mice models when administered orally.
5. Bioavailability of KRH-2731·5HCl is 37% when administered orally to rats at a dose of 10 mg/kg.

## Conclusions

KRH-2731·5HCl is an orally bioavailable small CXCR4 antagonist with a potent anti-X4 HIV-1 activity, both *in vitro* and *in vivo*, suggesting that it has a therapeutic potential in clinical use.

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