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Successful Efavirenz Dose Reduction in HIV Type 1–Infected Individuals with Cytochrome P450 2B6 *6 and *26

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Background. Efavirenz (EFV) is metabolized primarily by cytochrome P450 2B6 (CYP2B6), and high plasma concentrations of the drug are associated with a G→T polymorphism at position 516 (516G→T) of *CYP2B6* and frequent central nervous system (CNS)–related side effects. Here, we tested the feasibility of genotype-based dose reduction of EFV.

Methods. *CYP2B6* genotypes were determined in 456 human immunodeficiency virus type 1 (HIV-1)–infected patients who were receiving EFV treatment or were scheduled to receive EFV-containing treatment. EFV dose was reduced in *CYP2B6* 516G→T carriers who had high plasma EFV concentrations while receiving the standard dosage (600 mg). EFV-naive homozygous *CYP2B6* 516G→T carriers were treated with low-dose EFV. In both groups, the dose was further reduced when plasma EFV concentration remained high.

Results. *CYP2B6* 516G→T was identified in the **6* allele (found in 17.9% of our subjects) and a novel allele, **26* (found in 1.3% of our patients). All EFV-treated *CYP2B6* **6/*6* and **6/*26* carriers had extremely high plasma EFV concentrations (>6000 ng/mL) while receiving the standard dosage. EFV dose was reduced to 400 mg for 11 patients and to 200 mg for 7 patients with persistently suppressed HIV-1 loads. EFV-containing treatment was initiated at 400 mg in 4 *CYP2B6* **6/*6* carriers and one **6/*26* carrier. Two of them still had a high plasma EFV concentration while receiving that dose, and the dose was further reduced to 200 mg, with successful HIV-1 suppression. CNS-related symptoms improved with dose reduction in 10 of the 14 patients, although some had not been aware of the symptoms at initial dosage.

Conclusions. Genotype-based EFV dose reduction is feasible in *CYP2B6* **6/*6* and **6/*26* carriers, which can reduce EFV-associated CNS symptoms.

Efavirenz (EFV) is an important anti–HIV-1 agent in current combination treatment and is usually prescribed at a fixed dosage of 600 mg once daily [1, 2]. The plasma concentration of EFV varies widely in individuals, and the prevalence of CNS symptoms is higher in those with high concentrations [3]. EFV is metabolized mainly by cytochrome P450 2B6 (CYP2B6), and its concentration was reported to be associated with the *CYP2B6* 516G→T genetic polymorphism [4–8]. Previously, we reported that all Japanese patients with the 516TT genotype had extremely high EFV concentrations (>6000 ng/mL), without exception [4]. However, other studies reported some exceptional cases of subjects with the 516TT genotype with normal concentrations, although most of the

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516TT carriers had high concentrations [5–8]. The difference between our data and those of others may reflect polymorphisms other than 516G→T in *CYP2B6*. If this is the case, analysis of other polymorphisms and determination of the *CYP2B6* haplotype may be helpful in predicting EFV plasma levels. In the present study, we determined the *CYP2B6* haplotype of 456 HIV-1–infected patients and analyzed its relationship with EFV concentration in 111 of them. Furthermore, we reduced the EFV dose in 12 patients whose EFV concentrations had been high while receiving the standard dosage. We also used reduced doses of EFV in 5 EFV-naive patients in whom EFV concentration was predicted to become extremely high while receiving the standard dosage, on the basis of *CYP2B6* haplotype determination.

SUBJECTS, MATERIALS, AND METHODS

Patients. This analysis included 60 previously reported HIV-1–infected individuals at the International Medical Center of Japan (IMCJ) [4] and another group of 396 HIV-1–infected patients who were receiving treatment of the standard dosage (600 mg once daily) of EFV or were scheduled to begin receiving EFV-containing treatment at the following 11 hospitals in Japan: Hokkaido University (Sapporo), Sendai Medical Center (Sendai), Niigata University (Niigata), Higashi Saitama Hospital (Hasuda), IMCJ (Tokyo), Ishikawa Prefecture Central Hospital (Kanazawa), Nagoya Medical Center (Nagoya), Osaka National Hospital (Osaka), Hiroshima University (Hiroshima), Kyushu Medical Center (Fukuoka), and Kumamoto University (Kumamoto). The ethics committee of each hospital approved this study, and each participant gave written informed consent.

CYP2B6 *genotype.* DNA samples were extracted from peripheral blood specimens obtained from participants, and genotyping of *CYP2B6* 64C→T (*rs8192709*), 415A→G (*rs12721655*), 499C→G (*rs3826711*), 516G→T (*rs3745274*), 777C→A (*rs* number not available), 785A→G (*rs2279343*), 1375A→G (*rs* number not available), and 1459C→T (*rs3211371*) was performed by allele-specific fluorogenic 5′ nuclease chain reaction assay with predesigned primers and TaqMan MGB probes (TaqMan SNP Genotyping Assay; Applied Biosystems) or previously published primers and MGB probes [4]. In subjects confirmed to carry 499C→G, all 9 exons of the *CYP2B6* gene were amplified with previously published primers [9], and their DNA sequences were directly determined. For haplotype analysis of the *CYP2B6* allele, PCR amplification of the genomic region (3130 bp) containing exons 4 and 5 was performed using sense primer 5′-AACTGTACTCACTCCCAGAGT-3′ and antisense primer 5′-CTCCCTCTGTCTTTCATTCTGT-3′. The amplified PCR product was subjected to subcloning, and the DNA sequence of each clone was determined. For genotyping of *CYP2B6* 983T→C (*rs28399499*), new primers and probes were designed as follows: forward primer, 5′-GCCTGAAATGCCTCTTTAAAATGAGATTC-3′; reverse primer, 5′-GCGATGTGGGCCAATCAC-3′; VIC probe for 983T, 5′-CTGTTCAATCTCCC-3′; and FAM probe for 983C, 5′-CTGTTCAGTCTCCC-3′. The obtained genotyping results of *CYP2B6* 983T→C for >10 patients were confirmed by direct sequencing of exons 7 and 8 with use of primers published elsewhere [9].

Plasma EFV concentration. Samples of peripheral blood were collected during a daytime office visit (9–16 h after the patient took EFV) from the patients who had received EFV treatment at 600-mg dose at bedtime for >4 weeks. EFV concentration was measured by the reverse-phase high-performance liquid chromatography (HPLC) method [10]. For cases of EFV-dose reduction, plasma concentration was measured >2 weeks after the change in EFV dose. Differences in EFV concentrations between groups were examined for statistical significance with Student's t test. A P value <.05 denoted the presence of a statistically significant difference.

RESULTS

Novel* CYP2B6 *allele. The *CYP2B6* genotype was analyzed in 456 HIV-1–infected patients, including 442 Japanese, 8 other Asians, and 6 others. During the analysis, we noticed that some patients had the *CYP2B6* 499C→G polymorphism, substituting Ala for Pro at the 167th amino acid, which is already registered in the SNP Database, although the *CYP2B6* allele containing 499G had not been determined yet. TaqMan Genotyping Assay indicated that *CYP2B6* 449G was heterozygous with 499C in 12 individuals (2.6%), who were all Japanese (table 1). Direct sequencing of all the exons confirmed the results of TaqMan Genotyping Assay and showed that 8 subjects had 516GT, 785AG, and 1375AA genotypes; 3 had 516TT, 785GG, and 1375AA genotypes; and 1 had 516GT, 785AG, and 1375AG genotypes without any other mutation. Subcloning analysis of the PCR products confirmed that 499G always coexisted in the same allele with 516T and 785G (figure 1). Therefore, it was concluded that the novel haplotype containing 499C→G had 2 other single-nucleotide polymorphisms (SNPs): 516G→T and 785A→G. We formally registered this novel allele with the Human Cytochrome P450 Allele Nomenclature Committee, and it was designated "*CYP2B6 *26*" (http://www.cypalleles.ki.se/). With use of this nomenclature, the *CYP2B6* haplotype of the twelve 499C→G carriers were identified as eight **1/*26* heterozygotes, three **6/*26* heterozygotes, and one **23/*26* heterozygote (table 1). The allelic frequency of **26* was 1.3% in our study participants.

CYP2B6 *haplotype determination.* In 456 HIV-1–infected individuals, we determined the genotypes of 9 SNP positions (64C→T, 415A→G, 499C→G, 516G→T, 777C→A, 785A→G, 983T→C, 1375A→G, and 1459C→T) in *CYP2B6* (table 1). No *CYP2B6* genetic polymorphism was detected in 211 patients, and their haplotype was determined to be **1/*1*. The haplotypes

Table 1. *CYP2B6* haplotype and allele frequencies in study participants.

CYP2B6 status	*CYP2B6* genotype at nucleotide position								No. (%) of subjects	
	415	499	516	777	785	983	1375	1459	All[a]	Japanese
Haplotype:										
**1/*1*	AA	CC	GG	CC	AA	TT	AA	CC	211 (46.3)	205 (46.4)
**1/*2*	AA	CC	GG	CC	AA	TT	AA	CC	30 (6.6)	30 (6.8)
**1/*4*	AA	CC	GG	CC	AG	TT	AA	CC	43 (9.4)	42 (9.5)
**1/*5*	AA	CC	GG	CC	AA	TT	AA	CT	4 (0.9)	3 (0.7)
**1/*6*	AA	CC	GT	CC	AG	TT	AA	CC	104 (22.8)	101 (22.9)
**1/*23*	AA	CC	GG	CC	AA	TT	AG	CC	2 (0.4)	2 (0.5)
**1/*26*	AA	CG	GT	CC	AG	TT	AA	CC	8 (1.8)	8 (1.8)
**2/*4*	AA	CC	GG	CC	AG	TT	AA	CC	6 (1.3)	5 (1.1)
**2/*5*	AA	CC	GG	CC	AA	TT	AA	CT	1 (0.2)	1 (0.2)
**2/*6*	AA	CC	GT	CC	AG	TT	AA	CC	5 (1.1)	5 (1.1)
**4/*4*	AA	CC	GG	CC	GG	TT	AA	CC	5 (1.1)	5 (1.1)
**4/*6*	AA	CC	GT	CC	GG	TT	AA	CC	12 (2.6)	12 (2.7)
**5/*5*	AA	CC	GG	CC	AA	TT	AA	TT	1 (0.2)	1 (0.2)
**5/*6*	AA	CC	GT	CC	AG	TT	AA	CT	1 (0.2)	1 (0.2)
**6/*6*	AA	CC	TT	CC	GG	TT	AA	CC	19 (4.2)	17 (3.8)
**6/*26*	AA	CG	TT	CC	GG	TT	AA	CC	3 (0.7)	3 (0.7)
**23/*26*	AA	CG	GT	CC	AG	TT	AG	CC	1 (0.2)	1 (0.2)
Total									456	442
Allele:										
**1*	A	C	G	C	A	T	A	C	613 (67.2)	596 (67.4)
**2*	A	C	G	C	A	T	A	C	42 (4.6)	41 (4.6)
**4*	A	C	G	C	G	T	A	C	71 (7.8)	69 (7.8)
**5*	A	C	G	C	A	T	A	T	8 (0.9)	7 (0.8)
**6*	A	C	T	C	G	T	A	C	163 (17.9)	156 (17.6)
**23*	A	C	G	C	A	T	G	C	3 (0.3)	3 (0.3)
**26*	A	G	T	C	G	T	A	C	12 (1.3)	12 (1.4)
Total									912	884

[a] Including 442 Japanese, 8 other Asians (5 Thai, 2 Koreans, and 1 Filipino), 4 Hispanics, and 2 non-Hispanic whites.

of single-SNP carriers with 64CT, 785AG, 1375AG, and 1459CT were determined to be **1/*2*, **1/*4*, **1/*23*, and **1/*5*, respectively. Those of homozygous polymorphism carriers with 785GG only, 1459TT only, and both 516TT and 785GG were determined to be **4/*4*, **5/*5*, and **6/*6*, respectively. When the fact that **2* is the only allele harboring 64C→T is considered, patients with 64CT and 785AG; 64CT and 1459CT; and 64CT, 516GT, and 785AG were identified as **2/*4*, **2/*5*, and **2/*6* heterozygotes, respectively. Patients with both 516GT and 785GG genotypes but without other polymorphisms were determined to have **4/*6* heterozygotes. There were 104 patients (22.8%), including 101 Japanese, who held both 516GT and 785AG genotypes without other polymorphisms. There were 2 possible haplotypes, **1/*6* and **4/*9*, in this genotypic pattern. When the fact that **9* had not been reported in Japanese subjects was considered [11], we found that all 101 Japanese were **1/*6* heterozygotes. Haplotype analysis by subcloning of PCR products described above was performed in the 3 others, and their haplotype was determined as **1/*6*. One Japanese patient had 516GT, 785AG, and 1459CT genotypes without other polymorphisms, and there were 2 possible haplotypes, **1/*7* and **5/*6*, in this genotypic pattern. Because **7* had not been reported in Japanese subjects [11], the haplotype in this patient was determined to be **5/*6*. Overall, the allelic frequency of **6* was 17.9% in our study participants. The 415A→G, 777C→A, and 983T→C polymorphisms, which are the determinants of **8*, **3*, and **18*, respectively, were not observed in our subjects.

CYP2B6 *and EFV concentration.* We determined the *CYP2B6* haplotype in 251 patients at IMCJ and in 205 patients at the other 10 hospitals. Of the 251 genotype-analyzed patients at IMCJ, 101 were being treated or were beginning treatment with a standard dose of EFV during this study period (figure 2). Plasma EFV concentrations were measured in all 101 patients, including sixty-seven 516GG holders, twenty-eight 516GT holders, and six 516TT holders. To clarify the effect of the 516TT genotype, EFV concentration was also measured in ten 516TT holders undergoing treatment with the standard dose of EFV at other hospitals. The mean concentration (±SD)

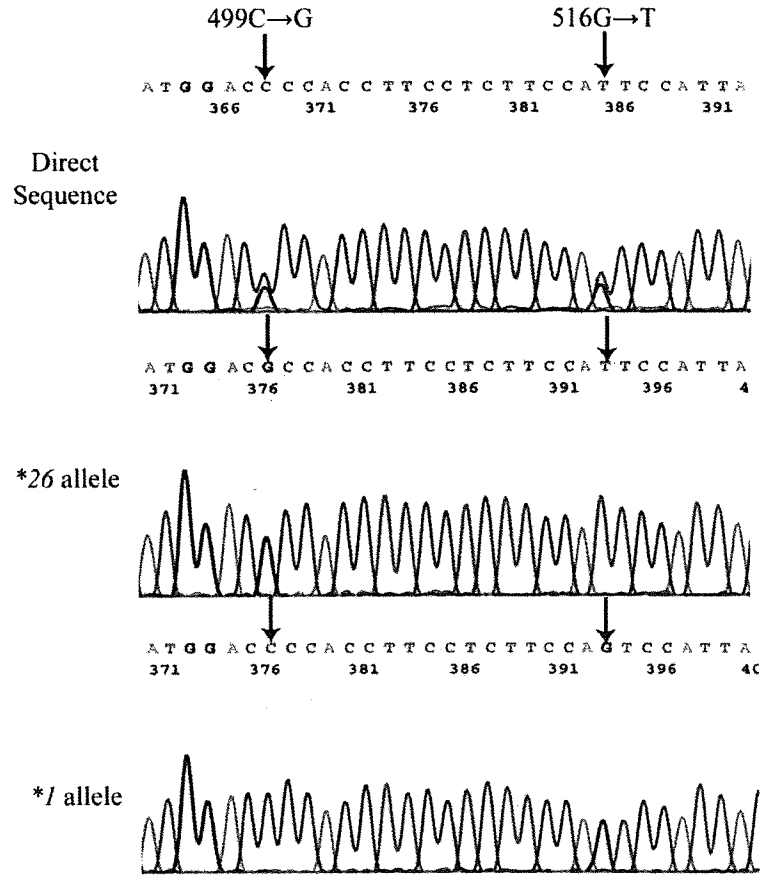

Figure 1. Direct *(top panel)* and subclonal *(middle and bottom panels)* sequences of *CYP2B6* in 499C→G carriers. The genotypes 499G, 516T, and 785G (not shown) exist in the same allele, newly designated as "*CYP2B6 *26*." The same results were obtained in all 9 patients with the 499CG, 516GT, and 785AG genotypes, and the patients were identified as eight **1/*26* carriers and one **23/*26* carrier. Although shown are the sense-strand sequences only, both strands were sequenced. Arrows indicate the variant nucleotide positions 499 and 516.

of EFV in all patients was 3740 ± 2800 ng/mL. When divided by the genotype of position 516, striking discreteness was observed (figure 3). All (95% CI 91.1%–100%) of the 16 carriers of 516TT genotype, including fourteen **6/*6* carriers and two **6/*26* carriers, had extremely high EFV concentrations (>6000 ng/mL). Their mean concentrations (9500 ± 2580 ng/mL) were many orders of magnitude higher than those of the other genotype carriers ($P < 10^{-4}$). There was no significant difference in EFV concentration between **6/*6* carriers and **6/*26* carriers. On the other hand, there were only 2 patients who had such high EFV concentrations among the other genotype carriers. One was a **1/*6* carrier (7140 ng/mL), and the other was a **1/*26* carrier (9710 ng/mL). Direct sequencing of all *CYP2B6* exons showed no polymorphism other than 499C→G, 516G→T, and 785A→G in these individuals. The mean concentrations of EFV of the twenty-eight 516GT carriers, including twenty-five **6*-heterozygotes (3320 ± 1240 ng/mL; $P < 10^{-4}$) and three **26*-heterozygotes (5470 ± 3840 ng/mL; $P < 10^{-4}$), were significantly higher than those of the sixty-seven 516GG genotype carriers (2450 ± 770 ng/mL). None (95% CI 0%–0.1%) of the 516GG carriers had a high EFV concentration (>6000 ng/mL). Considered together, it was concluded that high plasma EFV concentrations were associated with *CYP2B6 *6* and **26* and that *CYP2B6 *6/*6* and **6/*26* carriers had extremely high plasma EFV concentrations at standard dosage, without exception.

EFV dose reduction from 600 mg. To determine whether the EFV dose can be reduced in patients who have a high concentration while receiving the standard dose, a dose-reduction protocol was applied in 12 patients with high plasma concentrations (>6000 ng/mL [range, 6170–14,690 ng/mL]), including one **1/*26* heterozygote, nine **6/*6* homozygotes, and two **6/*26* heterozygotes. Before the dose reduction, plasma HIV-1 load was undetectable (<50 copies/mL) in all patients for >1 month with treatment of a standard antiretroviral regimen containing 600 mg of EFV. In these 12 patients,

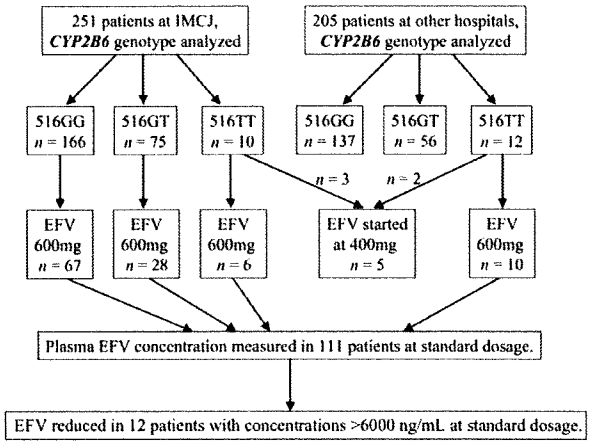

Figure 2. Flow diagram of study participants. The *CYP2B6* genotype was analyzed in 251 patients at the International Medical Center of Japan (IMCJ) and in 205 patients at other hospitals. Standard dosage of EFV was administered in 101 patients at IMCJ, including sixty-seven *CYP2B6* 516GG, twenty-eight 516GT, and six 516TT holders, whose EFV concentrations were measured. Ten 516TT holders at the other hospitals were administered standard dosages of EFV, and their EFV concentrations were also measured. A reduced-dose (400 mg) regimen of EFV was initiated in 5 other 516TT holders.

the EFV dose was reduced from 600 to 400 mg in 11 subjects and was further reduced to 200 mg in 7 of them who consented to further reduction. The plasma EFV concentrations decreased by approximately one-third (36%–46%), to 3720–6160 ng/mL, with dose reduction from 600 to 400 mg in 10 of 11 subjects, and further decreased by approximately one-half (51%–59%), to 1620–2960 ng/mL, with reduction from 400 to 200 mg in 6 of 7 subjects (figure 4). In one patient who had a markedly high EFV concentration (14,690 ng/mL) at the standard 600-mg dose, however, the concentration decreased unexpectedly by 69%, to 4500 ng/mL, with the reduction to 400 mg and further decreased by 82%, to 790 ng/mL, lower than the recommended range (>1000 ng/mL) [1], with the reduction from 400 to 200 mg. Therefore, the dose was increased in this patient back to 400 mg. In another patient who had reported severe dizziness during treatment with the standard dose (600 mg), the dose was reduced immediately to 200 mg at the patient's request. The plasma EFV concentration was also markedly high in this patient (14,360 ng/mL) during treatment with the standard dosage. However, it decreased by 83%, to 2410 ng/mL, with the dose reduction to 200 mg. Consequently, the final EFV dose was 400 mg in 5 subjects and 200 mg in 7 subjects. The determined dosage for each patient was continued for >6 months (the longest was 26 months for a patient who received the 200-mg dose), and the plasma HIV-1 load was continuously undetectable in all patients.

EFV initiation at 400-mg dose. Our analysis showed that *CYP2B6* **6/*6* and **6/*26* carriers had extremely high EFV concentrations, without exception (figure 3), and that dose reduction was possible in patients with high EFV concentration with retention of therapeutically effective anti–HIV-1 activity (figure 4). In the next phase of our study, we used an antiretroviral regimen containing a reduced dose (400 mg) of EFV in 5 EFV-naive patients (four **6/*6* homozygotes and one **6/*26* heterozygote). Before the introduction of low-dose EFV-containing regimen, the plasma HIV-1 loads had been undetectable during receipt of the previous protease inhibitor–containing regimen in all 5 patients. Their EFV concentrations were 4080–9450 ng/mL, and all such concentrations (95% CI, 99.5%–100%) were therapeutically adequate (>1000 ng/mL) at the 400-mg dose (figure 5). One **6/*6* homozygote developed severe dizziness, necessitating discontinuation of EFV-treatment at day 16. His EFV concentration was 5430 ng/mL. In one **6/*26* heterozygote, severe thrombocytopenia emerged, probably because of overdosage of rifabutin prescribed for the treatment of coinfection with *Mycobacterium intracellulare,* and EFV treatment was stopped at day 15. The EFV concentration was 5770 ng/mL. Two of the remaining 3 patients still had extremely high EFV concentrations (6760 and 9450 ng/mL) at the 400-mg dose, and their dose was subsequently reduced to 200 mg. The plasma EFV concentrations decreased to 2690 and 3660 ng/mL (i.e., by 60% and 61%, respectively). Consequently, 2 subjects

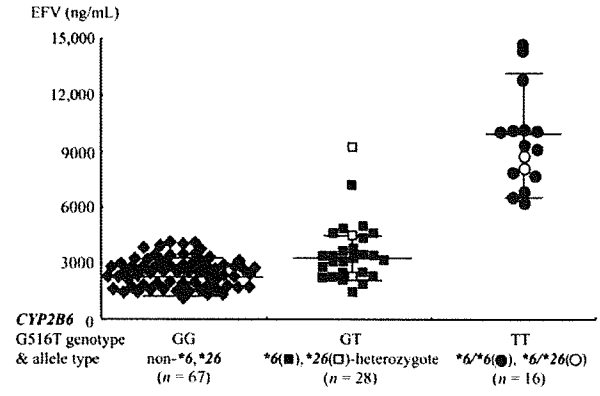

Figure 3. Plasma efavirenz (EFV) concentrations measured during EFV treatment with standard dose (600 mg). A total of 111 HIV-1–infected patients treated with EFV-containing regimens were divided into 3 groups on the basis of nucleotide genotype at *CYP2B6* position 516 (GG, GT, or TT), and their plasma EFV concentrations were compared. Blackened squares, **6* heterozygote with allele other than **26*; unblackened squares, *CYP2B6* 499C→G carriers (**26* heterozygote with allele other than **6*); blackened circles, **6* homozygote (**6/*6*); unblackened circles, *CYP2B6* 499C→G carriers (**6/*26* heterozygotes); blackened diamonds, other genotype carriers. Horizontal lines represent the mean (± SD) plasma EFV concentrations for each group.

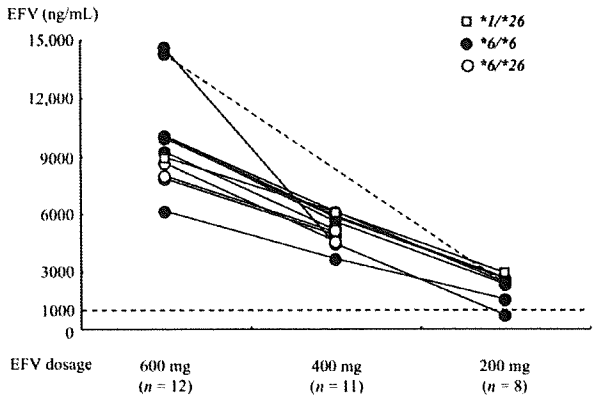

Figure 4. Dose reduction of efavirenz (EFV) in 12 patients whose concentrations were extremely high while receiving treatment with standard dose (600 mg). EFV dose was reduced from 600 to 400 mg in 11 patients and was further reduced, to 200 mg, in 7 patients. In one patient who had severe CNS symptoms while receiving treatment with standard dose, EFV dose was directly reduced to 200 mg (concentrations connected with a dotted line). The suggested minimum target concentration (1000 ng/mL) is indicated by the thin line.

discontinued the EFV-containing regimen, and 3 subjects continued low-dose EFV-containing regimen (400 mg for 1 patient and 200 mg for 2 patients). The low-dose regimen was continued for >6 months, and the plasma HIV-1 load was persistently undetectable in all 3 patients.

Improvement of CNS symptoms. As described above, the EFV dose was reduced from 600 to 400 and 200 mg as the final dose in 5 and 7 subjects, respectively (figure 4), and it was decreased from 400 mg as the initial dose to 200 mg for 2 other subjects (figure 5). To delineate the changes in CNS symptoms associated with the decrease in EFV concentration, a questionnaire survey of these 14 patients was conducted regarding 6 items: dizziness, strange dreams, depression, irritability, concentration problems, and sleep difficulty. More than 1 month after the dose had been reduced to the lowest dose, the patients were asked to judge the 6 CNS symptoms above at initial and final doses, with use of a 5-grade system ("none," "slight," "sometimes," "often," and "always"). Ten (71%) of the 14 patients had some of the aforementioned CNS symptoms during treatment with the initial dose (table 2). The most common symptom was dizziness (57%), followed by strange dreams (50%). Interestingly, all the symptoms improved after dose reduction in the 10 patients. Furthermore, dizziness and concentration problems disappeared during treatment with the final dose in one-half of the patients, although strange dreams and sleep difficulty were still reported by all the patients who had those difficulties at the initial dose. Finally, when the patients were asked whether they wanted to reincrease EFV to the previous dose, all 10 patients with CNS symptoms at the initial dose answered "no" (9 answered "absolutely no").

DISCUSSION

In this study, we identified a novel *CYP2B6* allele, **26*, which includes 499C→G, 516G→T, and 785A→G in 12 Japanese patients, and we showed that, without exception, all **6/*6* and **6/*26* carriers, all holding 516TT, had extremely high plasma EFV concentrations while receiving the standard dose (600 mg) [4]. In other reports, however, there were some exceptional subjects with 516TT who had normal concentrations of EFV, and the discreteness of the EFV concentration with the position 516 genotype was not as clear as it was in our patients [5–8]. This difference may be because some of the 516TT carriers had other *CYP2B6* alleles, such as **7* (containing 516G→T, 785A→G, and 1459C→T), **9* (containing 516G→T only), and **13* (containing 415A→G, 516G→T, and 785A→G). Those alleles could not be found in our subjects, and their effects on EFV concentration were not well described. Because numerous additional *CYP2B6* variants with impact on expression and/or function were recently reported [12–18], correct determination of *CYP2B6* haplotype seems indispensable for prediction of EFV plasma levels.

We reduced the EFV dose in 12 patients whose plasma EFV concentrations were extremely high while receiving the standard dose, and we initiated EFV treatment at a 400-mg dose in 5 EFV-naive **6/*6* and **6/*26* carriers. In most patients, the plasma EFV concentration decreased proportionally with the dose-reduction ratio. In 2 subjects, however, the concentrations decreased much more than expected, given the dose reduction

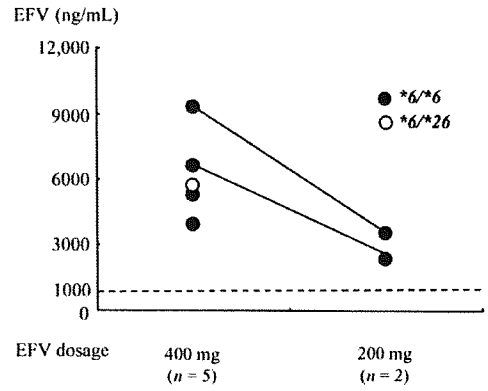

Figure 5. Introduction of low-dose efavirenz (EFV)-containing antiretroviral regimen to *CYP2B6 *6/*6* and **6/*26* carriers. Treatment was started in 4 EFV-naive carriers *CYP2B6 *6/*6* and one **6/*26* carrier, with 400-mg EFV-containing regimens. EFV dose was further reduced, to 200 mg, in 2 patients whose EFV concentrations were >6000 ng/mL while receiving treatment with the 400-mg dose.

Table 2. Changes in CNS-related symptoms after reduction of efavirenz dosage.

Symptom	No. (%) of subjects who reported symptom status during efavirenz treatment		
	Present[a] ($n=14$)	Improved[b]	Disappeared[b]
Dizziness	8 (57)	8 (100)	4 (50)
Strange dreams	7 (50)[c]	7 (100)[c]	0 (0)
Depression	5 (36)	5 (100)	1 (20)
Irritability	5 (36)	5 (100)	1 (20)
Concentration problem	4 (29)	4 (100)	2 (50)
Sleep difficulty	3 (21)	3 (100)	0 (0)
Any of the above	10 (71)[c]	10 (100)[c]	4 (40)

[a] Including the 4 grades "slight," "sometimes," "often," and "always" at the initial dosage. Includes 2 patients whose efavirenz treatment was originally 400 mg and was reduced to 200 mg.

[b] Percentage of those who initially reported "present."

[c] Including 1 patient whose efavirenz dose was originally 400 mg and was reduced to 200 mg.

ratio. Both of these patients had markedly high concentrations at standard dosage. Hasse et al. [19] reported a patient with excessively high plasma EFV concentration at standard dose, which decreased to one-thirtieth following dose reduction from 600 to 200 mg. Long-term exposure to such excessively high concentrations may induce CYP2B6 enzymatic expression in the liver, which could result in an unexpectedly large decrease in plasma EFV concentration by dose reduction if deinduction of the enzyme takes several weeks. At the 400-mg dose, the plasma concentrations of EFV were therapeutically adequate in all the treated **6/*6* and **6/*26* carriers in this study. Regarding the reduced dose, it is noteworthy that a phase II study during EFV development supported the use of a lower dose [20]. The same study indicated that the 600-mg dose of EFV is associated with a high rate of adverse events that could lead to discontinuation, which suggests that the lower dose of 400 mg may be almost as effective without the high discontinuation rate. In the present study, associated with the dose-reduction regimen, a significant number of patients experienced improvement of CNS symptoms, which was unexpected on the basis of previous reports [5, 21, 22]. Interestingly, some of these patients did not appreciate their clinical state and considered themselves to have no CNS-related symptoms during the standard-dose treatment. However, after the dose reduction, they reassessed the status and evaluated symptoms during the treatment with the standard dose as associated with CNS symptoms and indicated that the reduced dose of EFV relieved them of such symptoms. Because EFV-treated patients often stick to the regimen, previous reports of symptom questionnaires conducted during the standard treatment might have underestimated the EFV-associated CNS symptoms [5, 21, 22]. However, this finding might be confounded by placebo effect, because the patients were told that their EFV levels were high while receiving the initial dose and decreased throughout the dose-reduction protocol. Because of this possible placebo effect, a double-blind, placebo-controlled study would best address this question.

EFV dose reduction and initiation of EFV treatment at reduced dose is possible with therapeutic anti–HIV-1 potency retained in *CYP2B6 *6/*6* homozygotes and **6/*26* heterozygotes, which could relieve the patients of the EFV-associated CNS symptoms. It may also decrease the risk of development of EFV-resistant HIV-1 after mandatory treatment discontinuation, such as abdominal surgery [23], and reduce the treatment cost, an important issue in developing countries [24]. After dose reduction, however, careful monitoring is necessary until larger studies confirm the safety of reduced dose in such specific genotype carriers.

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Potential conflicts of interest. All authors: no conflicts.

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The Novel CXCR4 Antagonist KRH-3955 Is an Orally Bioavailable and Extremely Potent Inhibitor of Human Immunodeficiency Virus Type 1 Infection: Comparative Studies with AMD3100[▽]

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The previously reported CXCR4 antagonist KRH-1636 was a potent and selective inhibitor of CXCR4-using (X4) human immunodeficiency virus type 1 (HIV-1) but could not be further developed as an anti-HIV-1 agent because of its poor oral bioavailability. Newly developed KRH-3955 is a KRH-1636 derivative that is bioavailable when administered orally with much more potent anti-HIV-1 activity than AMD3100 and KRH-1636. The compound very potently inhibits the replication of X4 HIV-1, including clinical isolates in activated peripheral blood mononuclear cells from different donors. It is also active against recombinant X4 HIV-1 containing resistance mutations in reverse transcriptase and protease and envelope with enfuvirtide resistance mutations. KRH-3955 inhibits both SDF-1α binding to CXCR4 and Ca^{2+} signaling through the receptor. KRH-3955 inhibits the binding of anti-CXCR4 monoclonal antibodies that recognize the first, second, or third extracellular loop of CXCR4. The compound shows an oral bioavailability of 25.6% in rats, and its oral administration blocks X4 HIV-1 replication in the human peripheral blood lymphocyte-severe combined immunodeficiency mouse system. Thus, KRH-3955 is a new promising agent for HIV-1 infection and AIDS.

The chemokine receptors CXCR4 and CCR5 serve as major coreceptors of human immunodeficiency virus type 1 (HIV-1), along with CD4 as a primary receptor for virus entry (2, 15, 18, 19). SDF-1α, which is a ligand for CXCR4, blocks the infection of CXCR4-utilizing X4 HIV-1 strains (7, 34). On the other hand, ligands for CCR5 such as RANTES inhibit CCR5-utilizing R5 HIV-1 (10). These findings made chemokines, chemokine derivatives, or small-molecule inhibitors of chemokine receptors attractive candidates as a new class of anti-HIV-1 agents. Many CCR5 antagonists have been developed as anti-HIV-1 drugs. These include TAK-779 (Takeda Pharmaceutical Company) (5), TAK-652 (6), TAK-220 (45), SCH-C (Schering-Plough) (43), SCH-D (vicriviroc) (42), GW873140 (aplaviroc; Ono Pharmaceutical/Glaxo Smith Kline) (28), and UK-427,857 (maraviroc; Pfizer Inc.) (17). Of these, maraviroc was approved by the U.S. FDA in 2007 for the treatment of R5 HIV-1 in treatment-experienced adult patients, combined with other antiretroviral treatment. Several classes of CXCR4 antagonists have also been reported. The bicyclam AMD3100 showed antivirus activity against many X4 and some R5X4 HIV strains in peripheral blood mononuclear cells (PBMCs) but not against R5 strains (16, 40). The pharmacokinetics and antiviral activity of this compound were also evaluated in humans (21, 22). T22, [Tyr-5,12, Lys-7]polyphemusin II, which is an 18-mer peptide derived from horseshoe crab blood cells, was reported to specifically inhibit X4 HIV-1 strains (30). Studies on the pharmacophore of T140 (a derivative of T22) led to the identification of cyclic pentapeptides (46).

In 2003, we reported that KRH-1636 is a potent and selective CXCR4 antagonist and inhibitor of X4 HIV-1 (23). Although the compound was absorbed efficiently from the rat duodenum, it has poor oral bioavailability. Continuous efforts to find more potent CXCR4 antagonists that are bioavailable when administered orally allowed us to develop KRH-3955 by a combination of chemical modification of the lead compound and biological assays. In this report, we describe the results of a preclinical evaluation of KRH-3955, including its in vitro anti-HIV-1 activity, its in vivo efficacy in the human peripheral blood lymphocyte (hu-PBL)-severe combined immunodeficiency (SCID) mouse model, and its pharmacokinetics in rats in comparison with those of AMD3100.

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MATERIALS AND METHODS

Compounds. The synthesis and purification of KRH-3955, *N,N*-dipropyl-*N'*-[4-({[(1*H*-imidazol-2-yl)methyl][(1-methyl-1*H*-imidazol-2-yl)methyl]amino}methyl)benzyl]-*N'*-methylbutane-1,4-diamine tri-(2*R*,3*R*)-tartrate, were carried out by Kureha Corporation. The chemical structure of KRH-3955 is shown in Fig. 1. The CXCR4 antagonist AMD3100 and zidovudine (AZT) were obtained from Sigma. Saquinavir was obtained

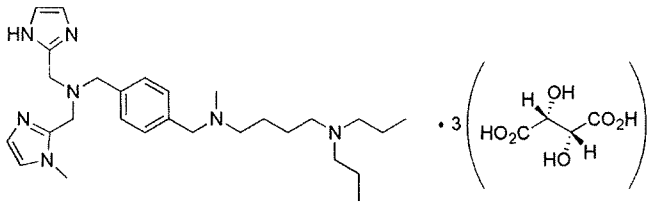

FIG. 1. Chemical structure of KRH-3955.

from the NIH AIDS Research and Reference Reagent Program, NIAID, Bethesda, MD. AMD070 and SCH-D were synthesized at Kureha Corporation.

Cells. Molt-4 no. 8 cells (24) were maintained in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO) and antibiotics (50 ng/ml penicillin, 50 ng/ml streptomycin, and 100 ng/ml neomycin; Invitrogen), which is referred to as RPMI medium. Chemokine receptor-expressing human embryonic kidney 293 (HEK293) cells (ATCC CRL-1573) and Chinese hamster ovary (CHO) cells (ATCC CCL-61) were maintained in minimal essential medium or F-12 (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics (50 ng/ml penicillin, 50 ng/ml streptomycin, and 100 ng/ml neomycin). PBMCs from HIV-1-seronegative healthy donors were isolated by Ficoll-Hypaque density gradient (Lymphosepal; IBL, Gunma, Japan) centrifugation (31) and grown in RPMI medium supplemented with recombinant human interleukin-2 (rhIL-2; Roche, Mannheim, Germany) at 50 U/ml.

Viruses. Viral stocks of HIV-1_{NL4-3}, HIV-1_{JR-CSF}, and HIV-$1_{89.6}$ were each produced in the 293T cell line by transfection with HIV-1 molecular clone plasmids pNL4-3 (1), pYK-JRCSF (25), and p89.6 (11), respectively, by the calcium phosphate method. The 50% tissue culture infective dose was determined by an end-point assay with PBMC cultures activated with immobilized anti-CD3 monoclonal antibody (MAb) (33, 51). Subtype B HIV-1 primary isolates 92HT593, 92HT599 (N. Hasley), and 91US005 (B. Hahn) and AZT-resistant HIV-1 (A018) (D. D. Richman) (26) were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. These clinical isolates were propagated in the activated PBMCs prepared as described above.

Anti-HIV-1 assays. Human PBMCs activated with immobilized anti-CD3 MAb (OKT-3; ATCC, Manassas, VA) in RPMI medium for 3 days were infected with 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 medium supplemented with rhIL-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) 7 to 10 days after infection. The cytotoxicities of the compounds were tested on the basis of the viability and proliferation of the activated PBMCs, as determined with Cell Proliferation Kit II (XTT) from Roche (36).

Susceptibility of multidrug-resistant HIV-1 to CXCR4 antagonists was also measured by using recombinant viruses in a single replication cycle assay (9, 49). HIV-1 resistance test vectors (RTVs) contain the entire protease (PR) coding region and the reverse transcriptase (RT) coding region, from amino acid 1 to amino acid 305, amplified from patient plasma and a luciferase expression cassette inserted in the *env* region. The RTVs in this study contain patient-derived PR and RT sequences that possess mutations associated with resistance to PR, RT, or both PR and RT. Env-pseudotyped viruses were produced by cotransfecting 293 cells with RTV plasmids and expression vectors encoding the Env protein of well-characterized X4-tropic laboratory strain HXB2, NL4-3, or NL4-3 containing the Q40H enfuvirtide (T20) resistance mutation introduced by site-direct mutagenesis. The virus stocks were harvested 2 days after transfection and used to infect U87 $CD4^+$ cells (kind gifted from N. Landau, NYU School of Medicine) expressing CXCR4 in 96-well plates, with serial dilutions of CXCR4 antagonists. Target cells were lysed, and luciferase activity was measured to assess virus replication in the presence and absence of inhibitors. Drug concentrations required to inhibit virus replication by 50% (IC_{50}) were calculated.

Immunofluorescence. Molt-4 cells or CXCR4-expressing HEK293 cells were treated with various concentrations of KRH-3955 or AMD3100 in RPMI medium or phosphate-buffered saline containing 1% bovine serum albumin and 0.05% NaN_3 (fluorescence-activated cell sorting [FACS] buffer). In washing experiments, cells were washed with RPMI medium or FACS buffer. The cells were Fc blocked with 2 mg/ml normal human immunoglobulin G (IgG) in FACS buffer and then stained directly with mouse MAbs 12G5-phycoerythrin (PE) and 44717-PE (R&D Systems, Inc., Minneapolis, MN) or rat MAb A145-fluorescein isothiocyanate (FITC) and indirectly with MAb A80. The A145 and A80 MAbs were produced in ascitic fluid of BALB/c nude mice, and IgG fractions were obtained from ascitic fluid by gel filtration chromatography with Superdex G200 (Amersham Pharmacia). Goat anti-rat IgG (heavy and light chains) labeled with FITC was purchased from American Corlex (47). After washing, the cells were analyzed on a FACScalibur (BD Biosciences, San Jose, CA) flow cytometer with CellQuest software (BD Biosciences).

DNA construction and transfection. Chemokine receptor-expressing CHO cells were generated as reported previously (23). Human CXCR4 cDNA was cloned into the pcDNA3.1 vector. Mutations were introduced by using the QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). All constructs were verified by DNA sequencing and transfected into 293 cells by using the Lipofectamine reagent (Invitrogen) (48). Stable transfectants were selected in the presence of 400 μg/ml G418 (Invitrogen). The COOH-terminal intracellular domain of CXCR4 (residues 308 to 352) was deleted in all mutants and the wild type. This deletion has no influence on HIV-1 infection or on SDF-1α binding and signaling but abolishes ligand-induced endocytosis (3).

Ligand-binding assays. Chemokine receptor-expressing CHO cells (5×10^6/0.2 ml per well) were cultured in a 24-well microtiter plate. After 24 h of incubation at 37°C, the culture medium was replaced with binding buffer (RPMI medium supplemented with 0.1% bovine serum albumin). Binding reactions were performed on ice in the presence of ^{125}I-labeled chemokines (final concentration of 100 pmol/liter; PeproTech Inc., Rocky Hill, NJ) and various concentrations of test compounds. After washing away of unbound ligand, cell-associated radioactivity was counted with a scintillation counter as described previously (23).

CXCR4-mediated Ca^{2+} signaling. Fura2-acetoxymethyl ester (Dojindo Laboratories, Kumamoto, Japan)-loaded CXCR4-expressing CHO cells were incubated in the absence or presence of various concentrations of KRH-3955 or AMD3100. Changes in intracellular Ca^{2+} levels in response to SDF-1α (1 μg/ml) were determined by using a fluorescence spectrophotometer as described previously (30).

Detection of KRH-3955 in blood after oral administration. The plasma concentration-time profile of R-176211 (distilled water was used as a vehicle), the free form of KRH-3955, was examined after a single oral administration of KRH-3955 at a dose of 10 mg/kg or intravenous administration at a dose of 10 mg/kg to male Sprague-Dawley rats (CLEA, Kanagawa, Japan). R-176211 in plasma was measured by liquid chromatography-tandem mass spectrometry. Pharmacokinetic parameters were calculated by using WinNonlin Professional (ver. 3.1; Pharsight Co.).

Infection of hu-PBL–SCID mice. Two groups of C.B-17 SCID mice (CLEA, Kanagawa, Japan) were administered a single dose of either KRH-3955 or tartrate (2% glucose solution was used as the vehicle) as a control orally (p.o.) and fed for 2 weeks. These mice were then engrafted with human PBMCs (1×10^7 cells/animal intraperitoneally [i.p.]) and after 1 day were infected i.p. with 1,000 infective units of X4 HIV-1_{NL4-3}. IL-4 (2 μg 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 collected from the peritoneal cavities and spleens of the infected mice and cultured in vitro for 4 days in RPMI medium supplemented with 20 U/ml rhIL-2. HIV-1 infection was monitored by measuring p24 levels in the culture supernatant. We used a selected donor whose PBMCs could be engrafted at an efficiency of >80% in C.B-17 SCID mice. Usually, 5×10^5 to 10×10^5 human $CD4^+$ T cells can be recovered from each hu-PBL–SCID mouse. Mice with no or low recovery of human $CD4^+$ T cells at the time of analysis were omitted. For ex vivo cultures, we used a quarter of the cells recovered from a mouse. The protocols for the care and use of the hu-PBL–SCID mice were approved by the Committee on Animal Research of the University of the Ryukyus before initiation of the present study.

RESULTS

Anti-HIV-1 activities of KRH-3955 in activated PBMCs. The inhibitory activity of KRH-3955 against X4 HIV-1 (NL4-3), R5X4 HIV-1 (89.6), and R5 HIV-1 (JR-CSF) was examined in activated human PBMCs from two different donors. KRH-3955 inhibited the replication of both X4 and R5X4 HIV-1 in activated PBMCs with 50% effective concentrations (EC_{50}) of 0.3 to 1.0 nM but did not affect R5 HIV-1 replication, even at concentration of up to 200 nM (Table 1). In contrast, the CCR5 antagonist SCH-D (vicriviroc) inhibited R5 HIV-1 rep-

TABLE 1. Anti-HIV-1 activity of KRH-3955 in activated PBMCs[a]

Virus	Donor	EC_{50} (nM)[b]					
		KRH-3955	AMD3100	AMD070	SCH-D	AZT	SQV
NL4-3	A	1.1	41	35	>1,000	11	9.0
X4	B	0.33	15	15	>1,000	8.0	29
89.6	A	0.38	44	55	>1,000	7.4	9.9
R5X4	B	ND[c]	ND	ND	ND	ND	ND
JR-CSF	A	>200	>200	>200	0.37	0.96	2.6
R5	B	>200	>200	>200	1.2	6.2	8.0
A018H (X4) (pre-AZT)	C	1.4	38	ND	ND	1.9	ND
A018G (X4) (post-AZT)	C	1.3	32	ND	ND	87,000	ND

[a] PBMCs from two different donors were used in each assay. Anti-HIV-1 activity was determined by measuring the p24 antigen level in culture supernatants.
[b] Assays were carried out in triplicate wells. The average of two to four experiments is shown.
[c] ND, not determined.

lication but inhibited neither X4 nor R5X4 HIV-1 replication (Table 1). The anti-HIV activity of KRH-3955 against the 89.6 virus from donor B was not determined because the virus did not replicate enough for calculation of the anti-HIV activity of KRH-3955 and other drugs. Notably, the anti-HIV-1 activity of KRH-3955 was much higher than that of AMD3100, a well-known X4 HIV-1 inhibitor, or AMD070, the other X4 inhibitor that is bioavailable when administered orally. KRH-3955 also inhibited the replication of clinical isolates of X4 HIV-1 (92HT599) and R5X4 HIV-1 (92HT593) with EC_{50} ranging from 4.0 to 4.2 nM (data not shown). Although both KRH-3955 and AMD3100 were effective against at least some R5X4 HIV-1 strains in activated PBMCs, neither KRH-3955 nor AMD3100 inhibited the infection of CD4/CCR5 cells by R5 or R5X4 HIV-1, even at a concentration of 1,660 nM (data not shown). Importantly, the 50% cytotoxic concentration of KRH-3955 in activated PBMCs (donor A) was 57 μM, giving a high therapeutic index (51,818) in the case of NL4-3 infection, which was higher than that of AZT (8,000 in the case of donor A). These results indicate that the compound is a selective inhibitor of HIV-1 that can utilize CXCR4 as a coreceptor. Since a CXCR4 antagonist should be used in combination with a CCR5 antagonist in a clinical setting, we next examined whether the combined use of both antagonists efficiently blocks mixed infection with X4 and R5 HIV-1. Combination of KRH-3955 and SCH-D at 4 plus 4 nM and 20 plus 20 nM blocked the replication of 50:50 mixtures of NL4-3 and JR-CSF by 91 and 96%, respectively (data not shown). Thus, KRH-3955 is a highly potent and selective inhibitor of X4 HIV-1.

Anti-HIV-1 activities of KRH-3955 in activated PBMCs from different donors. It has been observed that the anti-HIV-1 activity of compounds in PBMCs varies from donor to donor. Therefore, the anti-HIV-1 activity of KRH-3955 against X4 HIV-1 was examined in activated PBMCs from eight different donors. The levels of p24 antigen in NL4-3-infected cultures ranged from 17 to 120 ng/ml (Table 2). KRH-3955 inhibited the replication of NL4-3 with EC_{50} ranging from 0.23 to 1.3 nM and with EC_{90} ranging from 2.7 to 3.5 nM (Table 2), demonstrating that the anti-HIV-1 activity of KRH-3955 was independent of the PBMC donor.

Anti-HIV-1 activities of KRH-3955 against drug-resistant HIV-1 strains. To further assess the efficacy of KRH-3955, we used a single-cycle assay to evaluate the activity of KRH-3955 against a panel of recombinant viruses that express an X4-tropic envelope protein (HXB2) but contain PR and RT sequences containing a wide variety of mutations associated with resistance to PR inhibitors (PIs), nucleoside RT inhibitors (NRTIs), and non-NRTIs (NNRTIs). This assessment was also performed with recombinant viruses that express an X4-tropic envelope protein (NL4-3) that contains the Q40H mutation and displays resistance to T20 (an entry inhibitor). The results of these experiments demonstrate that both KRH-3955 and AMD3100 inhibited the infection of CD4/CXCR4 cells by these recombinant drug-resistant viruses, including viruses resistant to PIs, NRTIs, or NNRTIs; multidrug-resistant viruses; and T20-resistant viruses (Table 3). We also observed that KRH-3955 inhibited the replication of A018G, a highly AZT-resistant strain, in activated PBMCs with an EC_{50} of 1.3 nM (Table 1).

KRH-3955 selectively inhibits ligand binding to CXCR4. To investigate whether KRH-3955 specifically blocks ligand binding to CXCR4, the inhibitory effect of the compound on chemokine binding to CHO cells expressing CXCR4, CXCR1, CCR2b, CCR3, CCR4, or CCR5 was determined. KRH-3955 efficiently inhibited SDF-1α binding to CXCR4 in a dose-dependent manner (Fig. 2 and 3b), and the IC_{50} for SDF-1α binding was 0.61 nM, which is similar to its EC_{50} against HIV-1. Similar results were obtained when we used a Molt-4 T cell line as the CXCR4-expressing target cell (Fig. 3a). Interestingly, the inhibitory activity of AMD3100 against SDF-1α binding was much weaker than its anti-HIV-1 activity (Fig. 3), suggesting that the binding sites of these two compounds are different. In contrast, the compound did not affect the binding

TABLE 2. Anti-HIV-1 activity of KRH-3955 against NL4-3 infection of PBMCs from eight different donors

Donor	p24 level (ng/ml)	EC_{50} (nM)	EC_{90} (nM)
1	31	1.30	3.2
2	25	1.20	3.2
3	17	1.20	3.3
4	40	0.70	2.9
5	120	0.77	2.9
6	58	1.50	3.5
7	49	0.23	2.7
8	53	1.00	3.0
Mean ± SD	49 ± 32	0.99 ± 0.40	3.1 ± 0.30

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-

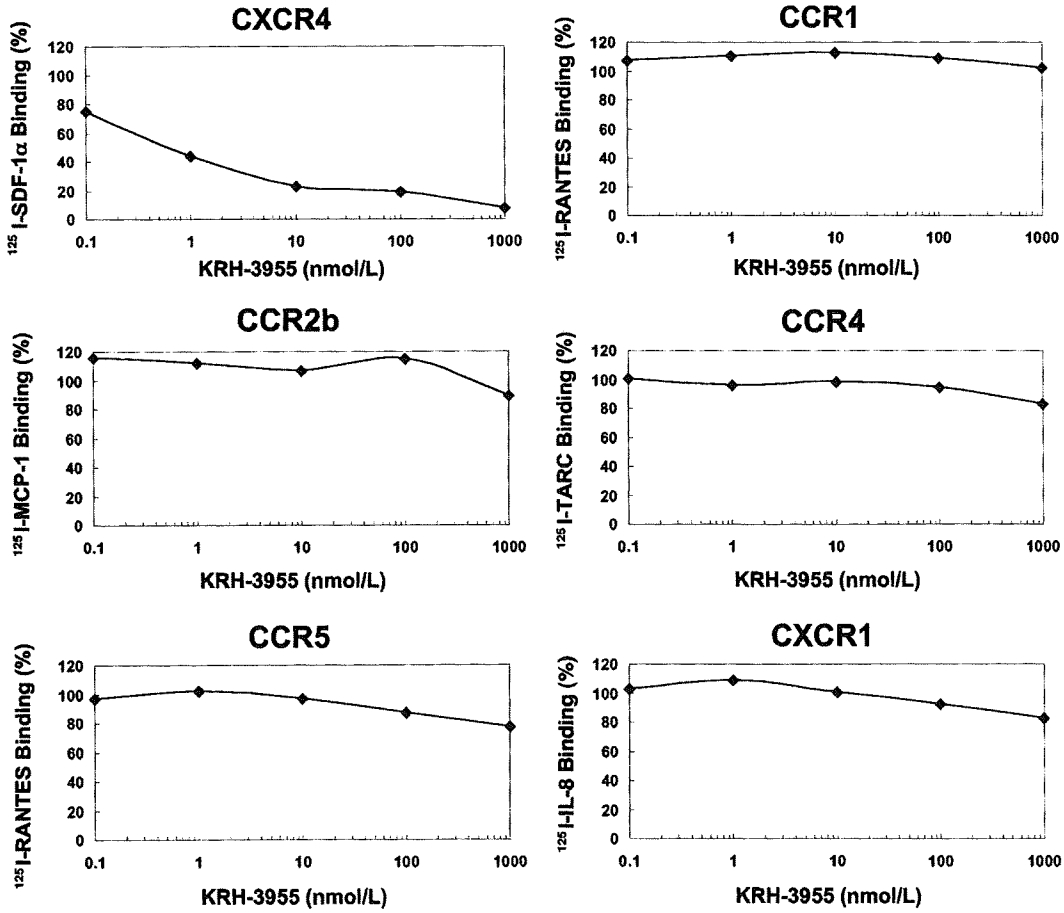

FIG. 2. Inhibitory effects of KRH-3955 on chemokine binding to CXCR4-, CCR1-, CCR2b-, CCR4-, CCR5-, or CXCR1-expressing CHO cells. Chemokine receptor-expressing CHO cells were incubated with various concentrations of KRH-3955 in binding buffer containing ^{125}I-labeled chemokine. Binding reactions were 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.

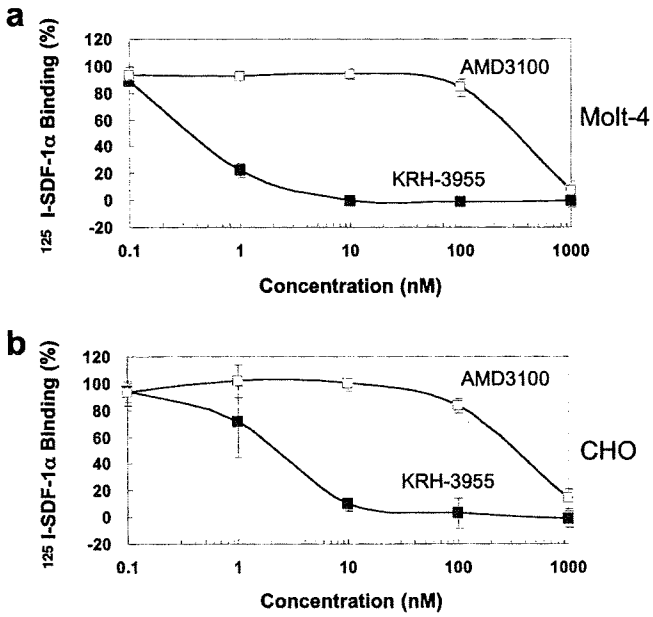

FIG. 3. Concentration-dependent inhibition by KRH-3955 of SDF-1α binding to (a) Molt-4 and (b) CXCR4-expressing CHO cells. CXCR4-expressing CHO cells were incubated with various concentrations of KRH-3955 (■) or AMD3100 (□) in binding buffer containing ^{125}I-labeled SDF-1α. Binding reactions were performed,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

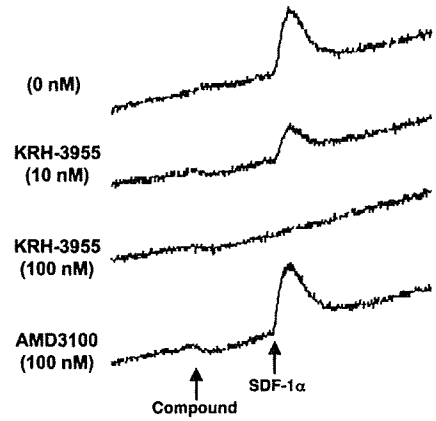

FIG. 4. Inhibitory effects of KRH-3955 on SDF-1α-induced Ca^{2+} mobilization in CXCR4-expressing CHO cells. Fura-2-acetoxymethyl ester-loaded CXCR4-expressing CHO cells were incubated in the presence or absence of various concentrations of KRH-3955 or AMD3100. Changes in intracellular Ca^{2+} levels 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

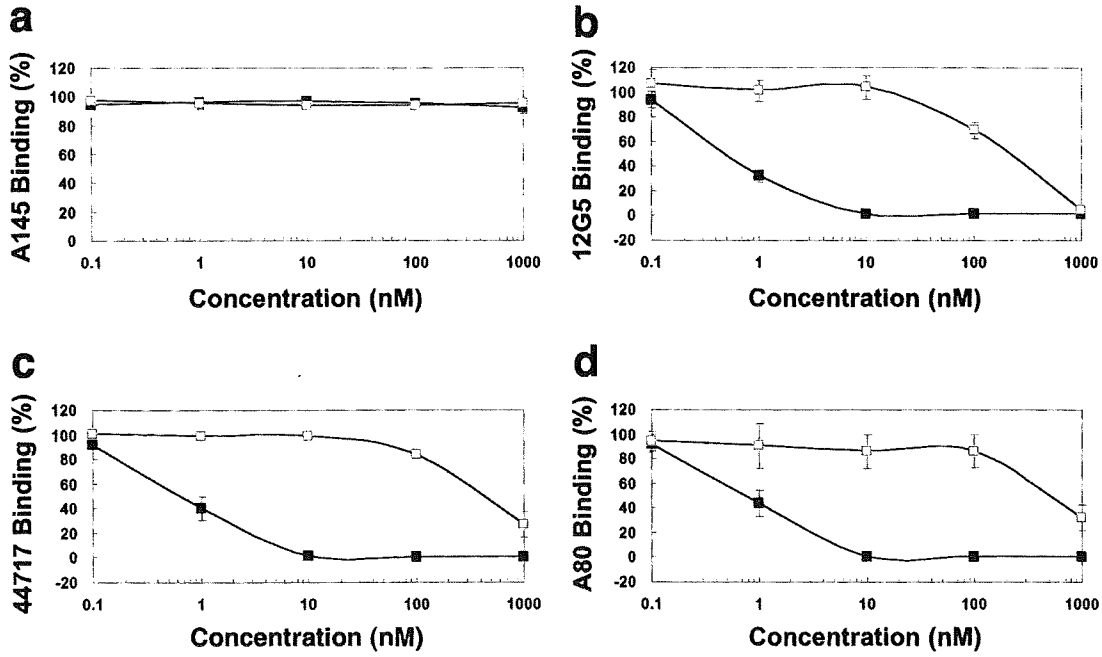

FIG. 5. Effect of KRH-3955 on the binding of four different MAbs to the CXCR4 receptor. Molt-4 cells were incubated with various concentrations of KRH-3955 (■) or AMD3100 (□). The cells were stained directly with MAbs 12G5 (recognizes ECL1 and ECL2 of CXCR4)-PE, 44717 (recognizes ECL2 of CXCR4)-PE, and 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)

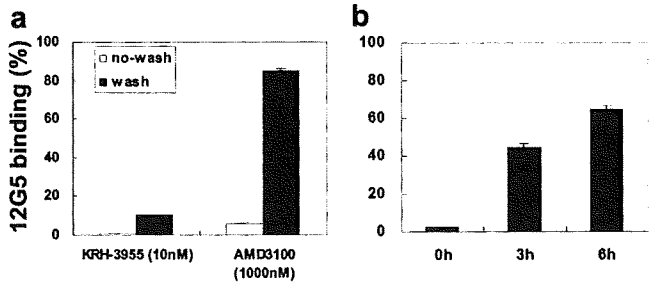

FIG. 6. Long-lasting inhibitory effects of KRH-3955 on the binding of MAb 12G5. (a) Molt-4 cells were treated with 10 nM KRH-3955 or 1,000 nM AMD3100 for 15 min. With (■) or without (□) washing, the cells were staining with MAb 12G5-PE and analyzed by flow cytometry. (b) Long-lasting inhibitory effect 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).

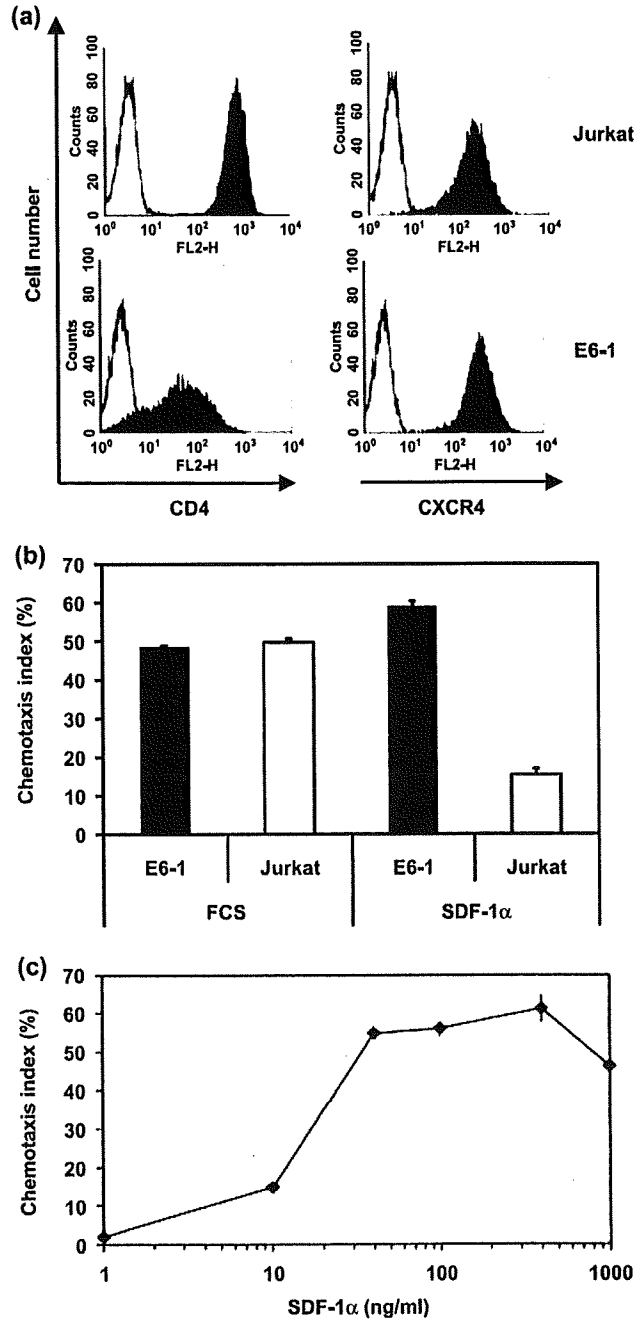

Fig. 1. A quantitative assay system for stromal cell-derived factor-1α (SDF-1α)-mediated chemotaxis. (a) Evaluation of CD4 and CXC chemokine receptor-4 (CXCR4) expression on Jurkat and its subline E6-1. The cells were stained with phycoerythrin-labeled anti-CXCR4 or anti-CD4 mouse monoclonal antibodies.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