HIV ペプチドのデザインと合成 第 44 回ペプチド討論会（富山）H19 年 11 月 7 日

27. ○井ノ口恵利子、鳴海哲夫、新居田歩、富田健嗣、大石真也、大野浩章、藤井信孝 パラジウム触媒カルボニル化反応を用いたトリフルオロメチルアルケンジペプチドイソスター及び類縁体の新規合成法の開発 第 27 回有機合成若手セミナー（京都）H19 年 11 月 15 日
28. ○富田健嗣、鳴海哲夫、新居田歩、大石真也、大野浩章、藤井信孝 シス型アミド結合等価体としてのZ型アルケンおよびE型フルオロアルケンジペプチドイソスターの合成と応用 第 26 回メディシナルケミストリーシンポジウム（相模大野）H19 年 11 月 28 日
29. ○加藤学、上田聡、井貫晋輔、大石真也、大野浩章、Barry Evans、Zi-xuan Wang、Stephen C. Peiper、藤井信孝 新規非ペプチド性ケモカイン受容体 CXCR4 アンタゴニストの創製研究 第 26 回メディシナルケミストリーシンポジウム（相模大野）H19 年 11 月 28 日
30. ○大石真也 ペプチド・蛋白質化学を基盤とする新興・再興感染症治療薬の創製 第 5 回バイオメディカル研究会（大阪）H20 年 2 月 13 日

H．知的財産権の出願・登録状況

1．特許取得

該当事項なし

2．実用新案登録

該当事項なし

3．その他

該当事項なし

厚生労働科学研究費補助金（政策創薬総合研究事業）

分担研究報告書

経口投与可能なCXCR4阻害剤に関する研究

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研究要旨: KRH-1636の誘導体で、経口吸収性を示すCXCR4阻害剤KRH-3955が、in vitroのウイルス感染実験において既存の抗HIV薬に対する耐性株を含めたX4 HIV-1（調べた範囲ではR5X4も含む）に対して低濃度（EC_{50}: 1-4 nM）で顕著な抗ウイルス活性を示した。さらに、hu-PBL-SCID miceを用いたHIV-1感染モデルにおいても単回経口投与で明確な抗ウイルス活性を示した。また、各種CXCR4変異体を用いてこの薬剤の作用するアミノ酸の同定も試みた。

A.　研究目的

本研究班の課題であるHIV吸着・膜融合過程を標的とする多剤耐性克服型HIV化学療法剤の開発に関して、共同研究者（株）クレハが開発したKRH-1636の誘導体で、経口吸収性を示すCXCR4阻害剤KRH-3955に関して行った研究成果を報告する。

B.　研究方法

（1）抗HIV-1活性測定：固定化抗CD3抗体で刺激し、IL-2存在下で増殖させたPBMCを標的細胞としてX4、R5、R5X4の各種HIV-1をMOI=0.001で感染させ、種々の濃度の薬剤存在下で7日から10日培養した。抗HIV-1活性は培養上清中のp24抗原量を市販のELISAを用いて測定後算出した。

（2）薬剤耐性HIV-1に対する抗ウイルス活性測定：CD4とCXCR4を導入したU87細胞を標的細胞として、HIV-1(HXB2またはNL4-3) Env発現ベクターとEnv領域にLuciferase遺伝子を導入したHIVゲノムベクター（薬剤耐性遺伝子搭載）から作製したシュードタイプウイルスを種々の濃度の薬剤存在下で感染させ、感染させた標的細胞抽出液のLuciferase活性を測定して抗ウイルス活性を算出した。

（3）hu-PBL-SCID miceを用いたHIV-1感染モデルにおける抗ウイルス活性測定：感染2週間前にKRH-3955を10 mg/kg単回経口投与した。分離したヒトPBMCをマウス腹腔に導入し、1日後にHIV-1 NL4-3を感染させた。感染7日後にマウス腹腔からPBMCを回収し、IL-2存在下で4日間培養した。抗HIV-1活性は培養上清中のp24抗原量を市販のELISAを用いて測定後算出した。

（4）SDF-1a結合阻害活性の測定：CXCR4を強制発現させたCHO細胞を用いて薬剤存在、非存在下で細胞に結合した^{125}I-SDF-1aの放射活性を測定した。

（5）各種抗CXCR4抗体結合阻害活性の測定：Molt-4細胞をon iceで薬剤処理した後細胞を洗浄し、抗体反応後結合したCXCR4抗体量をFACSにて定量した。

（6）CXCR4阻害剤との相互作用に影響を与えるCXCR4中のアミノ酸の同定：主に酸性アミノ酸をアラニンに置換したCXCR4点変異体を安定発現させた293細胞を使用し、CXCR4阻害剤が変異CXCR4と抗CXCR4抗体12G5との結合阻害活性に与える影響を測定することによって、阻害剤と相互作用するCXCR4中のアミノ酸を推定した。

（倫理面での配慮）

該当事項なし

C.　研究結果

（1）KRH-3955の抗HIV-1活性：KRH-3955は、用いたX4, R5X4 HIV-1の活性化PBMCにおける複製をEC_{50}: 1-4 nMというごく低濃度で抑制した（表1）。一方、

JR-CSF など R5 HIV-1 に対しては 1 mM においても顕著な抗ウイルス活性を示さなかった。また、活性化 PBMC や MT-4 細胞に対して 50 mM まで顕著な細胞毒性を示さなかった。

（2）KRH-3955 の薬剤耐性 HIV-1 に対する抗ウイルス 活性：KRH-3955 は、NRTI、NNRTI、PI 耐性、MDR（NRTI、NNRTI、PI 耐性）、T-20 耐性株のいずれに対しても親株である HXB2、NL4-3 とほぼ同程度の抗ウイルス活性を示した（表２）。

（3）hu-PBL-SCID mice を用いた HIV-1 感染モデルにおける抗ウイルス活性：感染前に単回投与した KRH-3955 は、hu-PBL-SCID mice 腹腔内における NL4-3 の感染・複製をほぼ完全に抑制した（表３）。

（4）SDF-1a 結合阻害活性：コントロールとして使用した CXCR4 阻害剤 AMD3100、AMD070、KRH のもうひとつの誘導体である KRH-3148 とともに検討した。KRH-3955、KRH-3148、AMD3100、AMD070 の SDF-1a 結合に対する IC_{50} (nM)はそれぞれ、0.8、2.2、281.1、3.7 であった。KRH-3955 の IC_{50} 値は、MT-4 細胞に HIV-1IIIB を感染させる系における化合物の EC_{50} 値によく対応していた。一方、AMD3100 の SDF-1a 結合阻害活性は HIV-1 複製阻害活性より顕著に弱く、AMD070 のそれは逆にやや強かった（図1）。

（5）各種抗 CXCR4 抗体結合阻害活性：KRH-3955 の CXCR4 に対する作用点を明らかにする実験の一つとして、これらの化合物が、各種抗 CXCR4 モノクローナル抗体の CXCR4 への結合を阻害するかを検討した。CXCR4 の N 末端を認識する抗体（A145）、レセプターの細胞外領域（ECL）１と２を認識する抗体（12G5）、ECL2 を認識する抗体（44717）、ECL3 を認識する抗体（A80）の４種類の抗体を用いた。KRH-3955 は N 末端を認識する抗体以外の抗体の CXCR4 発現細胞（Molt-4）への結合を強く阻害した。一方、コントロールとして用いた KRH-3148、CXCR4 阻害剤 AMD3100、AMD070 は、ECL１と２を認識する抗体である 12G5 の結合は抑制したが、それ以外の抗体結合の阻害は弱いかほとんど認められなかった。なお、3955、3148 で CXCR4 発現細胞を処理して 37□でインキュベートしても A145 の結合量が変化しないことから、これらの CXCR4 阻害剤には CXCR4 をダウンモジュレートする活性はないことも明らかになった（図２）。

（6）CXCR4 阻害剤との相互作用に影響を与える CXCR4 アミノ酸の同定： CXCR4 の細胞外領域、膜貫通領域と推定される中で細胞外領域に近接する領域に存在する主に酸性アミノ酸をアラニンに置換した点変異体を作製し 293 細胞に導入して安定発現株を樹立した。CXCR4 阻害剤が変異 CXCR4 と抗 CXCR4 抗体 12G5 の結合阻害活性に与える影響を測定することによって、 阻害剤と相互作用する CXCR4 中のアミノ酸を推定した。その結果、KRH-3955 は Asp^{262}、His^{281} と、KRH-3148 は Asp^{171}、Asp^{262}、His^{281}、Trp^{283} と相互作用すると推定された（図３）。文献で報告されている AMD3100 と相互作用するアミノ酸は Asp^{171}、Asp^{262} であり、KRH-3955、KRH-3148 のそれらと一部重なっていることが判明した。

D. 考察

KRH-3955 が一部の R5X4 HIV-1 の PBMC への感染を抑制できたのは、使用した R5X4 株が CXCR4 を優先的に使用するタイプの株であるためと考えられた。今後の検討事項としては、薬剤耐性株を誘導・分離して CXCR4 への作用についての情報を蓄積すること、サルを用いた感染防御実験での有効性を示すことなどが挙げられる。

E. 結論

新規 CXCR4 阻害剤 KRH-3955 が、in vitro のウイルス感染実験において既存の抗 HIV 薬に対する耐性株を含めた X4 HIV-1（調べた範囲では R5X4 も含む）に対して低濃度（EC_{50}: 1-4 nM）で顕著な抗ウイルス活性を示した。さらに、hu-PBL-SCID mice を用いた HIV-1 感染モデルにおいても単回経口投与で明確な抗ウイルス活性を示した。

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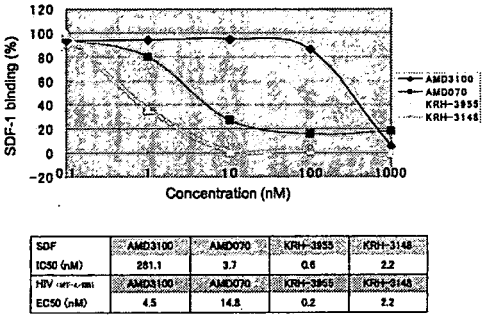
G. 知的財産権の出願・登録状況（予定を含む）

該当事項なし

Virus	Tropism	EC_{50} (nM) KRH-3955	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

表 1 . KRH-3955 の抗 HIV-1 活性



SDF	AMD3100	AMD070	KRH-3955	KRH-3148
IC50 (nM)	281.1	3.7	0.6	2.2
HIV	AMD3100	AMD070	KRH-3955	KRH-3148
EC50 (nM)	4.5	14.8	0.2	2.2

図 1. KRH-3955 の SDF-1a 結合阻害活性

Virus	EC_{50} (nM) KRH-3955	AMD3100
NL4-3	0.5	4.8
HXB2	0.6	6.2
NRTI-R (HXB2-env)	0.6	9.0
NNRTI-R (HXB2-env)	0.8	7.0
PI-R (HXB2-env)	0.7	9.2
MDR (HXB2-env)	0.7	5.3
T20-R (NL4-3-env)	0.4	2.3

表 2. KRH-3955 の薬剤耐性 HIV-1 阻害活性

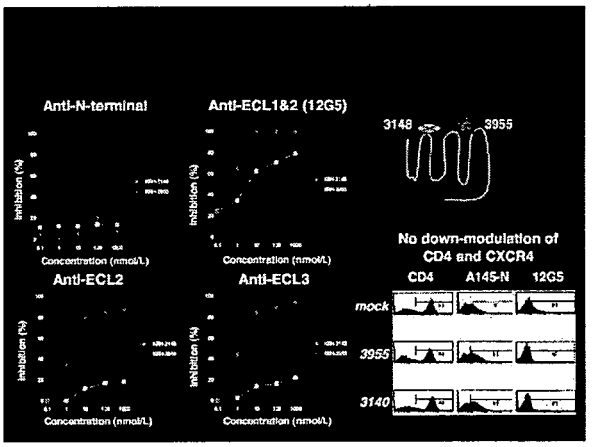


図 2. KRH-3955 の各種抗 CXCR4 抗体結合阻害活性

Group	mouse#	p24 produced (pg/ml)
Control	1	747
	2	10263
	3	<5
	4	5821
	5	1902
KRH-3955	6	<5
	7	<5
	8	<5
	9	<5
	10	36

Two groups of C.B-17 SCID mice (n= 5) were administrated with single dose of either KRH-3955 or tartrate as control, p.o, and fed for 2 weeks. Then these mice were engrafted with human PBMC (1 x 10⁷ cells/animal i.p.) and after one day these humanized mice were infected with 1000 infectious units of X4 HIV-1_{NL4-3} IL-4 (2 microgram/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 media containing 20 U/ml IL-2. HIV-1 infection was monitored by quatitation of cell culture supernatants by p24 ELISA kits. Means from duplicate determinations were shown. <5, under detection level.

表 3 . KRH-3955 の hu-PBL-SCID mice モデルにおける抗 HIV-1 活性

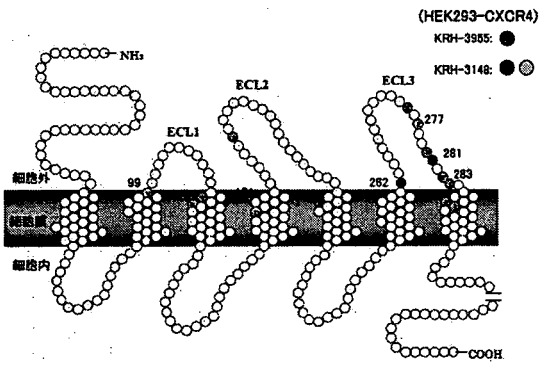


図 3. CXCR4 阻害剤との相互作用に影響を与える CXCR4 アミノ酸の同定

研究成果の刊行に関する一覧表

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研究成果の刊行に関する一覧表

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雑誌

発表者氏名	論文タイトル	発表誌名	巻号	ページ	出版年
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JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2008, p. 792–795 Vol. 46, No. 2
0095-1137/08/$08.00+0 doi:10.1128/JCM.01470-07

Dual-Reporter Phenotypic Assay for Human Immunodeficiency Viruses[▽]

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Received 22 July 2007/Returned for modification 27 September 2007/Accepted 5 December 2007

We have established a novel human immunodeficiency virus (HIV) tandem-reporter assay using HIV receptor-transduced NP-2 cells with long terminal repeat-controlled β-galactosidase, inserted internal ribosome entry site, and secretary alkaline phosphatase genes. This assay allows users to detect replication of clinical isolates, indicating its useful application as an HIV phenotypic assay.

Assays for detecting the emergence of resistant variants, as well as evaluating clinical efficacy, provide useful information regarding chemotherapy for human immunodeficiency virus (HIV) infection. To date, two types of assay systems have been developed and approved, namely, genotypic and phenotypic assays (13, 31). Genotypic assays detect genetic mutations that are associated with drug resistance and lead to rapid and sensitive detection of the emergence of resistant variants (9), although they only provide estimated resistance profiles (16, 30). Lists of significant resistance-associated mutations in reverse transcriptase (RT), protease, and envelope genes are maintained by some organizations and Universities, such as the International AIDS Society-USA (http://iasusa.org/resistance mutations); Los Alamos National Laboratory, Los Alamos, NM (http://resdb.lanl.gov/Resist_DB); and the Stanford University, Stanford, CA (http://hivdb.stanford.edu/index.html). PCR-based genotypic assays are heavily dependent on the primers used. Therefore, some biases must unfortunately be presumed, since most primer-matched sequences are preferentially amplified, resulting in some discordance with phenotypic assays (21, 33) that are time-consuming and require tedious procedures because isolation of replication-competent viruses is required. To date, phenotypic assays for clinical isolates have been mainly performed in experiments with a p24 production assay in phytohemagglutinin-stimulated peripheral blood mononuclear cells (27, 32, 34).

For more rapid and simple phenotypic assays, recombinant viruses containing the region responsible for resistance have been utilized instead of isolated viruses (14, 18, 35). However, since protease resistance mutations are introduced simultaneously with gag mutations (4, 25), cloning of the entire gag and protease coding region is occasionally required. Recently, mutations for 3′-azido-3′-deoxythymidine (AZT) resistance have been identified in the connection and/or RNase H subdomain (5, 7, 26), which no commercially available genotypic and phenotypic assays include for the analysis. Moreover, the mechanism of resistance to a fusion inhibitor, enfuvirtide, is a complex issue, since mutations in not only the gp41 coding region (6, 24) but also the V3 region (29) and the CD4-binding site (2) of gp120 influence the susceptibility, indicating that patient-derived viruses are ideally required for evaluation of drug susceptibility.

Recently, Hachiya et al. established a simple and rapid phenotypic assay using MAGIC5 cells (CCR5-transduced MAGI cells) (11). This system efficiently isolates clinical HIV variants and has proven to be useful for evaluating drug susceptibility (12). However, the expression of transduced receptors on MAGIC5 cells declines during prolonged culture, as described for MAGI cells (17). Therefore, in order to obtain HIV isolates efficiently and perform the assay, relatively fresh cells are required. More recently, we established a tetrazolium-based colorimetric assay for monitoring replications of not only CXCR4 (X4)-tropic but also CCR5 (R5)-tropic HIVs and drug susceptibilities (17). We reported that NCK45 cells stably express HIV receptors on their cell surface and provide reproducible results (17). Since this assay depends on the cytopathic effect induced by HIV, it appears to be insufficient for assessing infections with no or a few cytopathic variants. Furthermore, it requires 7 days of culture to obtain the drug susceptibility. In the present study, we have established a novel single long terminal repeat (LTR)-driven tandem two-reporter system using the internal ribosome entry site (IRES) (15), which enables the evaluation of drug susceptibility within 2 days for various HIVs, including clinical isolates.

To construct an LTR-driven reporter vector, an amplified LTR region (the −138 to +89 region of the transcriptional start site of HIV-1 molecular clone pNL4-3) was inserted into pβgal-Basic (Clontech Laboratories, Inc., Palo Alto, CA) between the NheI and HindIII sites (pLTR-βgal). The 5′ region (HindIII to EcoRV) of the β-galactosidase gene was replaced with the responsible β-galactosidase fragment with nuclear localization signal sequence (MPKKKRK) amplified from genomic DNA of MAGI cells (20). Fragments of IRES and secretory alkaline phosphatase (SEAP) were amplified from pIRES2-EGFP and pSEAP2-Basic (Clontech Laboratories, Inc.), respectively. A puromycin-resistance gene (Puro^r) under the control of the phosphoglycerate kinase promoter as a selection marker was inserted at the SalI site of the vector (pLTR-β-Gal/SEAP-Puro^r), as shown in Fig. 1A. All fragments were verified by sequencing.

The pLTR-β-Gal/SEAP-Puro^r plasmid was transfected into NCK45 cells (CD4, CXCR4, and CCR5-transduced NP-2 cells

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▽ Published ahead of print on 19 December 2007.

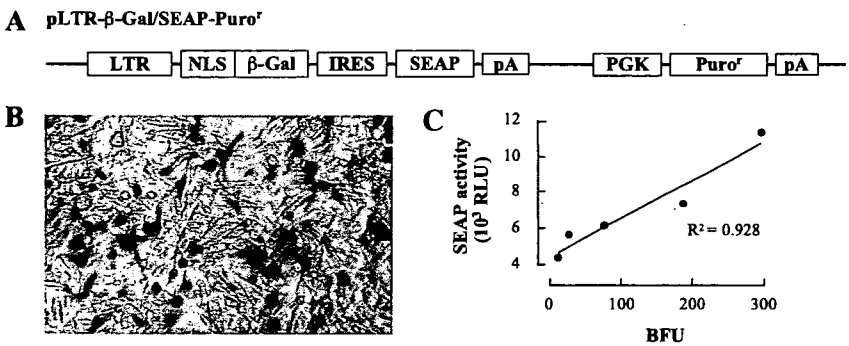

FIG. 1. Establishment of a cell line with β-galactosidase (β-Gal) and SEAP genes driven by an LTR. (A) Schematic diagram of the vector used in the present study, which simultaneously expresses genes for β-Gal and SEAP under the control of the HIV-1 LTR promoter (pLTR-β-Gal/SEAP-Puro^r). The enhancer region (positions −138 to +89) of the LTR, the nuclear localization signal (NLS) derived from the T-antigen of simian virus 40, the IRES, the phosphoglycerate kinase promoter (PGK), and the polyadenylation signal (pA) are also shown. (B) Microscopic image of X-Gal-stained NCK45-β-Gal/SEAP cells at 48 h after virus inoculation. (C) Correlation between β-Gal and SEAP activities in culture supernatants. NCK45-β-Gal/SEAP cells were infected with HIV at various infectious doses and incubated for 48 h. Culture supernatants were examined for their SEAP activities and expressed as relative right units (RLU). BFU, blue-cell-forming units.

derived from a glioma) (17, 36) to detect intracellular Tat expression through the LTR-driven tandem reporter genes. At 48 h after transfection, the cells were cultured in Dulbecco modified Eagle medium (Sigma, St. Louis, MO) supplemented with 5% heat-inactivated fetal calf serum, 0.5 mg of G418 disulfate (Nacalai Tesque, Kyoto, Japan)/ml, 0.2 mg of hygromycin B (Calbiochem, La Jolla, CA)/ml, and 10 μg of puromycin (Sigma)/ml and designated NCK45-β-Gal/SEAP cells. The expression levels of receptors on NCK45 cells confirmed by a flow cytometer (17) were 97, 83, and 99%, while those on H9 cells as a control were 65, 73, and 0.3% for CD4, CXCR4, and CCR5, respectively.

To evaluate anti-HIV agents, NCK45-β-Gal/SEAP cells (5×10^4 cells/ml) in Dulbecco modified Eagle medium-based culture medium supplemented with 5% fetal calf serum, penicillin, and streptomycin were seeded onto 96-well plates. On the following day, the cells were inoculated with sample viruses at 60 blue-cell-forming units (BFU)/well, incubated for 48 h, and then cultured in the presence of various concentrations of drugs. After 48 h of culture, the cells were fixed with 1% formaldehyde and 0.2% glutaraldehyde for 3 min, washed three times with phosphate-buffered saline, and incubated with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for 2 h (Fig. 1B). To evaluate the SEAP activities, the culture supernatants were harvested and analyzed by using a Great EscAPe SEAP chemiluminescent detection kit (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's protocol. Samples were measured by using a Wallac 1450 MicroBeta Jet Luminometer (Perkin-Elmer, Wellesley, MA). For comparison, MAGI-CCR5 cells were analyzed as previously described (17).

The activities of various anti-HIV agents toward NCK45-β-Gal/SEAP cells were compared to those in the MAGI assay. The BFU and SEAP activities were well correlated with the viral input (Fig. 1C; correlation coefficient $R^2 = 0.928$). We tested various anti-HIV agents (Table 1): DS5000, an adsorption inhibitor; AZT and 2′,3′-dideoxycytidine (ddC), RT inhibitors; T-140 (37), a CXCR4 antagonist; and TAK-779, a CCR5 antagonist (1). The antiviral activities of the compounds determined by each reporter in NCK45-β-Gal/SEAP cells were comparable to those obtained using MAGI cells, although some were statistically significant (Table 1). Intracellular nucleoside/nucleotide metabolisms, especially thymidine kinase (10), and expression levels of receptors (19) may alter the 50% effective concentrations of the AZT and CCR5 antagonists, respectively.

NCK45-β-Gal/SEAP cells also supported the replication of various clinical isolates, as well as laboratory strains. The clin-

TABLE 1. Comparison of anti-HIV activities in MAGI and NCK45-β-Gal/SEAP cells

Compound	Target	Mean EC_{50} (μM)[a] ± SD						CC_{50} (μM)[b] (NCK45-β-Gal/SEAP)
		HIV-1_{IIIB}			HIV-1_{Ba-L}			
		MAGI	NCK45-β-Gal/SEAP		MAGI	NCK45-β-Gal/SEAP		
			β-Gal	SEAP		β-Gal	SEAP	
DS5000	gp120	0.14 ± 0.025	0.076 ± 0.026	0.07 ± 0.0034	0.36 ± 0.052	0.49 ± 0.069	0.42 ± 0.15	>100
AZT	RT	0.031 ± 0.013*	0.0043 ± 0.0022	0.0035 ± 0.0007	0.05 ± 0.029	0.0035 ± 0.0007	0.0094 ± 0.0063	>100
ddC	RT	0.4 ± 0.16	0.53 ± 0.12	0.42 ± 0.14	0.48 ± 0.17	0.72 ± 0.067	0.67 ± 0.2	>100
T-140	CXCR4	0.006 ± 0.0006	0.006 ± 0.0002	0.0025 ± 0.0008	>100	>100	>100	>100
TAK-779	CCR5	>100	>100	>100	0.003 ± 0.0019*	0.035 ± 0.0088	0.027 ± 0.0098	>100

[a] EC_{50}, 50% effective concentration. Data represent mean values of at least three independent experiments. HIV-1_{IIIB} and HIV-1_{Ba-L} utilize CXCR4 (X4) and CCR5 (R5) as coreceptors, respectively. *, The EC_{50} values obtained from MAGI and NCK cells (both β-galactosidase and SEAP) were statistically significant (Student t test, $P < 0.01$).

[b] CC_{50}, 50% cytotoxic concentration. The CC_{50} was determined by the MTT method after 2 days exposure of compounds as described previously (11).

TABLE 2. Drug susceptibility against HIV clinical isolates in MAGI and NCK45-β-Gal/SEAP cells

Compound	Cell line	Detection	EC_{50} (μM)[a]					
			KMT/R5X4	IVR405/R5X4	IVR406/R5X4	IVR409/R5X4	IVR416/R5X4	IVR417/R5
DS5000	MAGI	β-Gal	0.54	0.43	0.18	0.51	0.16	1.4
	NCK45-β-Gal/SEAP	β-Gal	0.6	0.22	0.14	0.46	0.22	1.9
		SEAP	0.28	0.27	0.19	0.28	0.16	1.5
AZT	MAGI	β-Gal	0.0027	>1.0	0.68	0.055	0.046	0.0033
	NCK45-β-Gal/SEAP	β-Gal	0.0038	>1.0	1.0	0.061	0.018	0.004
		SEAP	0.0044	>1.0	0.41	0.032	0.013	0.0015
ddC	MAGI	β-Gal	1.3	0.55	1.4	1.2	1.0	1.3
	NCK45-β-Gal/SEAP	β-Gal	1.0	0.32	1.0	1.3	0.75	0.79
		SEAP	2.0	0.24	2.1	1.5	0.52	0.77

[a] EC_{50}, 50% antiviral effective concentration. Amino acid substitutions in the RT region were as follows: none for KMT, M41L/E44D/D67G/V118I/Q151M/L210W/T215Y for IVR405, M41L/E44D/D67N/V118I/M184I/L210W/T215Y for IVR406, M41L/E44D/M184V/L210W/T215Y for IVR416, and D67N/V106AV/M184V/T215F for IVR407. Coreceptor usage is indicated in each subheading after the slash: R5X4, CCR5 and CXCR4 dualtropic virus; R5, CCR5-tropic virus.

ical isolates used in the present study were kindly provided by Y. Maeda (Kumamoto University School of Medicine, Kumamoto, Japan) and S. Oka (AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan). The drug susceptibilities of not only R5-tropic isolates but also X4-tropic and dualtropic isolates were comparable to those in the MAGI assay (Table 2). IVR405 and IVR406 were highly resistant to AZT, whereas IVR409 and IVR416 showed moderate resistance to AZT, and KMT and IVR417 were susceptible to AZT. These susceptibilities were confirmed by the MAGI assay.

In the present study, we established an IRES-mediated tandem-reporter assay using NCK45 cells for the rapid and simultaneous evaluation of the antiviral activities of compounds. This assay enables the evaluation of various HIV strains and clinical isolates within 3 days, including the cell preparation procedure. Secreted SEAP from HIV-infected NCK45 cells is also useful for monitoring the isolation of viruses without cell destruction and enables the continuous propagation of the isolates during isolation. Since NCK45-β-Gal/SEAP cells are susceptible to various viruses, including clinical strains, depending on the experimental purpose any HIV variants may be used for a reference virus, e.g., an isolate prior to the therapy. The LTR promoter for the dual reporters used in the present study includes four CpG methylation-sensitive sites, although Tat can transactivate the HIV-1 LTR regardless of the methylation state (3, 28). Thus, reporter genes in NCK45 cells are less affected by gene silencing.

Compared to the commercially available phenotypic assays, our established system may be useful especially for inhibitors targeting new molecules, including envelope proteins and integrase. A fusion inhibitor T-20 can suppress variants refractory to most of approved RT and protease inhibitors (22, 23). Both phenotypic and genotypic assays for T-20 are needed to evaluate the clinical outcome of patients with T-20-containing regimens. An approved CCR5 antagonist, maraviroc, may also suppress such refractory variants (8). Needless to say, to assess the drug susceptibility experimentally and/or clinically, a combination of established databases accumulated with various assays appears to be useful and important.

In conclusion, the tandem reporters β-Gal and SEAP can evaluate the exact viral infectivity and proportional LTR activation level by repetitive analyses of culture supernatants, respectively. This IRES-mediated tandem-reporter system may be applicable to other reporter genes, e.g., the luciferase gene. Thus, our assay system may provide simple, rapid and stable results for HIV phenotypic assays.

We thank J. Overbaugh for providing the HeLa-CD4/CCR5-LTR/β-Gal cells through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (Bethesda, MD).

This study was supported in part by a grant for the Promotion of AIDS Research from the Ministry of Health and Welfare of Japan (to M.M. and E.K.); a grant for Research for Health Science Focusing on Drug Innovation from the Japan Health Science Foundation (to E.K.); and a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (to E.K.). K.K. and T.N. are supported by the 21st Century COE Program of the Ministry of Education, Culture, Sports, Science, and Technology.

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JOURNAL OF VIROLOGY, Jan. 2008, p. 764–774 Vol. 82, No. 2
0022-538X/08/$08.00+0 doi:10.1128/JVI.01534-07

Broad Antiretroviral Activity and Resistance Profile of the Novel Human Immunodeficiency Virus Integrase Inhibitor Elvitegravir (JTK-303/GS-9137)▽†

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Received 13 July 2007/Accepted 22 October 2007

Integrase (IN), an essential enzyme of human immunodeficiency virus (HIV), is an attractive antiretroviral drug target. The antiviral activity and resistance profile in vitro of a novel IN inhibitor, elvitegravir (EVG) (also known as JTK-303/GS-9137), currently being developed for the treatment of HIV-1 infection are described. EVG blocked the integration of HIV-1 cDNA through the inhibition of DNA strand transfer. EVG inhibited the replication of HIV-1, including various subtypes and multiple-drug-resistant clinical isolates, and HIV-2 strains with a 50% effective concentration in the subnanomolar to nanomolar range. EVG-resistant variants were selected in two independent inductions, and a total of 8 amino acid substitutions in the catalytic core domain of IN were observed. Among the observed IN mutations, T66I and E92Q substitutions mainly contributed to EVG resistance. These two primary resistance mutations are located in the active site, and other secondary mutations identified are proximal to these primary mutations. The EVG-selected IN mutations, some of which represent novel IN inhibitor resistance mutations, conferred reduced susceptibility to other IN inhibitors, suggesting that a common mechanism is involved in resistance and potential cross-resistance. The replication capacity of EVG-resistant variants was significantly reduced relative to both wild-type virus and other IN inhibitor-resistant variants selected by L-870,810. EVG and L-870,810 both inhibited the replication of murine leukemia virus and simian immunodeficiency virus, suggesting that IN inhibitors bind to a conformationally conserved region of various retroviral IN enzymes and are an ideal drug for a range of retroviral infections.

Three unique and essential HIV enzymes, protease (PR), reverse transcriptase with RNase H (RT), and integrase (IN), appear to be ideal targets for the development of inhibitors of human immunodeficiency virus (HIV) replication. Anti-HIV drugs targeting PR (PR inhibitors [PIs]) and RT (nucleoside/nucleotide RT inhibitors [NRTIs] and nonnucleoside RT inhibitors [NNRTIs]) have been approved for use in the treatment of HIV infection. Combinations of these drugs used in highly active antiretroviral therapy can effectively suppress HIV replication in vivo to undetectable levels and have led to significant declines in HIV-associated mortality (28, 40). However, the emergence of drug-resistant HIV variants can attenuate the efficacy of antiretroviral treatment. Some primary infections also result from the transmission of HIV strains that possess drug-resistant genotypes and phenotypes (9). To suppress these drug-resistant variants, new anti-HIV drugs that block new targets are urgently needed.

IN, a 32-kDa protein resulting from the proteolytic cleavage of the *gag-pol* precursor, plays an essential role in the integration of proviral DNA into the host genome. As LaFemina et al. previously reported that there is no human homologue of HIV IN (31), it is an attractive target for the development of new antiretroviral therapeutic agents without adverse effects. IN consists of three domains: an N-terminal zinc finger domain and a C-terminal DNA-binding domain flank a central catalytic core domain (CCD) that plays a critical role in its enzymatic activity (13, 14). Following reverse transcription, IN exerts at least two functions: the cleavage of two conserved nucleotides from the 3′ ends of both strands of the viral cDNA (3′ processing) (1) and, subsequently, the ligation of the viral cDNA into the host genome (strand transfer) (14). Gap filling of the interfaces between the viral and host genomic DNA is then completed using the host DNA repair machinery via a mechanism that is not yet fully understood. The completion of integration results in a fully functional provirus, which can then be used to initiate viral DNA transcription.

Several compounds that inhibit IN activity have been described, including diketo acid (DKA) derivatives such as L-731,988 (24) and S-1360 (16), both of which have potent

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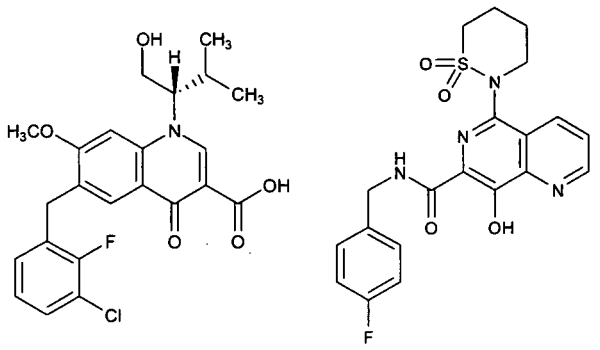
▽ Published ahead of print on 31 October 2007.



elvitegravir (EVG) L-870,810

FIG. 1. Structure of EVG and L-870,810. A dihydroquinoline carboxylic acid derivative, EVG, and a naphthyridine carboxamide derivative, L-870,810 (a representative IN inhibitor), are shown.

antiviral activity. Crystal structure analysis has indicated that 1-(5-chloroindol-3-yl)-3-hydroxy-3-(2*H*-tetrazol-5-yl)-propenone, an S-1360 derivative, binds to the CCD, the putative active site of IN (19). In vitro resistance selection experiments with several IN inhibitors demonstrated that mutations in the CCD of IN play a significant role in the generation of IN inhibitor-resistant viral variants. In vitro selection of HIV-1 in the presence of the DKA IN inhibitors L-731,988 and S-1360 resulted in the emergence of viral variants carrying IN mutations associated with resistance. These mutations, including T66I, S153Y, and M154I, are located in close proximity to the catalytic triad residues (D64, D116, and E152) in the CCD of IN (16, 24). In contrast, L-870,810 (Fig. 1), which has previously demonstrated potent antiviral activity in HIV-1-infected patients in a monotherapy study (33), induced unique IN mutations, including V72I, F121Y, T125K, and V151I, when HIV was selected with the compound in vitro (23). These mutations are also located in the active site of IN, suggesting that a common mechanism may be involved in the acquisition of resistance to IN inhibitors.

Although no IN inhibitors are currently approved for clinical use (41), two IN inhibitors, elvitegravir (EVG) (formerly known as JTK-303/GS-9137, being codeveloped by Gilead Sciences and Japan Tobacco) (Fig. 1) (43, 56) and raltegravir (MK-0518, developed by Merck) (22), are currently being investigated in clinical studies of HIV-1-infected patients. In a phase II study, antiretroviral treatment-experienced patients using 125 mg EVG (boosted with ritonavir) along with an active optimized background regimen showed >2-$\log_{10}$ declines in their viral loads that were durable through week 24 (56).

Here, we describe the antiviral activity, mechanism of action, and resistance profile of EVG in vitro. EVG exerted potent anti-HIV activity against not only wild-type strains but also drug-resistant clinical isolates. Interestingly, EVG also showed antiviral activity against murine leukemia virus (MLV) and simian immunodeficiency virus (SIV). These results imply that IN inhibitors are ideal agents for the treatment of a range of retroviral infections. During the selection of EVG-resistant viral variants, novel IN mutations emerged. Combinations of these mutations conferred resistance to EVG and reduced susceptibility to other IN inhibitors, suggesting that there is a common mechanism underlying the resistance to IN inhibitors. One such mechanism may be conformational changes induced by multiple mutations located in the active site of IN.

MATERIALS AND METHODS

Antiviral agents. Zidovudine (AZT) and dextran sulfate (DS5000) (average molecular weight, 5,000) were purchased from Sigma (St. Louis, MO). Efavirenz (EFV) (NNRTI) and nelfinavir (NFV) (PI) were used for the control inhibitor. EVG (43), L-731,988 (42), L-870,810 (23), and S-1360 (16) were synthesized as described previously. The structures of EVG and L-870,810 are depicted in Fig. 1.

Cells and viruses. MT-2 and MT-4 cells were grown in RPMI 1640 medium. 293T cells were grown in Dulbecco's modified Eagle's medium. These media were supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin. HeLa-CD4/CCR5-LTR/β-gal cells (5) were kindly provided by J. Overbaugh through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases (Bethesda, MD), and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 200 μg/ml hygromycin B, 10 μg/ml puromycin, and 200 μg/ml geneticin. Peripheral blood mononuclear cells (PBMC) were obtained from healthy HIV-1-seronegative donors by centrifugation through Ficoll-Hypaque density gradients. PBMC were stimulated with 20 U/ml interleukin-2 (Shionogi, Osaka, Japan) and 0.5 μg/ml phytohemagglutinin (Sigma) for 3 days and then used for assays as described previously (30).

Three laboratory strains, HIV-1_{IIIB}, HIV-2_{EHO}, and HIV-2_{ROD}, were used in this study. Various subtypes of drug-naïve clinical isolates of HIV-1 (four isolates of subtype B and seven isolates of non-B subtypes) were employed. Four drug-resistant clinical isolates of HIV-1, including IVR401, IVR409, IVR411, and IVR415, were kindly provided by S. Oka (AIDS Clinical Center, International Medical Center of Japan, Tokyo, Japan).

Determination of HIV drug susceptibility. Inhibitory effects of compounds on HIV infection were determined using multinuclear activation of a galactosidase indicator (MAGI) assay, as previously described (37). Inhibitory effects on HIV-1 clinical isolates were measured by p24 production, and cytotoxicity was measured by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay, as described previously (30). Antiviral activities and cytotoxicities of inhibitors are presented as the concentrations that block viral replication by 50% (50% effective concentration [EC_{50}]) and that suppress the viability of target cells by 50%, respectively.

Quantification of HIV-1 DNA species. MT-2 cells (5×10^5 cells) were infected with HIV-1_{IIIB} at a multiplicity of infection (MOI) of 0.1 in the absence or presence of various inhibitors. Infected cells were washed after incubation for 2 h at 37°C. At 24 h postinfection, DNA was extracted using DNAzol reagent (Invitrogen, Carlsbad, CA).

Quantification of integrated HIV-1 DNA and the two-long-terminal-repeat (2-LTR) circle was performed by real-time quantitative PCR as described previously (4). To normalize DNA species among inhibitors, β-globin amplification was used as an internal control (51). Reactions were analyzed by using the ABI Prism 7500 sequence detector (PE Applied Biosystems, Foster City, CA), and results were then normalized and expressed as relative HIV-1 DNA species compared to a "no-inhibitor" control.

In vitro strand transfer assay. An oligonucleotide-based strand transfer assay was performed as previously described (8), with some modifications. Briefly, preprocessed oligonucleotide H-U5V1-2 (5′-ATGTGGAAAATCTCTAGCA-3′), derived from the U5 end of the HIV-1 LTR, was labeled at the 5′ end with [γ-^{32}P]ATP. Radiolabeled H-U5V1-2 was annealed to H-U5V2 (5′-ACTGCTA GAGATTTTCCACAT-3′) and then used for assays. Recombinant HIV-1 IN derived from HIV-1 NL4-3 (wild type) or EVG-selected mutants was prepared using an *Escherichia coli* expression system. The strand transfer assay was performed with 1 μM IN and 150 nM substrate DNA in 20 mM MOPS (morpholinepropanesulfonic acid) buffer with 30 mM $MgCl_2$ incubated in either the presence or absence of IN inhibitors at 37°C for 60 min. Reaction products were analyzed by electrophoresis on 25% polyacrylamide gels and quantified using a BAS-2500 imaging system (Fuji Photo Film, Tokyo, Japan). The concentration of IN inhibitor that inhibited the production of strand transfer products by 50% (50% inhibitory concentration [IC_{50}]) compared to the control was determined.

Selection of EVG-resistant HIV-1 variants in vitro. MT-2 cells (2×10^5 cells) were infected with HIV-1_{IIIB} and then cultured in the presence of 0.5 nM (see Fig. 3A) or 0.1 nM (see Fig. 3B) EVG. Cultures were incubated at 37°C until an

TABLE 1. Antiviral activities against laboratory HIV strains[a]

Strain	Mean EC_{50} (nM) ± SD		
	AZT	EVG	L-870,810
$HIV\text{-}1_{IIIB}$	7.1 ± 1.3	0.7 ± 0.3	6.3 ± 0.3
$HIV\text{-}2_{EHO}$	22 ± 9.1	2.8 ± 0.8	11 ± 1.9
$HIV\text{-}2_{ROD}$	19 ± 4.7	1.4 ± 0.7	8.6 ± 0.4

[a] Antiviral activity was determined using the MAGI assay. Data shown are means and standard deviations obtained from at least three independent experiments.

extensive cytopathic effect (CPE) was observed, and the culture supernatant was then harvested for further passage in fresh MT-2 cells. The concentration of EVG was increased when a significant CPE was observed. At the indicated passages (see Fig. 3A and B), proviral DNA was extracted from infected MT-2 cells and then subjected to PCR, followed by direct population-based sequencing. Susceptibility to EVG at the indicated passages was determined using the MAGI assay (see Fig. 3A) or p24 production (see Fig. 3B).

Recombinant HIV-1 clones. An HIV-1 infectious clone, pNL101 (38), kindly provided by K.-T. Jeang (NIH, Bethesda, MD), was used to generate recombinant HIV-1 clones. Wild-type HIV-1 ($HIV\text{-}1_{WT}$) was constructed by replacing the *pol* coding region (nucleotide positions 2006 of the ApaI site to 5122 of the NdeI site of pNL101) with HIV-1 strain BH10. The *pol* coding region contains a silent mutation at nucleotide 4232 (TTTAGA to TCTAGA) resulting in the generation of a unique XbaI site. Recombinant HIV-1 IN infectious clones were generated using a modified pNL101-based vector, $pNLRT_{WT}$. In brief, mutations were introduced into the XbaI-NdeI region (891 bp) of $pSLInt_{WT}$, which encodes nucleotides 4232 to 5122 of pNL101, using an oligonucleotide-based site-directed mutagenesis method (54). Next, the XbaI-NdeI fragments were inserted into pBNΔInt, which encodes nucleotides 5122 (NdeI) to 5785 (SalI) of pNL101. Finally, the XbaI-SalI region (1,554 bp) was inserted into pNL101. Each infectious clone was transfected into 293T cells. The following day, MT-2 cells were added, and the supernatants were harvested when an extensive CPE was observed.

Replication kinetics of resistant HIV-1 variants. MT-2 cells (10^5 cells) were infected with each virus preparation (500 MAGI units) for 4 h. The infected cells were then washed and cultured in the presence or absence of EVG. The culture supernatants were harvested on day 5 after infection, and p24 levels were quantified using a Retro-Tek HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA) (ZeptoMetrix, Buffalo, NY).

Evaluation of antiretroviral activities of IN inhibitors. The MLV-based retroviral vector pRCV/LIG (15) and plasmid pcDNA-VSVG, encoding the vesicular stomatitis virus envelope glycoprotein (a generous gift from H. Miyoshi, RIKEN Bioresource Center, Tsukuba, Japan), were employed to generate viral particles. These plasmids were cotransfected into an MLV-derived Gag-Pol-expressing packaging cell line, GP293 (Clontech, Palo Alto, CA). After 48 h of transfection, culture supernatants were filtered through a 0.45-μm membrane and stored at −80°C until use.

An HIV-1-based luciferase expression vector, pBC-LIG; pCMVΔ8/9, encoding the HIV-1 viral proteins including IN; and pcDNA-VSVG were transfected into 293T cells to generate pseudotyped HIV-1. The viruses were used to infect 293T cells (10^5 cells per well in 12-well plates) at an MOI of 0.02 in the absence or presence of inhibitors. After 48 h of transduction, luciferase activity was determined using a luciferase assay system (Promega, Madison, WI) and an LB 9507 luminometer (Berthold, Bad Wildbad, Germany).

An SIV molecular clone, pMA239 (46), containing the full SIVmac239 genome, was a kind gift from E. Ido, Institute for Virus Research, Kyoto University. pMA239 was used to generate viral stocks as previously described (6). Antiviral activities of IN inhibitors against SIVmac239 were determined using the MAGI assay as described above.

Molecular modeling studies. A three-dimensional model of EVG in complex with HIV-1 IN CCD was prepared by PyMOL software, version 0.97, using previously reported data (44). Amino acid residues involved in resistance to EVG were displayed within this model.

RESULTS

Anti-HIV activities of IN inhibitors. The antiviral activity of EVG against $HIV\text{-}1_{IIIB}$, $HIV\text{-}2_{EHO}$, and $HIV\text{-}2_{ROD}$ was first evaluated by the MAGI assay. EVG showed potent antiviral activity against three laboratory strains of HIV, with EC_{50} values in the subnanomolar to nanomolar range (Table 1). Next, we evaluated the activity of EVG against wild-type clinical isolates representing various subtypes of HIV-1. EVG suppressed the replication of all HIV-1 subtypes tested, with an antiviral EC_{50} ranging from 0.10 to 1.26 nM (Table 2). Moreover, EVG suppressed the replication of HIV-1 clinical isolates carrying NRTI, NNRTI, and PI resistance-associated genotypes, as did a control IN inhibitor, the compound L-870,810 (see Table S1 in the supplemental material). The cytotoxicities of these inhibitors were also determined using an MTT colorimetric assay. Mean values for the concentration that suppresses the viability of target cells by 50% for EVG and L-870,810 in PBMC obtained from three independent donors were 4.6 ± 0.5 μM and 2.7 ± 0.6 μM, respectively. Thus, EVG can suppress various HIV strains, including diverse HIV-1 subtypes and clinical isolates carrying multiple mutations associated with resistance to currently approved antiretroviral drugs.

TABLE 2. Antiviral activities of EVG against various subtypes of HIV-1[a]

Subtype	Isolate	EC_{50} (nM)	
		AZT	EVG
A	RW/92/016	7.91	0.41
B	96USHIPS7	8.41	0.26
	BR/92/021	2.13	0.76
	BR/93/017	1.10	0.18
	BR/93/022	11.7	1.13
C	BR/92/025	2.84	0.10
D	UG/92/046	7.26	0.50
E	CMU02	9.07	1.26
F	BR/93/020	25.3	0.74
G	JV1083	11.1	0.35
O	BCF01	1.52	1.17

[a] Antiviral activity was determined using p24 ELISA.

Mechanism of anti-HIV activity of EVG. First, we performed a "time-of-addition" experiment as described previously (30), with some modifications. MT-4 cells were infected with $HIV\text{-}1_{IIIB}$ at an MOI of 0.5. One hour after infection, infected cells were extensively washed, and compounds were added, including an NNRTI (EFV at 100 nM), a PI (NFV at 500 nM), or EVG (100 nM). Amounts of p24 antigen were determined at 31 h postinfection. The antiviral activity of EFV gradually decreased from 6 h postinfection and disappeared at 12 h postinfection, whereas the antiviral activity of EVG decreased from 10 h postinfection and was no longer detected by 12 h postinfection. On the other hand, the PI NFV effectively blocked the infection up to 12 h postinfection and still exerted approximately 20% inhibitory activity up to 24 h postinfection. These results strongly suggest that EVG inhibits the HIV replication at a step that occurs after reverse transcription but before proteolytic cleavage, consistent with the integration step.

To elucidate the mode of action of EVG on HIV-1 replication, the levels of intracellular HIV-1 DNA species were determined using real-time quantitative PCR (Fig. 2A). MT-2 cells were infected with $HIV\text{-}1_{IIIB}$ in the presence or absence

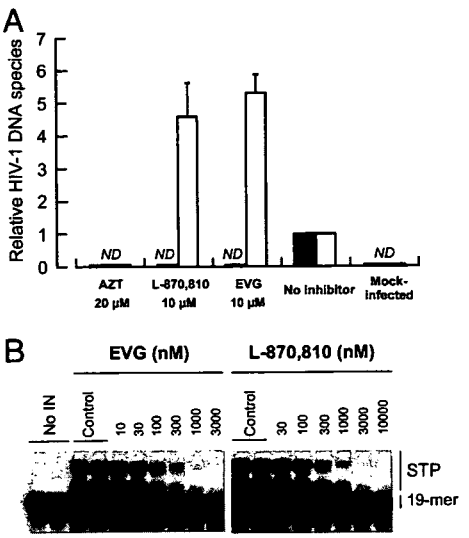


FIG. 2. Mechanism of action of EVG. (A) Quantification of HIV-1 DNA species. MT-2 cells were infected with HIV-1_{IIIB} in the presence or absence of AZT, L-870,810, and EVG. Unintegrated (2-LTR) (white bars) and integrated (black bars) forms of proviral DNA were quantified by real-time PCR and normalized to the β-globin gene after 24 h of infection. The data are represented as means and standard deviations of value relative to that of the no-inhibitor control from three independent experiments. ND means that the signals were not detected even after 40 cycles of amplification. (B) Inhibitory effect of IN inhibitors on strand transfer activity. Gel electrophoresis shows strand transfer products (STP) generated from preprocessed donor DNA substrate (19-mer) covalently bound to acceptor DNA.

of a CD4-gp120 binding inhibitor, DS5000; an NRTI, AZT; an IN inhibitor, L-870,810; and EVG. Unintegrated (2-LTR) and integrated forms of reverse-transcribed HIV-1 genomic DNA were quantified after 24 h of infection and then normalized with β-globin DNA. In the presence of 20 μM AZT, neither 2-LTR nor integrated forms were detected as expected. Similar results were also observed with 20 μM DS5000 (data not shown). In the presence of 10 μM L-870,810, integrated provirus was undetectable, while relative 2-LTR levels increased about 5-fold (4.6-fold ± 1.0-fold). Similar results were observed with 10 μM EVG (2-LTR) (5.3-fold ± 0.5-fold), indicating that EVG exerts anti-HIV activity by blocking the integration step.

To further characterize the mechanism by which EVG inhibits the integration step, the effect of EVG on strand transfer was assessed by characterizing its ability to inhibit the activity of recombinant wild-type HIV-1 IN enzyme in an oligonucleotide-based strand transfer assay (Fig. 2B). EVG and L-870,810 both inhibited the synthesis of strand transfer products with IC_{50} values of 54 nM and 118 nM, respectively. Taken together, these results indicate that like L-870,810, EVG blocks integration via the inhibition of IN-mediated strand transfer.

Selection of EVG-resistant HIV-1 variants in vitro. To determine the in vitro resistance profile of EVG, EVG-resistant viral variants were selected using a dose escalation method, and the susceptibilities of the resulting selected variants to EVG (EC_{50}) were determined. Selection of resistant HIV-1_{IIIB} was initiated with 0.5 nM EVG (Fig. 3A). At passage 12 (P-12), where the concentration of EVG was 4 nM, 2 amino acid substitutions, glutamine-to-proline at IN codon 146 (Q146P) and asparagine-to-aspartic acid at IN codon 232 (N232D), were observed (Fig. 3A). An N232D substitution was previously reported to be an IN polymorphism in HIV-1 (34). The EVG EC_{50} of a P-24 variant containing a Q146P- and N232D-substituted variant was 6.2 nM. At P-32 (32 nM EVG), a T66I IN substitution was newly observed, whereas the N232D substitution had reverted to the baseline sequence. The EVG EC_{50} against a P-36 variant was 64 nM. An S147G IN substitution was detected at P-48 (128 nM EVG), and the EVG EC_{50} further increased to 635 nM. In addition, a Q95Q/K IN substitution (mixture of Q and K) and an E138E/K IN substitution were newly identified at P-54 (256 nM EVG). These mixtures, Q95Q/K and E138E/K, fully emerged in the viral pools by P-64 and P-80, respectively. The EVG EC_{50} at P-68 (1,024 nM EVG) was greater than 1,000 nM.

An independent EVG selection experiment, again using HIV-1_{IIIB}, was performed but began at 0.1 nM EVG (Fig. 3B). An E92E/Q mixture in the IN coding region was first detected at P-30 (10 nM EVG) and was predominantly E92Q by P-38 (20 nM EVG). Additional IN substitutions, H51H/Y and S147S/G, emerged at P-60 (640 nM EVG), and an E157E/Q mixture emerged at P-70 (1,280 nM EVG); the viral pools at the terminal passage P-80 (1,280 nM EVG) had the IN sequence H51Y/E92Q/S147G/E157E/Q (Fig. 3B). The emergence of each of these mutations correlated with an increase in the EVG EC_{50} of the resulting viral pools (Fig. 3). Other than the N232D polymorphism, all of these mutations are located in the CCD of IN.

Phenotypic analysis of IN recombinant viruses. (i) EVG-selected mutations. To characterize which mutations are responsible for EVG resistance, infectious HIV-1 clones containing single IN substitutions (H51Y, T66I, E92Q, Q95K, E138K, Q146P, S147G, or E157Q) that were observed to emerge under selection with EVG were generated (Fig. 3 and Table 3). Mutations were classified into two groups based on the level of resistance: mutations that conferred more than 10-fold reduced susceptibility compared to the wild type were defined as primary mutations, and mutations conferring less than 10-fold reduced susceptibility were defined as secondary mutations. T66I and E92Q substitutions conferred significantly reduced susceptibility to EVG (37- and 36-fold reduced, respectively, relative to the wild type), whereas the Q146P and S147G substitutions conferred more moderate reductions in EVG susceptibility (11-fold reduced), indicating that these four IN mutations are primary mutations involved in resistance to EVG. In contrast, H51Y, Q95K, and E157Q substitutions all conferred smaller reductions in EVG susceptibility (each less than 6.3-fold reduced compared to the wild type), suggesting that these substitutions are secondary resistance mutations. Interestingly, the E138K mutation alone conferred no reduction in susceptibility to either EVG or L-870,810. Thus, several distinct mechanisms of resistance may be represented by these different IN mutations.

Multisubstituted clones observed during EVG selection experiments were also generated. HIV-$1_{T66I/Q146P}$ showed high-level resistance to EVG (119-fold reduced susceptibility) (Table 3). Combinations of S147G with T66I/Q146P or E92Q further enhanced resistance, 412- and 356-fold, respectively.

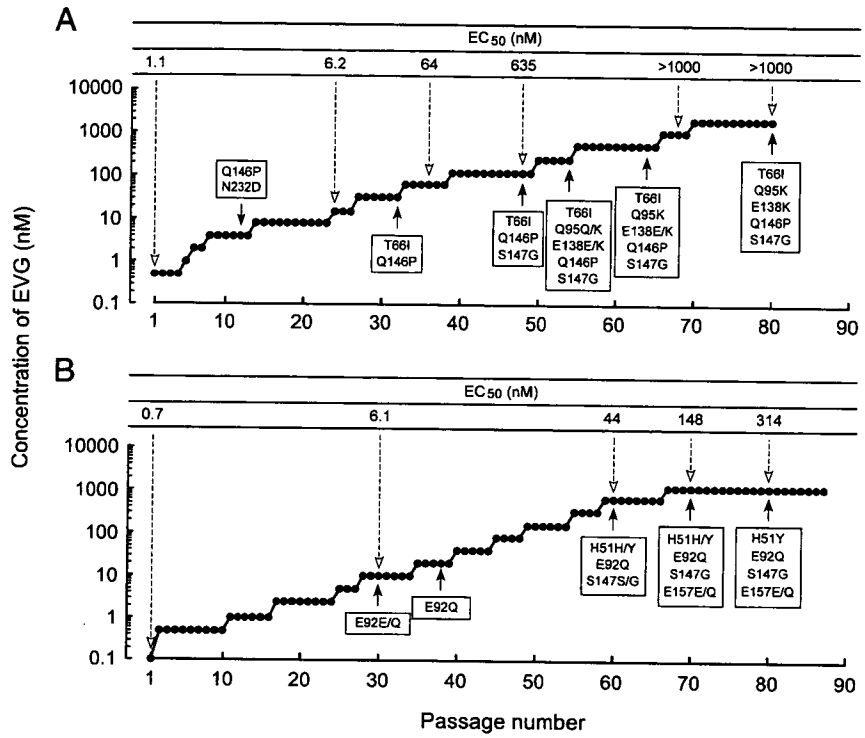


FIG. 3. Induction of EVG-resistant HIV-1. Data from MT-2 cells are shown. The initial concentrations of EVG were 0.5 nM (A) and 0.1 nM (B). Results are from two identical but independent experiments. At the indicated passage number (black arrowheads), proviral DNA extracted from infected MT-2 cells was sequenced. Amino acid substitutions are shown. The EC_{50} values of HIV-1 variants selected by EVG at the indicated passage number (white arrowheads) were determined using MAGI assay (A) or the production of p24 in MT-2 cells (B).

The triple mutant HIV-$1_{H51Y/E92Q/S147G}$ showed high-level resistance to EVG (700-fold reduced susceptibility). Interestingly, the addition of the secondary mutation H51Y, which on its own reduced EVG susceptibility only 3.6-fold, substantially enhanced resistance relative to that observed for the double mutant HIV-$1_{E92Q/S147G}$. HIV-$1_{T66I/Q95K/Q146P/S147G}$, HIV-$1_{T66I/Q95K/E138K/Q146P/S147G}$, and HIV-$1_{H51Y/E92Q/S147G/E157Q}$ mutants all showed high-level resistance to EVG, with EC_{50} values greater than 1,000 nM in all cases. These results indicate that the T66I and E92Q mutations provided the highest change (*n*-fold) in EVG susceptibility as individual resistance mutations and that the additional substitutions identified further enhance the level of resistance to EVG when combined with these primary mutations.

(ii) L-870,810-selected mutations. Infectious HIV-1 clones containing mutations (V72I, F121Y, T125K, and V151I) previously shown to be associated with resistance to L-870,810 (23) and two mutations, L74M and G163R, observed in our selection using L-870,810 (data not shown) were generated. Among these variants, HIV-1_{F121Y} and HIV-1_{V151I} demonstrated reduced susceptibility to both L-870,810 and EVG (Table 3). V151I has been observed in some HIV-1 clinical isolates and may be an IN polymorphism (34). Moreover, the effect of V151I on susceptibility to L-870,810 has been controversial (23, 29). This discrepancy might arise from the viral strain or plasmid backbone used, so further experiments to clarify the effect of V151I on IN inhibitor susceptibility are needed. HIV-$1_{F121Y/T125K}$ showed significant resistance to both L-870,810 and EVG (68-fold and 177-fold reduced susceptibility, respectively). HIV-$1_{V72I/F121Y/T125K/V151I}$ showed high-level resistance to both IN inhibitors (EC_{50} greater than 1,000 nM).

(iii) DKA IN inhibitor-selected mutations. Highlighting the potential for related mechanisms of IN inhibitor resistance and cross-resistance, the T66I mutation has also been observed to be selected by DKA IN inhibitors such as L-708,906 and S-1360. Additional mutations, L74M and S153Y, in combination with T66I were also observed to be selected by these DKA IN inhibitors (16, 17). L74M also emerged during L-870,810 selection in our studies (data not shown) but conferred no change in susceptibility to L-870,810 when present alone and only low-level resistance (3.0-fold) to EVG (Table 3). The combination of T66I and L74M conferred slightly higher resistance to EVG (45-fold) than did T66I alone but only moderate resistance to L-870,810 (7.1-fold). Another IN mutant, HIV-$1_{T66I/S153Y}$, observed in L-708,906 selection experiments (24) showed high-level resistance to EVG (260-fold) but low-level resistance to L-870,810 (5.0-fold). These results suggest that the mechanism of EVG resistance may have some similarities to that of DKA IN inhibitors.

Taken together, these results suggest that a variety of IN mutations may be selected by EVG and other IN inhibitors. Most of the IN inhibitor resistance mutations are observed to cluster in the CCD of IN. The resulting mutations and their combinations have the capacity to confer various levels of resistance and potential cross-resistance to EVG and other IN inhibitors. Given their location in the CCD, many of these mutations may act via a common mechanism. The observed development of IN inhibitor resistance mutations resembles