the C-terminal half of RT: the connection subdomain and RNase H domains, certain mutations in this region have been recently found to be associated with resistance to NRTIs and NNRTIs [7–20]. Despite the accumulation of data on the prevalence and mechanisms of mutations in the C-terminal half of RT, most data are from subtype B viruses and little information is available on those of non-B subtype. Since amino acid sequence diversity in the *pol* gene is 10–15% among subtypes [21–23] and the subtype have an impact on drug resistance mutations [19–31], there is a need to determine whether inter-subtype diversity influences the spectrum of drug resistance mutations in the C-terminal half of RT as well.

Circulating recombinant form (CRF) 01_AE is the most predominant subtype in Viet Nam [32–34] and accounting for 83% of all HIV-1 infections in Southeast Asia [28–34]. Recently, Delviks-Frankenberry demonstrated that the substitution A400T, a common polymorphism in RNase H of CRF01_AE, is responsible for the high AZT resistance [16], although A400T usually emerged after AZT exposure in subtype B [19]. As well as A400T, we found a high frequency of G335D and A371V in treatment-naïve CRF01_AE patients [20]. Although G335D and A371V are assumed as common polymorphisms in CRF01_AE in the Stanford HIV Drug Resistance Database [http://hivdb.stanford.edu/index.html, accessed as late as July 20th 2010], they are thought to be associated with AZT resistance in subtype B [7,11]. However, the role of substitutions G335D and A371V in drug resistance to NRTIs has not been well characterized.

In the present study, we first investigated drug resistance mutations of CRF01_AE HIV-1 including the connection subdomain and RNase H domain of RT from HIV-1-infected patients failing ART. In addition, since we again found high frequencies of the double mutation of G335D and A371V in this population, we examined phenotypic resistance levels of these mutations by using mutant recombinant viruses containing G335D, A371V or both with or without TAMs, to determine the impacts of these mutations on drug resistance.

2. Materials and methods

2.1. Study population

HIV-1-infected patients who had taken antiretroviral therapy for more than 6 months at the National Hospital for Tropical Diseases (NHTD) in Hanoi between October 1, 2007 and June 30, 2008, were enrolled in this study. Each participant provided a written informed consent. Plasma viral load (pVL) was measured by the Cobas AmpliPrep-Cobas TaqMan system (Roche Diagnostics, Tokyo, Japan) and plasma samples were stored at −80 °C for genotypic resistance testing. When pVL was >1000 copies/ml, the patient was defined as treatment failure and the frozen plasma was shipped to the National Center for Global Health and Medicine (NCGM) in Tokyo for genotypic resistance testing.

The study protocol was approved by the institutional ethical review boards of NTHD and NCGM (IMCJ-H18-360) and by the ethics committee of the Vietnamese Ministry of Health (#1468,1469/QD-BYT dated April 19, 2007).

2.2. Reagents and cells

AZT, stavudine (d4T) and didanosine (ddI) were purchased from Sigma (St. Louis, MO). Lamivudine (3 TC) and tenofovir (TDF) were purchased from Moravek Biochemicals, Inc. (Brea, CA). Abacavir (ABC) was generously provided by GlaxoSmithKline (Philadelphia, PA). Cos-7 and MAGIC-5 cells (CCR5-transduced HeLa-CD4/LTR-β-Gal cells) were cultured and used as described previously [35].

2.3. Genotypic resistance and subtype analysis

Drug resistance genotyping was carried out by in-house protocols in NCGM. In brief, total RNA was extracted from plasma with a High Pure Viral RNA kit (Boehringer Mannheim, Mannheim, Germany), followed by reverse transcription-polymerase chain reaction (PCR) with a One Step RNA PCR kit (TaKaRa Shuzo, Otsu, Japan). Nested PCR was subsequently conducted with a Prime STAR Max Premix kit (TaKaRa Shuzo, Otsu, Japan) to amplify nearly the entire RT region (codons 1–560) and protease region. The primer sets for amplification of the N-terminal half of RT (codons 1–318) were T1-AE [5′-AGGGGGAATTGGAGGTTT; nucleotides (nt) 2393–2410] and T4-AE (5′-TTCTGTTAGTGCTTTGGTT; nt 3422–3404) for the first PCR, and T12-AE (5′-CCAGTAAAATTAAAGC-CAG; nt 2574–2592) and T15-AE (5′-TCCCAC-TAACTTCTGTATGTC; nt 3335–3315) for the second PCR. The primer sets for amplification of the C-terminal half of RT (codons 319–560) were 3120F-AE (5′-TCTGATTTAGAAA-TAGGGCAG; nt 3120–3140) and 4428R-AE (5′-GTGTGC AATCTAATTGCCATAT; nt 4428–4407) for the first PCR, and 3240F-AE (5′-GGATATGAACTCCATCCTGA; nt 3240–3259) and 4316R-AE (5′-GTGGCAAATTAAAATCACTAGCC; nt 4316–4295) for the second PCR. Primer sets for amplification of protease were PR01-AE (5′-CCAACAGCCCCACCAGC; nt 2152–2168) and PR02AE (5′-ATTTTCAGGCCCAATT TTTGA; nt 2711–2691) for the first PCR, and PR03-AE (5′-AGCAGGAGCAGAAAGACAAGG; nt 2213 to) and PR04-AE (5′-CTGGCTTTAATKTTACTGGTA; nt 2592–2572) for the second PCR. The PCR products were purified with QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and subjected to direct sequencing with an ABI PRISM 3730 automated DNA sequencer (Applied Biosystems, Foster City, CA). Amino acid sequences were deduced with the Genetyx-Win program version 8.0 (Software Development, Tokyo).

Resistance-associated mutations in the N-terminal half of RT were identified according to the International AIDS Society Resistance-USA Panel revised in December 2009 [36] and subtypes of HIV-1 in RT gene were determined by software "Genotyping/NCBI" using BLAST algorithm [http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi]. Resistant mutations in the connection subdomain and the RNase H domain of RT in the previous reports were determined if greater than three-fold increase of EC$_{50}$ compared to that of NL4-3 was noted in the reports. Since all sequences of the study participants belonged to CRF01_AE subtype, data on frequencies of each mutation in the C-terminal half of RT in CRF01_AE and subtype B in treatment-naïve patients was obtained from the Stanford HIV Drug Resistance Database [http://hicdb.stanford.edu/index.html, accessed as late as July 20th, 2010] for reference. Nucleotide sequences of the C-terminal half of CRF01_AE RT from 38 patients have been deposited in the DDBJ database (accession numbers AB545813–AB545850).

2.4. Construction of recombinant HIV-1 harboring G335D and/or A371V with or without TAMs

To examine the influence of G335D and A371V on drug susceptibility to NRTIs, we constructed mutant HIV-1 recombinants that included G335D, A371V or both with or without TAMs. TAM-1 virus was constructed as combination of M41L, L210W and T215Y and TAM-2 as combination of D67N, K70R and T215F. Mutant recombinant plasmid clones of the virus were generated by oligonucleotide site-directed mutagenesis as described previously [10], using pBS-RT_{WT}, which contains the entire RT coding sequences (amino acid position 14–560) and three silent restriction sites (XmaI, NheI and XbaI from the 5′ to 3′ end of RT at codons 15, 267, and 560). After site-directed mutagenesis, the mutated RT was ligated into pNL4-3, which contains the entire genome of HIV-1 and the same silent restriction sites as pBS-RT_{WT}. The infectious virus was generated by transfection of each molecular clone into Cos-7 cells, harvested and stored at −80 °C until use. Infectivity was measured as blue cell-forming units (BFU) of MAGIC-5 cells. All mutations in recombinant viruses were confirmed by full-length sequencing of the entire RT coding region.

2.5. Drug susceptibility assay

Susceptibility to NRTIs was determined by using MAGIC-5 cells as described previously [35] in more than three experiments. MAGIC-5 cells were infected with diluted virus stock (100 BFU) in the presence of increasing concentrations of RTIs, cultured for 48 h, fixed and stained with X-Gal (5-bromo-4chloro-3-indolyl-βD-galactopyranoside). The stained cells were counted under a light microscope. Drug concentrations that reduced the cell count to 50% of that of the drug-free control (EC_{50}) were determined by referring to the dose–response curve.

2.6. Statistical analysis

Data are expressed as mean ± SD. The Student's t-test was used to compare two groups of continuous variables and a *p*-value less than 0.05 was considered statistically significant. Statistical analyses were performed using SPSSII software package for Windows, version 11.0J (SPSS Japan Inc, Tokyo, Japan).

3. Results

3.1. Characteristics of patients failing antiretroviral therapy

A total of 416 individuals on ART were consecutively enrolled in the present study and their pVLs were assayed between October 1, 2007 and June 30, 2008 at the NTHD in Hanoi. Among them, 49 individuals were confirmed as treatment failure by the definition described above and assigned for genotypic resistance analysis. The characteristics of the 49 individuals are listed in Table 1. All patients had received AZT or d4T plus 3TC combined with NVP, EFV or lopinavir/ritonavir (LPV/r) at the time of enrollment. The most frequently used combination was AZT, 3TC, and NVP, followed by d4T, 3TC and NVP. Protease inhibitors (PIs) were used by 17 (34.7%) patients, while the Vietnamese national ART guideline recommends d4T, 3TC plus 1 NNRTI for the first line regimen [2]. This was probably due to the inclusion of patients who had started ART when the guideline had not been issued yet. The median duration of ART exposure was 2.98 years (IQR 2.17–4.00).

3.2. Genotypic resistance patterns including C-terminal domain of RT

We successfully amplified the N-terminal half of RT and protease of all the 49 patients and C-terminal region of RT of 38 patients. The proportion of patients with at least one NRTI resistance mutation in the N-terminal half of RT was 89.8%.

Table 1
Characteristics of patients failing antiretroviral therapy.

		n = 49	(%)
Sex, *n* (%)			
males		33	(67.3)
females		16	(32.7)
Median Age, years (range)		31	(21–50)
Risk of HIV-1 infection (multiple choice), *n* (%)			
sexual contact		46	(93.9)
intravascular drug use		20	(40.8)
CD4 count, median cells/mm^3 (IQR)		145	(84–195)
Plasma viral load, median log copies/ml (IQR),		4.23	(3.59–4.94)
Duration of prior ART, median years (IQR)		2.98	(2.17–4.00)
Experienced ART, *n* (%)			
NRTI	AZT	39	(79.6)
	d4T	24	(49.0)
	3TC	49	(100)
	ddI	7	(14.3)
	ABC	2	(4.1)
	TDF	2	(4.1)
NNRTI	NVP	43	(87.8)
	EFV	15	(30.6)
PI	IDV	12	(24.5)
	SQV	6	(12.2)
	LPVr	3	(6.1)

IQR: interquartile range. ART: antiretroviral therapy. NRTI: nucleoside reverse transcriptase inhibitor. NNRTI: non-nucleoside reverse transcriptase inhibitor. PI: protease inhibitor.

Of those, M184V was the most common (81.6%) and TAMs were also observed frequently in 71.4%: M41L (22.4%), D67N (24.5%), K70R (18.4%), L210W (14.3%), T215F (16.3%), T215Y (28.6%), K219E (12.2%) and K219Q (6.1%), whereas K65R (6.1%), L74V (4.1%), Y115F (2.0%) and mutations driven by Q151M complex (4.1%) were relatively rare. Similar to previous reports on drug resistance in CRF01_AE [28–30], mutations classified into TAM type 2 (TAM-2): D67N, K70R, T215F and K219E/Q, were more frequently observed than those of TAM type 1 (TAM-1): M41L, L210W and T215Y/F (30.6% v.s. 26.5%), except for a patient having only T215F. With regard to codon 215, T215F were more frequently seen with other TAM-2 mutations (six out of eight sequences that contain T215F), concurring with the previous reports showing the introduction of T215F into TAM-2 backbone increase relative fitness in the presence of AZT but resulted in decreased viral fitness in TAM-1 backbone [37]. The resistance mutations of NNRTIs in the N-terminal half of RT were detected in 79.6%. The most frequent NNRTI-resistance mutations were Y181C/I/V (32.7%), K103N (26.5%) and G190A (26.5%). In 17 PI experienced patients, no major mutations were found, but 9 minor mutations were detected: L10I/V (11.8%), I13V (88.2%), G16E (11.8%), K20R (17.6%), M36I (100%), L63P (29.4%), H69K (100%), V82I (11.8%) and I93L (8.2%). However, the mutations in protease are considered as consensus amino acids in most non-B subtype HIV-1 (I13V, M36I and H69K) or common polymorphic mutations (L10V, G16E, K20R, L63P, V82I and I93L) and could not be determined as mutations that emerged after treatment.

The frequencies of mutations in the C-terminal half of the RT reported previously as NRTI or NNRTI resistance [7–20] are described in Table 2. As shown, G335D (100%), N348I (36.8%), A371V (100%), A376S (5.3%), E399D (28.9%) and A400T (97.4%) were detected in the patients failing ART. However, as we reported previously [20], G335D and A371V were also commonly observed in untreated patients infected with non-B subtype HIV-1 and the frequencies of G335D and A371V in CRF01_AE subtype shown in the Stanford HIV Drug Resistance Database are 95.2% and 97.1%, respectively, while those are rare in subtype B (G335D: 1.3%, A371V: 3.2%). A400T is also one of the known polymorphisms in CRF01_AE [16]. Therefore, it is unlikely that G335D, A371V and A400T in this population were selected by ART exposure or involved in the resistance mutations.

Table 2
Frequencies of mutations associated with RTI-resistance in the connection and RNase H domain of reverse transcriptase of HIV-1.

Mutations[b]	Study participants (Treatment failure)		Stanford database[a] (RTI-naïve)	
	CRF01_AE		CRF01_AE	Subtype B
	n = 38			
	%	(n)	%	%
G333	100	(38)		
D	0	(0)	0	0.7
E	0	(0)	0	7.5
G335	0	(0)		
C	0	(0)	0	0.5
D	100	(38)	92.0	1.3
N348	57.9	(22)		
I	36.8	(14)	0	0.5
T	5.3	(2)	0	0
A360	97.4	(37)		
I	0	(0)	0	0
V	0	(0)	0	0.7
S	2.6	(1)	1.1	0
V365	100	(38)		
I	0	(0)	0	3.2
T369	94.7	(36)		
I	0	(0)	0	0
A	2.6	(1)	19.3	3.3
V	2.6	(1)	2.8	1.2
A371	0	(0)		
V	100	(38)	97.1	3.2
A376	94.7	(36)		
S	5.3	(2)	1.7	5.8
E399	*68.4*	*(26)*		
D	*28.9*	*(11)*	*2.6*	*14*
K	*2.6*	*(1)*	*0*	*0.1*
A400	0	(0)		
T	97.4	(37)	89.2	25.3
L	2.6	(1)	0	1
Q475	100	(38)		
A	0	(0)	0	0
Q509	97.4	(37)		
L	0	(0)	0	0
R	2.6	(1)	0	0

[a] Available from http://hicdb.stanford.edu/index.html.

[b] Resistance mutations reported previously [8–21] are indicated in bold. Resistance was defined as greater than three fold increase of EC_{50} compared to that of NL4-3.

3.3. Drug susceptibility assay for mutant recombinant HIV-1

To address whether G335D or A371V have an impact on NRTI susceptibility depending on the pattern of TAMs, we constructed recombinant viruses containing G335D and/or A371V in the background of TAM-1 or TAM-2 by site-directed mutagenesis. As shown in Table 3, G335D, A371V or their double mutant did not increase the resistance levels to all NRTIs by themselves. In contrast, as shown in Table 4, variants with G335D, A371V or both exhibited higher resistance to AZT in the background of TAM-1 (8.2- to 23.2-fold) and the increased resistance level was the greatest in the double mutant G335D/A371V. Although G335D/A371V showed statistical increase in resistance to all the other NRTIs except 3TC, the fold increase from TAM-1 mutant was the greatest in AZT (Table 4). Similar to TAM-1 background, G335D, A371V or G335D/A371V with TAM-2 exhibited considerable increase in susceptibility to AZT (52.7-, 21.1-, 52.6-fold, respectively). In addition, there were marginal changes in d4T susceptibility (Table 5) in the three patterns of the mutants, G335D, A371V or G335D/A371V. In TAM-2 background, we also found G335D alone increased susceptibility to ABC (4.2-fold) and to TDF (2.4-fold), and that G335D/A371V increased susceptibility to ddI (7.2-fold), ABC (3.1-fold) and

Table 3
Drug susceptibilities of HIV-1 variants with G335D or A371V.

Mutation[a]	EC_{50} (μM)[b] (fold increase)											
	AZT		d4T		ddI		3TC		ABC		TDF	
Wild Type	0.050 ± 0.002		2.55 ± 0.07		1.90 ± 0.17		0.45 ± 0.035		2.48 ± 0.21		0.020 ± 0.0023	
335D	0.052 ± 0.004	(1)	3.19 ± 0.14	(1.3)	4.56 ± 0.20	(2.4)	0.45 ± 0.022	(1)	2.71 ± 0.17	(1.1)	0.018 ± 0.0019	(0.9)
371V	0.047 ± 0.003	(0.9)	3.26 ± 0.17	(1.3)	5.30 ± 0.02	(2.8)	0.55 ± 0.027	(1.2)	2.32 ± 0.09	(0.9)	0.027 ± 0.0014	(1.3)
335D/371V	0.052 ± 0.010	(1)	3.52 ± 0.06	(1.4)	3.38 ± 0.21	(1.8)	0.65 ± 0.023	(1.5)	2.39 ± 0.12	(1)	0.025 ± 0.0031	(1.2)

AZT, zidovudine; d4T, stavudine; ddI, didanosine; 3TC, lamivudine; ABC, abacavir; TDF, tenofovir.

[b] Data are mean ± SD from at least three independent experiments. Fold increase was the relative change in EC_{50} value compared with that of HIV-1 WT.

[a] See Materials and Methods for the construction of clones.

TDF (5.2-fold). Of note, the increased resistance levels to AZT, d4T, ddI and TDF were greater in G335D/A371V in TAM-2 background than that in TAM-1 background. Our data suggest double mutant G335D/A371V in TAM-2 background would have the most impact on NRTI susceptibility.

4. Discussion

In the present study, we described the drug resistance mutations in the entire RT of CRF01_AE HIV-1-infected Vietnamese patients who had high pVL levels despite 6-month ART. According to the criteria used for evaluation of drug resistance proposed by Shafer et al. [38,39], correlations between mutations and treatment should be confirmed by extensive resistance surveillance. However, limited sequences of CRF01_AE in the connection subdomain and RNase H domain of the RT have been available so far especially from treatment-experienced patients [40]. Santos et al. [19] previously compared amino acid variations between treatment-naïve and treatment-experienced patients in connection subdomain (280 naïve vs. 230 treated) and RNase H domain (334 naïve vs. 234 treated). Although their study included substantial number of patients, larger number of cases belonged to subtype B (80–82% of treatment-experienced patients) and the unique characteristics of CRF01_AE, accounting for only 10% of their study, could not be fully assessed. Since our present study focused on CRF01_AE sequence alone, the data provide direct information on the evaluation of drug resistance mutations in CRF01_AE, although sequences before ART initiation were not available. The largest study to date exploring treatment-related mutation in RT C-terminal site in CRF01_AE infection is the report from Thailand by Saeng-aroon et al. [40], in which significantly higher frequencies of N348I, E399D, P537S and I542M in treatment-exposed patients than treatment-naïve patients (76 naïve vs. 49 treated) was noted. Although the former two mutations have already known to be associated with exposure to NRTI or NNRTI and were detected in our treatment-experienced patients, the results of P537S and I542M were different from us: no patients in our study had P537S and I542M. Further studies are required to determine the prevalence of drug resistance mutations in the C-terminal half of RT in CRF01_AE.

Among the mutations previously reported as drug resistance in the connection subdomain and RNase H domain of RT, we found no mutations except G335D, N348I, A371V, A376S, E399D and A400T in treatment-experienced individuals with CRF01_AE infection. Of these mutations, N348I is one of the most extensively assessed mutations in the RT connection domain and has been established as multiclass resistance to both NRTIs and NNRTIs by being identified in clinical isolates in treatment-experienced individuals in subtype B and by *in vitro* drug susceptibility assay [9,10,12,13]. Since N348I is rare in treatment-naïve of both subtype B and CRF01_AE, N348I observed in 35.8% of CRF01_AE sequences in our study was considered to be treatment-related. The wide use of NVP in Viet Nam might be one of the causes of the higher prevalence of N348I in this population than in subtype B. In addition to N348I, E399D has been thought to be associated with resistance to AZT and to EFV when combined with K103R and 179D [41,42]. Although our results of E399D prevalence of in treatment-exposed patients (28.9%) was relatively higher than those in the Stanford database (9%), it was similar to the previous study by Saeng-aroon et al. of treatment-exposed patients with CRF01_AE infection (32.7%) and considered to be selected after treatment. In contrast, A376S detected in this study was not clearly identified as a treatment-related mutation because the frequency (5.3%) was similar to those of treatment-naïve

Table 4
Drug susceptibilities of HIV-1 variants with G335D or A371V in the TAM-1 background.

Mutation	EC_{50} (μM) (fold change)											
	AZT		d4T		ddI		3TC		ABC		TDF	
Wild Type	0.050 ± 0.002		2.55 ± 0.07		1.90 ± 0.17		0.45 ± 0.035		2.48 ± 0.21		0.020 ± 0.0023	
TAM-1	0.200 ± 0.016	**(4)**	4.78 ± 0.30	(1.9)	5.35 ± 0.79	(2.8)	2.37 ± 0.017	**(5.3)**	4.20 ± 0.25	(1.7)	0.043 ± 0.0030	(2.2)
TAM-1/335D	0.411 ± 0.028	**(8.2)**[a]	6.63 ± 0.05	(2.6)	5.71 ± 0.57	(3.0)	2.14 ± 0.099	**(4.8)**	3.17 ± 0.23	(1.3)	0.024 ± 0.0026	(1.2)
TAM-1/371V	0.473 ± 0.052	**(9.4)**[a]	6.07 ± 0.12	(2.4)	6.30 ± 0.48	**(3.3)**	2.45 ± 0.110	**(5.5)**	3.88 ± 0.32	(1.6)	0.046 ± 0.0018	(2.3)
TAM-1/335D/371V	1.160 ± 0.078	**(23.2)**[a]	9.01 ± 0.20	**(3.5)**[a]	7.87 ± 0.35	**(4.1)**[a]	2.40 ± 0.016	**(5.4)**	7.57 ± 0.57	**(3.1)**[a]	0.056 ± 0.0004	(2.8)

Boldface indicates an increase greater than threefold.

[a] Increases in fold change were significant compared to TAM-1 without G335D or A371V.

Table 5
Drug susceptibilities of HIV-1 variants with G335D or A371V in the TAM-2 background.

Mutation	EC_{50} (μM) (fold increase)											
	AZT		d4T		ddI		3TC		ABC		TDF	
Wild Type	0.050 ± 0.002		2.55 ± 0.07		1.90 ± 0.17		0.45 ± 0.035		2.48 ± 0.21		0.020 ± 0.0023	
TAM-2	0.3960 ± 0.076	**(7.9)**	6.18 ± 0.11	(2.4)	6.71 ± 0.57	**(3.5)**	2.57 ± 0.089	**(5.7)**	2.97 ± 0.29	(1.2)	0.033 ± 0.0026	(1.7)
TAM-2/335D	2.6390 ± 0.396	**(52.7)**[a]	7.97 ± 0.47	**(3.1)**[a]	5.74 ± 0.63	**(3)**	2.37 ± 0.082	**(5.3)**	10.43 ± 0.41	**(4.2)**[a]	0.049 ± 0.0014	(2.4)[a]
TAM-2/371V	1.0600 ± 0.131	**(21.1)**[a]	8.29 ± 0.23	**(3.3)**[a]	6.00 ± 0.64	**(3.2)**	2.58 ± 0.072	**(5.8)**	3.43 ± 0.21	(1.4)	0.036 ± 0.0012	(1.8)
TAM-2/335D/371V	2.6340 ± 0.132	**(52.6)**[a]	13.71 ± 0.76	**(5.4)**[a]	13.76 ± 0.51	**(7.2)**[a]	2.45 ± 0.062	**(5.5)**	7.57 ± 0.21	**(3.1)**[a]	0.105 ± 0.0030	**(5.2)**[a]

Boldface indicates an increase greater than threefold.

[a] Increases in fold change were significant compared to TAM-2 without G335D or A371V.

subtype B (5.8%) and CRF01_AE (1.7%) infected individuals in the Stanford database. On the other hand, G335D, A371V and A400T were found in almost all the patients in our study. Although these three mutations are thought to be related to NRTI resistance in subtype B [7,11,16], they are common polymorphisms of wild-type CRF01_AE HIV-1 with prevalence of more than 90% in our previous study [20] and in the Stanford database. Therefore, we conclude that G335D, A371V and A400T detected in the present study were not selected after treatment but had existed before the introduction of treatment. Consequently, N348I was the only drug resistance mutation in the C-terminal half of RT observed in our cohort of treatment-experienced Vietnamese infected with CRF01_AE HIV-1.

Our results demonstrated that common CRF01_AE polymorphisms G335D and A371V play considerable role in drug resistance to NRTIs. Recent studies suggested that each of G335D or A371V is associated with drug resistance; G335D emerged after AZT exposure exhibits greater AZT resistance (8 to 53-fold over WT) when combined with TAM [11] and A371V selected in the background of D67N and K70R by high concentrations of AZT *in vitro* shows strong resistance to AZT in the presence of TAMs [7]. In agreement with those reports, our results showed that mutant containing G335D or A371V did not increase the resistance levels to NRTIs by themselves but they conferred higher resistance when combined with TAMs, especially to AZT (8.2–52.7 fold increase). Furthermore, we found that the dual mutation G335D/A371V had the greater impact than each single mutation on resistance in the presence of TAM. As G335D and A371V always appear together in treatment-naïve CRF01_AE, this finding is more critical for CRF01_AE HIV-1 infection than for subtype B infection. In addition, the fold change increased by G335D and A371V was greater with TAM-2 than that with TAM-1. Since TAM-2 is more frequent in CRF01_AE than in subtype B [28–30], this data is important for CRF01_AE HIV-1. Although the impact of G335D and A371V was the greatest in AZT resistance and seemed to be minor in other NRTIs' resistance, the fold-increase in TDF of G335D/A371V plus TAM-2 variant were above the clinical cut-off values [43], which can cause treatment failure. As TDF is often used in second line ART [2], this data is crucial for decisions on the next therapeutic strategies for CRF01_AE HIV-1-infected patients failing first line ART. Since our recombinant viruses were created with pBS-RT_{WT}, which was derived from subtype B RT but not from CRF01_AE RT, our results cannot be applied directly to CRF01_AE infection. CRF01_AE/B recombinants have been emerged and highly prevalent in Southeast Asian countries [32,44,45] and the breakpoint analysis showed some CRF01_AE/B recombinants consisted of subtype B N-terminal site and CRF01_AE C-terminal sites [45]. Therefore, our data suggests the potential influence of those CRF01_AE/B recombinants as well as CRF01_AE strain on the selection of second line therapy in Southeast Asia.

In summary, we reported the frequencies of drug resistance mutations in the connection subdomain and RNase H domain of RT in CRF01_AE HIV-1-infected Vietnamese who experienced ART. Then we demonstrated that the combination of G335D and A371V, a common pattern of polymorphisms in wild-type CRF01_AE, confer significant resistance to various NRTIs in the presence of TAMs. Our findings emphasize the important role of polymorphisms in C-terminal half of RT in CRF01_AE HIV-1 on drug resistance, especially in consideration of the second line therapy. Further investigation is needed on drug resistance mutations in widely prevailing non-subtype B HIV-1.

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References

[1] C.F. Gilks, S. Crowley, R. Ekpini, S. Gove, J. Perriens, Y. Souteyrand, D. Sutherland, M. Vitoria, T. Guerma, K. De Cock, The WHO public-health approach to antiretroviral treatment against HIV in resource-limited settings, Lancet 368 (2006) 505–510.

[2] Ministry of Health of Viet Nam, Decision by the Minister of Health on the Issuance of the Guidelines for HIV/AIDS Diagnosis and Treatment: Guidelines for HIV/AIDS Diagnosis and Treatment. Health Publishing House, 2005, No: 06/2005QD-BYT.

[3] UNAIDS/WHO, AIDS epidemic update ASIA regional summary 2007 Available from: http://data.unaids.org/pub/Report/2008/jc1527_epibriefs_asia_en.pdf (2008).

[4] UNAIDS/WHO, Epidemiological fact sheets on HIV/AIDS and sexually transmitted infections Available from: http://apps.who.int/GlobalAtlas/predefinedReports/EFS2008/index.asp (2008).

[5] Ministry of Health of Viet Nam, The third country report on following up the implementation to the declaration of commitment on HIV and AIDS Available from: http://data.unaids.org/pub/Report/2008/viet_nam_2008_country_progress_report_en.pdf (2008).

[6] D.E. Bennett, M. Myatt, S. Bertagnolio, D. Sutherland, C.F. Gilks, Recommendations for surveillance of transmitted HIV drug resistance in countries scaling up antiretroviral treatment, Antivir. Ther. 13 (2008) 25–36.

[7] J.H. Brehm, D. Koontz, J.D. Meteer, V. Pathak, N. Sluis-Cremer, J.W. Mellors, Selection of mutations in the connection and RNase H domains of human immunodeficiency virus type 1 reverse transcriptase that increase resistance to 3′-azido-3′-dideoxythymidine, J. Virol. 81 (2007) 7852–7859.

[8] K.A. Delviks-Frankenberry, G.N. Nikolenko, R. Barr, V.K. Pathak, Mutations in human immunodeficiency virus type 1 RNase H primer grip enhance 3′-azido-3′-deoxythymidine resistance, J. Virol. 81 (2007) 6837–6845.

[9] K.A. Delviks-Frankenberry, G.N. Nikolenko, P.L. Boyer, S.H. Huges, J. M. Coffin, A. Jere, V.K. Pathak, HIV-1 reverse transcriptase connection subdomain mutations reduce template RNA degradation and enhance AZT excision, Proc. Natl. Acad. Sci. USA 105 (2008) 10943–10948.

[10] A. Hachiya, E.N. Kodama, S.G. Sarafianos, M.M. Schuckmann, Y. Sakagami, M. Matsuoka, M. Takiguchi, H. Gatanaga, S. Oka, Amino acid mutation N348I in the connection subdomain of human immunodeficiency virus type 1 reverse transcriptase confers multiclass resistance to nucleoside and nonnucleoside reverse transcriptase inhibitors, J. Virol. 82 (2008) 3261–3270.

[11] G.N. Nikolenko, K.A. Delviks-Frankenberry, S. Palmer, F. Maldarelli, M.J. Fivash Jr., J.M. Coffin, V.K. Pathak, Mutations in the connection domain of HIV-1 reverse transcriptase increase 3′-azido-3′-deoxythymidine resistance, Proc. Natl. Acad. Sci. USA 104 (2007) 317–322.

[12] S.H. Yap, C.W. Sheen, J. Fahey, M. Zanin, D. Tyssen, V.D. Lima, B. Wynhoven, M. Kuiper, N. Sluis-Cremer, P.R. Harrigan, G. Tachedjian, N348I in the connection domain of HIV-1 reverse transcriptase confers zidovudine and nevirapine resistance, PLoS Med. 4 (2007) e335.

[13] M. Ehteshami, G.L. Beilhartz, B.J. Scarth, E.P. Tchesnokov, S. McCormick, B. Wynhoven, P.R. Harrigan, M. Götte, Connection domain mutations N348I and A360V in HIV-1 reverse transcriptase enhance resistance to 3′-azido-3′-deoxythymidine through both RNase H-dependent and -independent mechanisms, J. Biol. Chem. 283 (2008) 22222–22232.

[14] S. Zelina, C.W. Sheen, J. Radzio, J.W. Mellors, N. Sluis-Cremer, Mechanisms by which the G333D mutation in human immunodeficiency virus type 1 reverse transcriptase facilitates dual resistance to zidovudine and lamivudine, Antimicrob. Agents Chemother. 52 (2008) 157–163.

[15] J.H. Brehm, J.W. Mellors, N. Sluis-Cremer, Mechanism by which a glutamine to leucine substitution at residue 509 in the ribonuclease H domain of HIV-1 reverse transcriptase confers zidovudine resistance, Biochemistry 47 (2008) 14020–14027.

[16] K.A. Delviks-Frankenberry, G.N. Nikolenko, F. Maldarelli, S. Hase, Y. Takebe, V.K. Pathak, Subtype-specific differences in the human immunodeficiency virus type 1 reverse transcriptase connection subdomain of CRF01_AE are associated with higher levels of resistance to 3′-azido-3′-deoxythymidine, J. Virol. 83 (2009) 8502–8513.

[17] S.D. Kemp, C. Shi, S. Bloor, P.R. Harrigan, J.W. Mellors, B.A. Larder, A novel polymorphism at codon 333 of human immunodeficiency virus type 1 reverse transcriptase can facilitate dual resistance to zidovudine and L-2′,3′-dideoxy-3′-thiacytidine, J. Virol. 72 (1998) 5093–5098.

[18] M. Ntemgwa, M.A. Wainberg, M. Oliveira, D. Moisi, R. Lalonde, V. Micheli, B.G. Brenner, Variations in reverse transcriptase and RNase H domain mutations in human immunodeficiency virus type 1 clinical isolates are associated with divergent phenotypic resistance to zidovudine, Antimicrob. Agents Chemother. 51 (2007) 3861–3869.

[19] A.F. Santos, R.B. Lengruber, E.A. Soares, A. Jere, E. Sprinz, A.M. Martinez, J. Silveira, F.S. Sion, V.K. Pathak, M.A. Soares, Conservation patterns of HIV-1 RT connection and RNase H domains: identification of new mutations in NRTI-treated patients, PLoS ONE 3 (2008) e1781.

[20] A. Hachiya, K. Shimane, S.G. Sarafianose, E.N. Kodama, Y. Sakagami, F. Negishi, H. Koizumi, H. Gatanaga, M. Matsuoka, M. Takiguchi, S. Oka, Clinical relevance of substitutions in the connection subdomain and RNase H domain of HIV-1 reverse transcriptase from a cohort of antiretroviral treatment-naïve patients, Antiviral. Res. 82 (2009) 115–121.

[21] L. Buonaguro, M.L. Tornesello, F.M. Buonaguro, Human immunodeficiency virus type 1 subtype distribution in the worldwide epidemic: pathogenetic and therapeutic implications, J. Virol. 81 (2007) 10209–10219.

[22] R. Kantor, Impact of HIV-1 pol diversity on drug resistance and its clinical implications, Curr. Opin. Infect. Dis. 19 (2006) 594–606.

[23] R. Kantor, D.A. Katzenstein, B. Efron, A.P. Carvalho, B. Wynhoven, P. Cane, J. Clarke, S. Sirivichayakul, M.A. Soares, J. Snoeck, C. Pillay, H. Rudich, R. Rodrigues, A. Holguin, K. Ariyoshi, M.B. Bouzas, P. Cahn, W. Sugiura, V. Soriano, L.F. Brigido, Z. Grossman, L. Morris, A.M. Vandamme, A. Tanuri, P. Phanuphak, J.N. Weber, D. Pillay, P.R. Harrigan, R. Camacho, J.M. Schapiro, R.W. Shafer, Impact of HIV-1 subtype and antiretroviral therapy on protease and reverse transcriptase genotype: results of a global collaboration, PLoS Med. 2 (2005) e112.

[24] J. Snoeck, R. Kantor, R.W. Shafer, K. Van Laethem, K. Deforche, A.P. Carvalho, B. Wynhoven, M.A. Soares, P. Cane, J. Clarke, C. Pillay, S. Sirivichayakul, K. Ariyoshi, A. Holguin, H. Rudich, R. Rodrigues, M.B. Bouzas, F. Brun-Vézinet, C. Reid, P. Cahn, L.F. Brigido, Z. Grossman, V. Soriano, W. Sugiura, P. Phanuphak, L. Morris, J. Weber, D. Pillay, A. Tanuri, R.P. Harrigan, R. Camacho, J.M. Schapiro, D. Katzenstein, A.M. Vandamme, Discordances between interpretation algorithms for genotypic resistance to protease and reverse transcriptase inhibitors of human immunodeficiency virus are subtype dependent, Antimicrob. Agents Chemother. 50 (2006) 694–701.

[25] E. Caride, R. Brindeiro, K. Hertogs, B. Larder, P. Dehertogh, E. Machado, C.A. de Sá, W.A. Eyer-Silva, F.S. Sion, L.F. Passioni, J.A. Menezes, A.R. Calazans, A. Tanuri, Drug-resistant reverse transcriptase genotyping and phenotyping of B and Non-B subtypes (F and A) of human immunodeficiency virus type I found in Brazilian patients failing HAART, Virology 275 (2000) 107–115.

[26] B. Montes, L. Vergne, M. Peeters, J. Reynes, E. Delaporte, M. Segondy, Comparison of drug resistance mutations and their interpretation in patients infected with non-B HIV-1 variants and matched patients infected with HIV-1 subtype B, J. Acquir. Immune. Defic. Syndr. 35 (2004) 329–336.

[27] V. Novitsky, C.W. Wester, V. DeGruttola, H. Bussmann, S. Gaseitsiwe, A. Thomas, S. Moyo, R. Musonda, E. Van Widenfelt, R.G. Marlink, M. Essex, The reverse transcriptase 67N 70R 215Y genotype is the predominant TAM pathway associated with virologic failure among HIV Type 1C-infected adults treated with ZDV/ddI-Containing HAART in Southern Africa, AIDS Res. Hum. Retrovir. 23 (2007) 868–878.

[28] K. Ariyoshi, M. Matsuda, H. Miura, S. Tateishi, K. Yamada, W. Sugiura, Patterns of point mutations associated with antiretroviral drug treatment failure in CRF01_AE (subtype E) infection differ from subtype B infection, J. Acquir. Immune. Defic. Syndr. 33 (2003) 336–342.

[29] L.Y. Hsu, R. Subramaniam, L. Bacheler, N.I. Paton, Characterization of mutations in CRF01_AE virus isolates from antiretroviral treatment-naive and -experienced patients in Singapore, J. Acquir. Immune. Defic. Syndr. 38 (2005) 5–13.

[30] W.C. Yam, J.H. Chen, K.H. Wong, K. Chan, V.C. Cheng, H.Y. Lam, S.S. Lee, B.J. Zheng, K.Y. Yuen, Clinical utility of genotyping resistance test on determining the mutation patterns in HIV-1 CRF01_AE and subtype B patients receiving antiretroviral therapy in Hong Kong, J. Clin. Virol. 35 (2006) 454–457.

[31] C. Sukasem, V. Churdboonchart, W. Sukeepaisarncharoen, W. Piroj, T. Inwisai, M. Tiensuwan, W. Chantratita, Genotypic resistance profiles in antiretroviral-naive HIV-1 infections before and after initiation of first-line HAART: impact of polymorphism on resistance to therapy, Int. J. Antimicrob. Agents 31 (2008) 277–281.

[32] T.H.L. Nguyen, P. Recordon-Pinson, V.H. Pham, N.T. Uyen, T.T. Lien, H.T. Tien, I. Garrigue, M.H. Schrive, I. Pellegrin, M.E. Lafon, J.P. Aboulker, F. Barré-Sinoussi, H.J. Fleury, HIV type 1 isolates from 200 untreated individuals in Ho Chi Minh City (Vietnam): ANRS 1257 study. Large predominance of CRF01_AE and presence of major resistance mutations to antiretroviral drugs, AIDS Res. Hum. Retrovir. 19 (2003) 925–928.

[33] T.T.H. Tran, I. Maljkovic, S. Swartling, D.C. Phung, F. Chiodi, T. Leitner, HIV-1 CRF01_AE in intravenous drug users in Hanoi, Vietnam, AIDS Res. Hum. Retrovir. 20 (2004) 341–345.

[34] A. Ishizaki, H.C. Nguyen, P.V. Thuc, V.T. Nguyen, K. Saijoh, S. Kageyama, K. Ishigaki, J. Tanuma, S. Oka, H. Ichimura, Profile of HIV Type 1 infection and genotypic resistance mutations to antiretroviral drugs in treatment-naive HIV type 1-infected individuals in Hai Phong, Viet Nam, AIDS Res. Hum. Retrovir. 25 (2009) 175–182.

[35] A. Hachiya, S. Aizawa-Matsuoka, M. Tanaka, Y. Takahashi, S. Ida, H. Gatanaga, Y. Hirabayashi, A. Kojima, M. Tatsumi, S. Oka, Rapid and simple phenotypic assay for drug susceptibility of human immunodeficiency virus type 1 using CCR5-expressing HeLa/CD4$^+$ cell clone 1–10 (MAGIC-5), Antimicrob. Agents Chemother. 45 (2001) 495–501.

[36] V.A. Johnson, F. Brun-Vézinet, B. Clotet, H.F. Gunthard, D.R. Kuritzkes, D. Pillay, J.M. Schapiro, D.D. Richman, Update of the drug resistance mutations in HIV-1: December 2009, Top. HIV Med. 17 (2009) 138–145.

[37] Z. Hu, F. Giguel, H. Hatano, P. Reid, J. Lu, D.R. Kuritzkes, Fitness comparison of thymidine analog resistance pathways in human immunodeficiency virus type 1, J. Virol. 80 (2006) 7020–7027.

[38] R.W. Shafer, S.Y. Rhee, D. Pillay, V. Miller, P. Sandstrom, J.M. Schapiro, D.R. Kuritzkes, D. Bennett, HIV-1 protease and reverse transcriptase mutations for drug resistance surveillance, AIDS 21 (2007) 215–223.

[39] D.E. Bennett, R.J. Camacho, D. Otelea, D.R. Kuritzkes, H. Fleury, M. Kiuchi, W. Heneine, R. Kantor, M.R. Jordan, J.M. Schapiro, A.M. Vandamme, P. Sandstrom, C.A. Boucher, D. van de Vijver, S.Y. Rhee, T.F. Liu, D. Pillay, R.W. Shafer, Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update, PLoS ONE 4 (2009) e4724.

[40] S. Saeng-aroon, N. Tsuchiya, W. Auwanit, P.I. Ayuthaya, P. Pathipvanich, P. Sawanpanyalert, A. Rojanawiwat, M. Kannagi, K. Ariyoshi, W. Sugiura, Drug-resistant mutation patterns in CRF01_AE cases that failed d4T+3TC+nevirapine fixed-dosed, combination treatment: follow-up study from the Lampang cohort, Antivir. Res. 87 (2010) 22–29.

[41] S. Gupta, S. Fransen, E. Paxinos, W. Huang, R. Dua, E. Stawiski, C. Petropoulos, N. Parkin, Infrequent occurrence of mutations in the C-terminal region of reverse transcriptase modulates susceptibility to RT inhibitors, Antivir. Ther. 11 (2006) S143.

[42] E. Poveda, C. de Mendoza, T. Pattery, M. González, J. Villacian, V. Soriano, Phenotypic impact of resistance mutations on etravirine susceptibility in HIV patients with prior failure to nonnucleoside analogues, AIDS 22 (2008) 2395–2398.

[43] B. Winters, E. Van Craenenbroeck, K. Van der Borght, P. Lecocq, J. Villacian, L. Bacheler, Clinical cut-offs for HIV-1 phenotypic resistance estimates: update based on recent pivotal clinical trial data and a revised approach to viral mixtures, J. Virol. Methods 162 (2009) 101–108.

[44] B. Wang, K.A. Lau, L.Y. Ong, M. Shah, M.C. Steain, B. Foley, D.E. Dwyer, C.B. Chew, A. Kamarulzaman, K.P. Ng, N.K. Saksena, Complex patterns of the HIV-1 epidemic in Kuala Lumpur, Malaysia: evidence for expansion of circulating recombinant form CRF33_01B and detection of multiple other recombinants, Virology 367 (2007) 288–297.

[45] K.K. Tee, C.K. Pon, A. Kamarulzaman, K.P. Ng, Emergence of HIV-1 CRF01_AE/B unique recombinant forms in Kuala Lumpur, Malaysia, AIDS 19 (2005) 119–126.

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High Performance Liquid Chromatography Using UV Detection for the Simultaneous Quantification of the New Non-nucleoside Reverse Transcriptase Inhibitor Etravirine (TMC-125), and 4 Protease Inhibitors in Human Plasma

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Etravirine (TMC-125, ETV) is a second-generation non-nucleoside reverse transcriptase inhibitor (NNRTI) that demonstrates potent activity against NNRTI-resistant strains of human immunodeficiency virus type-1 (HIV-1). Thus, ETV has been used in combination with ritonavir-boosted protease inhibitor (PI) and integrase inhibitor for therapy-experienced HIV-1-infected patients. On the other hand, as ETV is a substrate and inducer of cytochrome P450 3A4 (CYP3A4), ETV may induce metabolism of PI and alter the concentrations of co-administered PIs. In order to ensure optimal drug efficacy and prevention of resistance, it is essential to monitor plasma concentrations of ETV and PIs. Here we describe the application of HPLC with UV detection for the simulataneous assay of ETV and 4 PIs, darunavir (DRV), atazanavir (ATV), ritonavir (RTV) and lopinavir (LPV). In this study, the calibration curve of each drug was linear with the average accuracy ranging from 93.6 to 110.9%. Both intra- and interday coefficients of variation for each drug were less than 11.6%. The mean recovery of all drugs ranged from 88.0 to 97.5%. The limit of quantification was 0.04, 0.04, 0.04, 0.05 and 0.07 μg/ml for ETV, DRV, ATV, RTV and LPV, respectively. These results demonstrate that our HPLC-UV method can be used for routine determination of plasma concentrations of ETV and 4 PIs in clinical settings.

Key words etravirine; HPLC; protease inhibitor; therapeutic drug monitoring

Etravirine (TMC-125, ETV) is a second-generation non-nucleoside reverse transcriptase inhibitor (NNRTI) that demonstrates potent activity against NNRTI-resistant strains of human immunodeficiency virus type-1 (HIV-1). According to the DUET studies (randomized, double-blind, placebo-controlled trials), overall, ETV was well tolerated in treatment-experienced patients infected with HIV-1, with its safety and tolerability profile generally comparable to placebo at week 24.[1,2] Additionally, 48-week data pooled from these studies showed greater virologic and immunologic responses compared with placebo.[3]

In the latest HIV treatment, ETV has been used in combination with ritonavir-boosted protease inhibitor (PI) and integrase inhibitor for therapy-experienced HIV-1-infected patients. On the other hand, as ETV is a substrate and inducer of cytochrome P450 3A4 (CYP3A4), ETV may induce metabolism of PI and alter the concentrations of co-administered PIs.[4] In order to ensure optimal drug efficacy and prevention of resistance, it is essential to monitor plasma concentrations of ETV and PIs.

Fayet *et al.*[5] and Quaranta *et al.*[6] succeeded in determining plasma concentrations of ETV and other drugs through the use of liquid chromatography-tandem mass spectrometry (LC-MS/MS). Rezk *et al.*[7] have also developed a method to measure plasma concentrations of ETV and PIs by LC-MS. LC-MS or LC-MS/MS assay is very sensitive and accurate. However, MS equipment is very expensive and unavailable in conventional hospital laboratories. Therefore, development of alternate methods is necessary.

Recently, D'Avolio *et al.*[8] reported a new HPLC method that employs a photo diode array (HPLC-PDA) for quantification of ETV and other antiretroviral drugs. This method is simple, reliable, and sensitive, using cost-effective instrumentation when compared with others.[5—7] However, this method requires a solid phase extraction. Furthermore, in general hospitals, a UV detector coupled with HPLC is more popular than a PDA detector. The HPLC-UV method is a user-friendly assay that is readily adaptable to standard laboratory equipment for routine therapeutic drug monitoring (TDM).

In this study, we propose the simultaneous quantitative assay of ETV and 4PIs, darunavir (DRV), atazanavir (ATV), ritonavir (RTV) and lopinavir (LPV) in a simple procedure that is derived from a previously established HPLC-UV method.[9] This method can be applied to pharmacokinetic studies of PIs and ETV, the newest NNRTI, and it is useful when evaluating the clinical significance of TDM for these drugs.

MATERIALS AND METHODS

Chemicals ETV and DRV were supplied by Tibotec Pharmaceuticals Ltd. (Eastgate Village, Eastgate, Little Island, Co., Cork, Ireland). LPV and RTV were generously provided by Abbott Laboratories (Abbott Park, IL, U.S.A). ATV was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (New Brunswick, NJ, U.S.A.). The internal standard (IS), 6,7-dimethyl-2,3-di(2-pyridyl)quinoxaline, was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Acetonitrile, methanol, ethyl acetate and *n*-hexane (Kanto

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Chemical, Tokyo, Japan) were HPLC grade. Sodium carbonate was purchased from Katayama Chemical (Osaka, Japan). Water was deionized and osmosed using a Milli-Q$^{®}$ system (Millipore, Bedford, MA, U.S.A.). All other chemicals were of analytical grade and have been described in our previous report.[9]

Standard Solutions Stock solutions of tested drugs and IS were prepared by accurately dissolving weighed amounts of each reference compound in water/ethanol (50 : 50, v/v) to yield concentrations of 191.0 μg/ml for ETV, 85.2 μg/ml for DRV, 502.0 μg/ml for ATV, 425.0 μg/ml for RTV, 95.1 μg/ml for LPV, and 588.0 μg/ml for IS. These stock solutions were stored at −80 °C until the day of analysis. Each stock solution was diluted in drug-free plasma to yield concentrations of 0.08, 0.14, 0.42, 1.04 and 4.17 μg/ml for ETV, 0.07, 0.12, 0.37, 0.93 and 3.72 μg/ml for DRV, 0.09, 0.15, 0.44, 1.10 and 4.38 μg/ml for ATV, 0.07, 0.12, 0.37, 0.93 and 3.71 μg/ml for RTV, 0.08, 0.14, 0.42, 1.04 and 4.15 μg/ml for LPV.

Chromatography The HPLC system consisted of a Waters pump (model 515), a 717 plus autosampler, and a 2487 dual λ absorbance detector coupled to the Empower$^{\text{TM}}$ software (Waters, Milford, MA, U.S.A.). The analytical column was a Radial-Pak Nova-Pak C$_{18}$ column (4 μm, 8×100 mm, Waters) protected by Guard-Pak Inserts Nova-Pak C$_{18}$ precolumn. Absorbance was measured at 205 nm, with separations performed at 30 °C. The mobile phase consisted of 39% 50 mM phosphate buffer (pH 6.2), 22% methanol and 39% acetonitrile. The assay run time was 30 min with a flow rate of 1.8 ml/min. Drugs were quantified by measuring the peak areas under the chromatograms.

Sample Preparation A total of 2 ml of ethyl acetate/*n*-hexane (50 : 50, v/v) containing the IS (1.18 μg/ml) and 1 ml of 0.5 M sodium carbonate were added to a 500 μl plasma sample. The mixture was vortexed and then centrifuged at 3500×***g*** for 5 min. The organic layer was separated and evaporated to dryness. The dried material was then dissolved in 100 μl of a mobile phase solution and centrifuged at 13000×***g*** for 5 min. Lastly, 25 μl of the upper solution was injected into the HPLC column.

The institutional review board of the National Hospital Organization Nagoya Medical Center approved this study. Plasma samples were prepared from patients after obtaining written informed consent.

Validation Intra- and interday precision values using this method were estimated by assaying control plasma containing five different concentrations of each drug five times on the same day and on three separate days to obtain the coefficient of variation (CV). Accuracy was determined as the percentage of the nominal concentration. Drug recovery from plasma was evaluated by analyzing triplicate samples with or without extraction. Plasma samples spiked with known amounts of both drugs and IS were extracted as usual. Blank plasma samples that contained only the IS were extracted and subsequently spiked with the same amount of analytes to give the 100% reference. The recovery was assessed by comparing the peak area ratio (analytes/IS) of extracts. The limit of quantification was defined as the lowest concentration for which both the CV% and the percent of deviation from the nominal concentration were less than 20%.

RESULTS

Plasma Sample Chromatograms Figure 1A is a chromatogram of a spiked plasma sample containing 3.77 μg/ml of DRV, 1.18 μg/ml of IS, 4.44 μg/ml of ATV, 3.76 μg/ml of RTV, 1.69 μg/ml of ETV and 4.21 μg/ml of LPV. Under the described chromatographic conditions, retention times were 3.4, 4.4, 8.3, 10.5, 11.7, 13.0 min for DRV, IS, ATV, RTV, ETV and LPV, respectively. At a detection wavelength of 205 nm, assays performed on drug-free human plasma demonstrated that there were no interfering peaks during the intervals of interest for the retention times (Fig. 1B).

Figure 2A is a chromatogram of a plasma sample from an HIV-1-infected patient treated with raltegravir, ETV, ATV, RTV and lamivudine. The patient was a Japanese male aged 37 years with a body weight of 72.6 kg. His CD4$^+$ T cell count was 302/μl with a viral load of 7200 copies/ml. ETV and other antiretroviral agents were administered for 7 d. The plasma concentration at trough was 0.30, 0.48 and 0.24 μg/ml for ETV, ATV and RTV, respectively.

Figure 2B shows a chromatogram of a plasma sample from an HIV-1-infected patient treated with raltegravir, ETV, DRV, RTV and lamivudine. The patient was a Brazilian male aged 49 years with a body weight of 83.0 kg. His CD4$^+$ T cell count was 157/μl with a viral load of 44 copies/ml. ETV

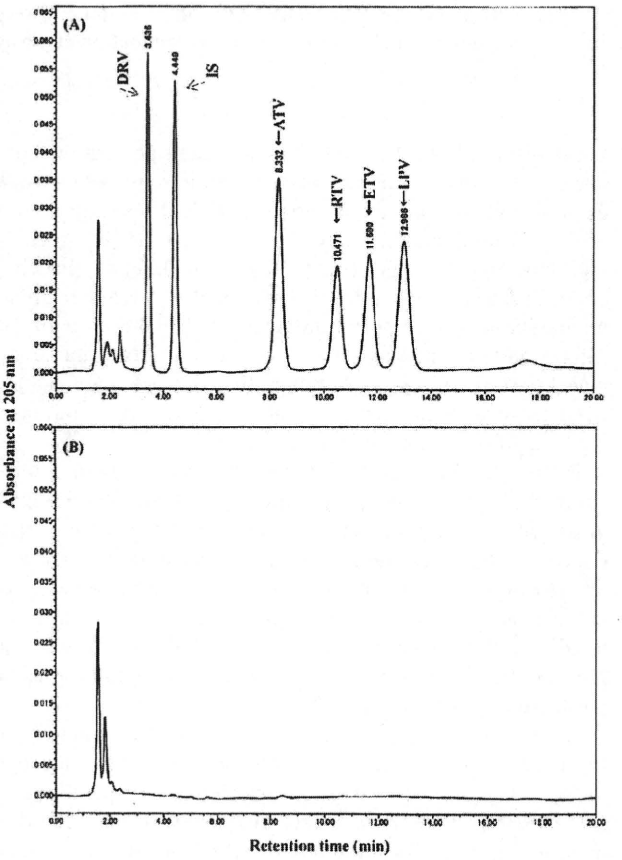

Fig. 1. Chromatograms Obtained after Extraction of (A) Spiked Plasma Sample Containing 3.77 μg/ml of DRV, 1.18 μg/ml of IS, 4.44 μg/ml of ATV, 3.76 μg/ml of RTV, 1.69 μg/ml of ETV and 4.21 μg/ml of LPV and (B) Drug-Free Human Plasma Sample from a Healthy Volunteer

DRV, darunavir; IS, internal standard; ATV, atazanavir; RTV, ritonavir; ETV, etravirine; LPV, lopinavir.

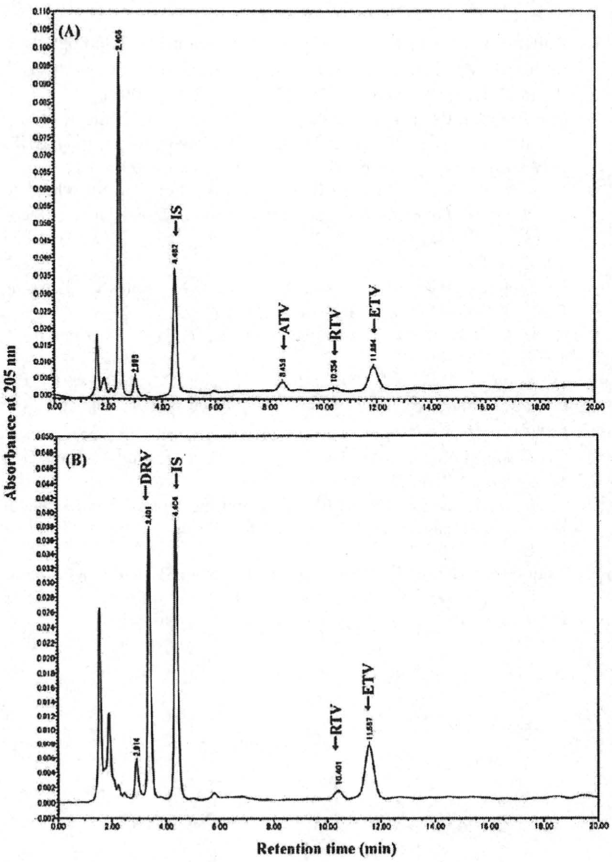

Fig. 2. Chromatograms Obtained after Extraction of a Plasma Sample from an HIV-1-Infected Patient (A) Treated with Raltegravir, ETV, ATV, RTV and Lamivudine, and (B) Treated with Raltegravir, DRV, ETV, RTV and Lamivudine

DRV, darunavir; IS, internal standard; ATV, atazanavir; RTV, ritonavir; ETV, etravirine

Table 1. Intra- and Interday Precision and Accuracy for ETV, ATV, RTV, LPV and DRV

	Expected (μg/ml)	Intraday ($n = 5$)		Interday ($n = 15$)		Accuracy (%)
		Measured (μg/ml)	CV (%)	Measured (μg/ml)	CV (%)	
ETV	0.08	0.09 ± 0.00	3.0	0.09 ± 0.00	4.9	110.9 ± 2.8
	0.14	0.14 ± 0.01	3.9	0.14 ± 0.01	3.9	99.4 ± 3.9
	0.42	0.42 ± 0.01	1.2	0.42 ± 0.01	3.0	99.8 ± 3.0
	1.04	1.03 ± 0.01	0.5	1.03 ± 0.01	1.4	98.9 ± 1.4
	4.17	4.17 ± 0.03	0.8	4.14 ± 0.05	1.3	99.2 ± 1.2
ATV	0.09	0.09 ± 0.00	2.2	0.09 ± 0.01	6.5	103.1 ± 6.7
	0.15	0.16 ± 0.00	2.6	0.16 ± 0.01	3.3	103.6 ± 3.4
	0.44	0.45 ± 0.01	1.4	0.44 ± 0.01	2.4	100.8 ± 2.5
	1.10	1.10 ± 0.00	0.4	1.12 ± 0.02	2.1	101.4 ± 2.1
	4.38	4.37 ± 0.01	0.3	4.38 ± 0.04	0.9	99.9 ± 0.9
RTV	0.07	0.07 ± 0.01	8.1	0.07 ± 0.01	8.0	93.6 ± 7.5
	0.12	0.12 ± 0.00	3.3	0.12 ± 0.01	4.4	100.3 ± 4.4
	0.37	0.37 ± 0.01	2.3	0.37 ± 0.01	3.9	99.6 ± 3.9
	0.93	0.93 ± 0.01	1.4	0.94 ± 0.02	2.2	101.1 ± 2.2
	3.71	3.72 ± 0.07	1.9	3.69 ± 0.06	1.7	99.6 ± 1.6
LPV	0.08	0.08 ± 0.01	11.6	0.09 ± 0.01	9.3	107.3 ± 10.0
	0.14	0.14 ± 0.00	2.7	0.14 ± 0.01	3.7	99.2 ± 3.7
	0.42	0.42 ± 0.01	2.7	0.42 ± 0.01	3.4	101.1 ± 3.9
	1.04	1.04 ± 0.01	0.8	1.05 ± 0.02	2.1	100.5 ± 2.1
	4.15	4.14 ± 0.02	0.4	4.16 ± 0.07	1.6	100.1 ± 1.6
DRV	0.07	0.07 ± 0.01	7.6	0.08 ± 0.01	9.9	108.4 ± 10.7
	0.12	0.12 ± 0.01	4.7	0.12 ± 0.00	3.9	99.7 ± 3.9
	0.37	0.37 ± 0.01	1.9	0.37 ± 0.01	2.8	100.8 ± 2.8
	0.93	0.93 ± 0.01	0.9	0.93 ± 0.02	1.9	100.5 ± 1.9
	3.72	3.70 ± 0.04	1.0	3.72 ± 0.03	0.9	100.1 ± 0.9

ETV, etravirine; ATV, atazanavir; RTV, ritonavir; LPV, lopinavir; DRV, darunavir; CV, coefficient of variation

and other antiretroviral agents were administered for 3 weeks. The plasma concentration at trough was 0.33, 3.30, and 0.29 μg/ml for ETV, DRV and RTV, respectively.

In chromatograms of Figs. 2A and B, the peaks of raltegravir and lamivudine were not detected.

Precision, Accuracy, Recovery, Linearity and Limit of Quantification The precision and accuracy for all tested drugs are shown in Table 1. The analyses show satisfactory precision with intra- and interassay coefficients of variation less than 11.6%. Accuracies ranged from 93.6 to 110.9%. The mean recovery of all drugs ranged from 88.0 to 97.5%. The regression coefficients of determination (R^2) values of the calibration curves for each drug were 0.99 or greater. The limit of quantification was 0.04, 0.04, 0.04, 0.05 and 0.07 μg/ml for ETV, DRV, ATV, RTV and LPV, respectively.

DISCUSSION

ETV has activity *in vitro* against viral strains with mutations that confer resistance to efavirenz and nevirapine.[1] The EC_{50} of ETV was < 100 nM (43.5 ng/ml) against clinically derived recombinant viruses resistant to at least one of the currently marketed NNRTIs. However, clinical investigations have yet to determine the therapeutic range of ETV concentrations that are associated with the desired therapeutic response. In addition, there is the potential problem for complex drug interactions due to the fact that ETV is a substrate and inducer of CYP3A4, as well as a substrate and inhibitor of 2C9 and 2C19.[1] In clinical treatment, ETV is co-administered with other antiretroviral agents including RTV-boosted PI. Therefore, too-low or too-high plasma concentrations of these drugs may decrease treatment efficacy or increase the risk of adverse effects. To solve these problems, a simple drug monitoring system for these agents is needed. Here we describe the application of HPLC with UV detection for simultaneously assaying ETV and 4 PIs. HPLC-UV equipment is frequently used in conventional hospital laboratories.

In this study, the calibration curve of each drug was linear with the average accuracy ranging from 93.6 to 110.9%. Both intra- and interday coefficients of variation for each drug were less than 11.6%. These results demonstrate that our HPLC-UV method has advantages in both reproduciblity and accuracy in measuring plasma concentration of ETV and 4 PIs in a single run.

In our clinical cases, the ETV plasma concentrations, measured at trough, were 0.30 or 0.33 μg/ml for the HIV-1-infected patients. These values were similar to the previously reported findings in DUET studies.[1,2] In each case, the trough concentration of DRV or ATV was more than the suggested minimum target trough value in the guideline.[1] The viral load has been decreasing in these patients and treatment success is expected in the future. We, thus, proposed maintaining the current daily dose of these drugs. Conversely, the peaks of co-administered raltegravir and lamivudine were not detected because these drugs were not extracted from plasma by our liquid liquid extraction technique. As these drugs are not metabolized by cytochrome P450, there are no drug interactions with ETV.

In conclusion, we have successfully constructed a protocol for the simultaneous quantification of ETV and 4 PIs by HPLC-UV. We believe our method enables accurate monitoring of ETV and co-administered PIs and may guide optimized administration of these drugs and prevent potential drug interactions and toxicity in treatment.

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REFERENCES AND NOTES

1) Madruga J. V., Cahn P., Grinsztejn B., Haubrich R., Lalezari J., Mills A., Pialoux G., Wilkin T., Peeters M., Vingerhoets J., de Smedt G., Leopold L., Trefiglio R., Woodfall B., *Lancet*, **370**, 29–38 (2007).
2) Lazzarin A., Campbell T., Clotet B., Johnson M., Katlama C., Moll A., Towner W., Trottier B., Peeters M., Vingerhoets J., de Smedt G., Baeten B., Beets G., Sinha R., Woodfall B., *Lancet*, **370**, 39–48 (2007).
3) Katlama C., Haubrich R., Lalezari J., Lazzarin A., Madruga J. V., Molina J. M., Schechter M., Peeters M., Picchio G., Vingerhoets J., Woodfall B., De Smedt G., *AIDS*, **23**, 2289–2300 (2009).
4) The Panel on Clinical Practices for Treatment of HIV Infection Convened by the Department of Health and Human Services (DHHS). Published at http://AIDSinfo.nih.gov, 1 December 2009.
5) Fayet A., Beguin A., Zanolari B., Cruchon S., Guignard N., Telenti A., Cavassini M., Gunthard H. F., Buclin T., Biollaz J., Rochat B., Decosterd L. A., *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.*, **877**, 1057–1069 (2009).
6) Quaranta S., Woloch C., Paccou A., Giocanti M., Solas C., Lacarelle B., *Ther. Drug Monit.*, **31**, 695–702 (2009).
7) Rezk N. L., White N. R., Jennings S. H., Kashuba A. D., *Talanta*, **79**, 1372–1378 (2009).
8) D'Avolio A., Baietto L., Siccardi M., Sciandra M., Simiele M., Oddone V., Bonora S., Di Perri G., *Ther. Drug Monit.*, **30**, 662–669 (2008).
9) Takahashi M., Yoshida M., Oki T., Okumura N., Suzuki T., Kaneda T., *Biol. Pharm. Bull.*, **28**, 1286–1290 (2005).
10) Product Information. INTELENCE (etravirine tablet). Tibotec Therapeutics. Bridgewater, NJ, U.S.A.: http://www.intelence-info.com/intelence/
11) Andries K., Azijn H., Thielemans T., Ludovici D., Kukla M., Heeres J., Janssen P., De Corte B., Vingerhoets J., Pauwels R., de Bethune M. P., *Antimicrob. Agents Chemother.*, **48**, 4680–4686 (2004).

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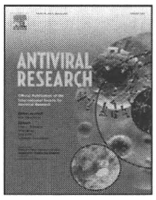

Characterization of HIV-1 resistance to a fusion inhibitor, N36, derived from the gp41 amino-terminal heptad repeat

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ABSTRACT

A transmembrane glycoprotein of HIV-1, gp41, plays a central role in membrane fusion of HIV-1 and host cells. Peptides derived from the amino- and carboxyl-terminal heptad repeat (N-HR and C-HR, respectively) of gp41 inhibit this fusion. The mechanism of resistance to enfuvirtide, a C-HR-derived peptide, is well defined; however the mechanism of resistance to N-HR-derived peptides remains unclear. We characterized an HIV-1 isolate resistant to the N-HR-derived peptide, N36. This HIV-1 acquired a total of four amino acid substitutions, D36G, N126K and E137Q in gp41, and P183Q in gp120. Among these substitutions, N126K and/or E137Q conferred resistance to not only N36, but also C34, which is the corresponding C-HR-derived peptide fusion inhibitor. We performed crystallographic and biochemical analysis of the 6-helix bundle formed by synthetic gp41-derived peptides containing the N126K/E137Q substitutions. The structure of the 6-helix bundle with N126K/E137Q was identical to that in wild-type HIV-1 except for the presence of a new hydrogen bond. Denaturing experiments revealed that the stability of the 6-helix bundle of N126K/E137Q is greater than in the wild-type. These results suggest that the stabilizing effect of N126K/E137Q provides resistance to N36 and C34.

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1. Introduction

An envelope glycoprotein (Env) of human immunodeficiency virus type 1 (HIV-1), gp120, interacts with CD4 and co-receptors, such as CCR5. This induces conformational changes of gp120 to activate gp41, which mediates viral membrane fusion to the host membrane (Eckert and Kim, 2001b). Briefly, after the fusion domain located in the N-terminal end of gp41 penetrates into the host cell membrane, the interaction of a trimer of the amino-terminal heptad repeat (N-HR) with the carboxyl-terminal HR (C-HR) of gp41, results in the formation of a 6-helix bundle in which the N- and C-HRs are arranged in a three-hairpin structure. Alternatively, the three N-HRs form a coiled-coil, and the three C-HRs are packed in an antiparallel manner into highly conserved, hydrophobic grooves on the surface of the coiled-coil (Chan et al., 1997). Peptides derived from the N- and C-HR regions inhibit fusion by blocking the interaction between the N- and C-HRs and preventing the formation of the 6-helix bundle fusogenic state of gp41 (Chan et al., 1998; Wild et al., 1993, 1992). One of the C-HR-derived peptides, enfuvirtide (T-20), effectively suppresses HIV-1 replication *in vivo* (Kilby et al., 1998; Lalezari et al., 2003; Lazzarin et al., 2003). Another C-HR-derived peptide, C34, which contains the four amino acids, W117, W120, D121, and I124, required to dock into a hydrophobic pocket termed the "deep pocket" of the trimer of the N-HR also exerts strong inhibition of HIV-1 fusion *in vitro* (Chan et al., 1997). In addition to peptides derived from the HIV-1 gp41 consensus amino acid sequence, several modified peptides have also been developed, including T-1249 (Eron et al., 2004), T-2635 (Dwyer et al., 2007), SC34EK (Nishikawa et al., 2009) and T-20$_{S138A}$ (Izumi et al., 2009).

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To date, resistant variants to C-HR-derived peptides, such as T-20 and C34, have been extensively investigated. Resistant variants to T-20 have emerged with mutations in the N-HR region, especially from L33 to L45, which is thought to be the binding site of T-20, both *in vitro* (Fikkert et al., 2002; Rimsky et al., 1998) and *in vivo* (Aquaro et al., 2006; Bienvenu et al., 2006; Cabrera et al., 2006; Labrosse et al., 2006; Menzo et al., 2004; Perez-Alvarez et al., 2006; Ray et al., 2007; Wei et al., 2002), suggesting that substitutions in the N-HR directly interfere with T-20 binding. Variants resistant to C34 also emerged in vitro with amino acid mutations in the N-HR region (Armand-Ugon et al., 2003; Lohrengel et al., 2005; Nameki et al., 2005). Taken together, mutations in the N-HR region play a key role in resistance to C-HR-derived peptides.

An N-HR-derived peptide N36 corresponding to the leucine/isoleucine zipper sequence of gp41 has anti-HIV-1 activity to a lesser extent when compared to T-20 and C34 (Dubay et al., 1992; Wild et al., 1994; Wild et al., 1992). It is believed that N36 easily aggregates in physiological solutions, resulting in reduced potency (Lu et al., 1995; Lu and Kim, 1997). Meanwhile, a stabilized and trimeric coiled-coil N peptide, IZN17, was developed and displayed 100-fold greater potency compared with N36 (Eckert and Kim, 2001a). Moreover, $(CCIZN17)_3$, a covalently stabilized trimer of IZN17s, represented 30-fold increase in potency compared with IZN17 (Bianchi et al., 2005). These studies demonstrated that appropriate engineering of N-HR-derived peptides could increase their inhibitory effect, suggesting that development of N-HR-derived peptides is one of the novel candidates for effective HIV-1 inhibitors.

In this study, we selected for and characterized HIV-1 variants resistant to N36 by virological, biochemical and X-ray crystallographic analyses, with the aim of elucidating further information regarding HIV-1 fusion.

2. Materials and methods

2.1. Cells and viruses

MT-2 and 293T cells were grown in RPMI 1640 medium and Dulbecco's modified Eagle medium (DMEM), respectively. HeLa-CD4-LTR-β-gal cells were kindly provided by M. Emerman through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease (Bethesda, MD, USA), and used for the drug susceptibility assay as described previously (Nameki et al., 2005). Recombinant infectious HIV-1 clones carrying various mutations were generated by pNL4-3 plasmid with site-directed mutagenesis as described previously (Nameki et al., 2005). Each molecular clone was transfected into 293T cells with *Trans*IT® (Mirus Bio LLC, Madison, WI, USA). After 48 h, the supernatants were harvested and stored at −80 °C until required.

2.2. Antiviral agents

The peptides used in this study were synthesized as described previously (Otaka et al., 2002). A reverse transcriptase inhibitor, 2′,3′-dideoxycytidine (ddC), and an adsorption inhibitor, dextran sulfate (DS5000) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.3. Determination of drug susceptibility

The peptide sensitivity of infectious clones was determined by the multinuclear activation of galactosidase indicator (MAGI) assay as described previously (Nameki et al., 2005). Briefly, the target cells (HeLa-CD4-LTR-β-gal; 10^4 cells/well) were plated in 96-well flat-bottomed microtiter culture plates. On the following day, the cells were inoculated with the HIV-1 clones (60 MAGI unit/well, giving 60 blue cells after 48 h of incubation) and cultured in the presence of various concentrations of drugs in fresh medium. Forty-eight hours after viral exposure, cells were stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and blue cells were counted in each well. The activity of test compounds was determined as the effective concentration that blocked HIV-1 replication by 50% (EC_{50}).

2.4. Induction of HIV-1 variants resistant to N36

MT-2 cells were exposed to wild-type HIV-1 ($HIV\text{-}1_{WT}$) and cultured in the presence of N36 at an initial concentration of 0.1 μM. Cultures were incubated at 37 °C until extensive cytopathic effect (CPE) was observed. The culture supernatants were used for further passage of MT-2 cells in the presence of increasing concentrations of N36 until massive CPE was seen at earlier stages of culture. Such dose-escalating culture was performed until resistant variants were obtained. This selection was carried out for a total of 25 passages. At the indicated passages, 10, 20, and 22, the sequence of the *env* region was determined by direct sequencing of the proviral DNA extracted from the infected MT-2 cells.

2.5. Viral replication kinetics assay

MT-2 cells (10^5 cells/mL) were infected with each virus preparation (500 MAGI unit) for 16 h. The infected cells were then washed and cultured in a final volume of 3 mL. The culture supernatants were collected from days 2–7 after infection, and the amount of p24 antigen was determined.

2.6. Circular dichroism (CD) spectroscopy

Each peptide was incubated at 37 °C for 30 min, with the final concentration of peptides were 10 μM in phosphate buffered saline (PBS) pH 7.4. CD spectra were recorded on a AVIV model 202 spectropolarimeter (AVIV) with a 1 mm path-length cuvette at 25 °C from an average of eight scans. The thermal stability was assessed by monitoring the change in the CD signal at 222 nm. The midpoint of the thermal unfolding transition, the melting temperature (T_m) of each complex was determined as described previously (Otaka et al., 2002).

2.7. Crystallization, data collection and refinement

Crystallization was performed using the hanging-drop vapor-diffusion method at 4 °C. The solution for crystallization was prepared by mixing 2 μL peptide solution (10 mg/mL each of N36 and $C34_{KQ}$ peptides) with 2 μL of mother liquor. The triangular prism-shaped crystals of the N36/$C34_{KQ}$ complex were grown in 80 mM ammonium chloride, 16% 2-methylpentan-2,4-diol and 25% isopropanol, which diffract to beyond 1.7 Å resolution and belong to space group C2 with unit-cell parameters $a = 88.63$, $b = 50.48$, $c = 56.15$ Å, $\beta = 90.88°$. X-ray diffraction data were collected at 100 K on a rotating copper-anode home X-ray source (MicroMax-007, Rigaku, Japan) equipped with an imaging plate detector (*R*-axis IV^{++}, Rigaku). The structure was solved by molecular replacement using the program MOLREP (Vagin and Teplyakov, 1997) with the model of a wild-type 6-helical bundle structure which was generated by symmetry operations from the PDB coordinate file 1AIK. Structure refinement was performed with the programs CNS (Brunger et al., 1998) and XtalView (McRee, 1999).

3. Results

3.1. Selection of N36-resistant HIV-1

In order to induce HIV-1 variants resistant to N36, escalating doses of N36 (from 0.1 μM) were applied to HIV-1$_{\text{WT}}$-infected MT-2 cells. At passage 11 (P-11), P-20 and P-22, when the concentration of N36 was 6.4, 12.8 and 25.6 μM, respectively, the sequence of the *env* region was determined by direct sequencing of the proviral DNA extracted from MT-2 cells as described previously (Fig. 1) (Nameki et al., 2005). Sequence analysis of HIV-1 at P-11 revealed that the aspartic acid residue at position 36 (D36) and the asparagine at position 126 (N126) of gp41 had been substituted for glycine (D36G) and lysine (N126K), respectively. At P-20 and P-22, E137Q in gp41 and P183Q in gp120 had emerged, respectively. Both N126K and E137Q substitutions were located in the C-HR which is thought to be the interactive site of N36 (Fig. 1A) (Chan et al., 1997). An N126K mutation was also induced in resistant viruses to C34 (Nameki et al., 2005), T-20 (Baldwin et al., 2004), modified C-HR-derived peptide, T-1249 (Eggink et al., 2008), and cell membrane-anchored C-peptide, maC46 (Hermann et al., 2009). In the bulk-sequencing, we found mixed substitution at N126K, AAG and AAA. Therefore, we cloned PCR products to a cloning vector pSL301 (Invitrogen, Carlsbad, CA, USA) and revealed that 6 and 3 clones were AAG and AAA, respectively, and that no other substitutions in the both HRs were observed.

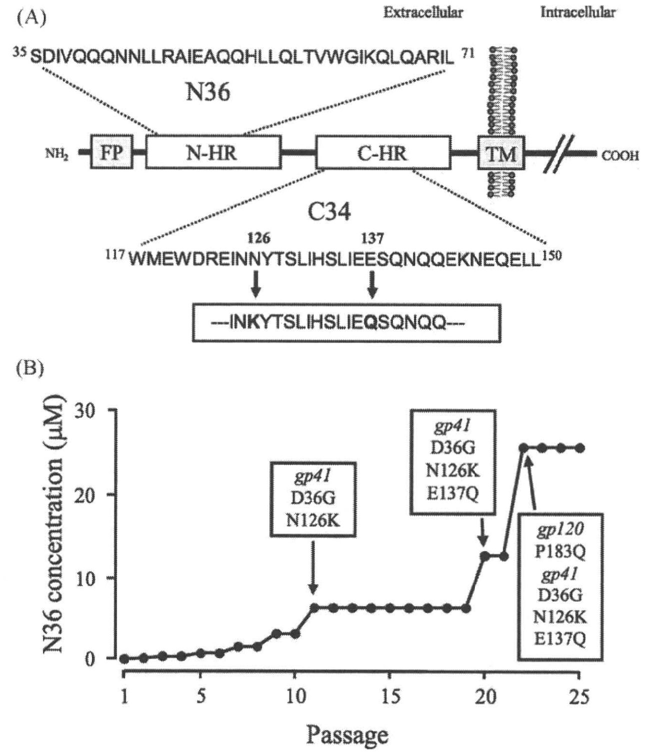

Fig. 1. Schematic view of HIV-1 gp41 (A) and induction of N36-resistant HIV-1 (B). The locations of the fusion peptide (FP), N-terminal heptad repeat region (N-HR), C-terminal heptad repeat region (C-HR), transmembrane domain (TM), and the gp41-derived peptides, N36 and C34, are shown (A). The residue numbers of each peptide correspond to their positions in gp41. The bold underlined letters in the box indicate the novel mutations that were observed in the C-HR of N36-resistant HIV-1 variants. (B) HIV-1$_{\text{WT}}$ was passaged in MT-2 cells with increasing concentrations of N36. The dose-escalating selection was carried out for a total of 25 passages, with compound concentrations ranging from 0.1 to 25.6 μM. At the indicated passages, proviral DNA from the lysates of infected cells were sequenced.

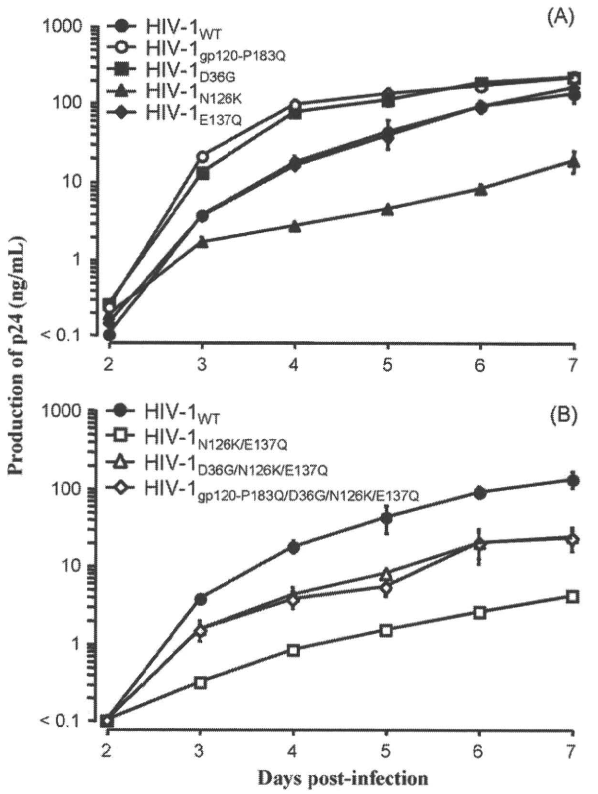

Fig. 2. Replication kinetics of N36-resistant variants. Replication kinetics of N36-resistant HIV-1 variants with a single (A) or combination of mutations (B). Supernatants were collected on days 2–7 from infected MT-2 cells and were subjected to determination of p24 production. Representative results show the mean and standard deviation of experiments performed independently three times.

3.2. Susceptibility of HIV-1 variants to N36

To identify which substitutions were responsible for N36 resistance, we generated seven recombinant viruses, and examined the susceptibility of N36 against these viruses with the MAGI assay. HIV-1$_{\text{N126K}}$, HIV-1$_{\text{E137Q}}$, HIV-1$_{\text{N126K/E137Q}}$, HIV-1$_{\text{D36G/N126K/E137Q}}$, and HIV-1$_{\text{gp120-P183Q/D36G/N126K/E137Q}}$ showed reduced susceptibility to N36 (Table 2). Since N126K emerged in HIV-1 variants resistant to C-HR-derived peptides (Baldwin et al., 2004; Nameki et al., 2005), all N126K containing viruses also demonstrated a 4–5-fold reduction in susceptibility to C34. The D36G substitution, observed in the majority of HIV-1 strains (Kuiken et al., 2009), and P183Q in gp120 demonstrated no resistance to all the inhibitors tested. P183Q which is located in the gp120 V2 region was observed in a wide range of HIV-1 subtypes including subtypes A, B, and C as well as the wild-type, indicating that P183Q is a polymorphism (Kuiken et al., 2009). These results indicate that the single mutations, N126K and E137Q, are involved in resistance to N36, but when both mutations were present, only a weak effect on resistance was observed.

3.3. Replication kinetics of N36-resistant variants

To address effects of the mutations on HIV-1 replication, we examined replication kinetics of HIV-1 variants through p24 production in culture supernatants. The N126K substitution had an adverse effect on replication kinetics, while E137Q exhibited no effect on replication kinetics compared to HIV-1$_{\text{WT}}$ (Fig. 2A). The variant, HIV-1$_{\text{N126K/E137Q}}$ had markedly reduced replication kinetics, however the D36G mutation moderately restored these kinetics

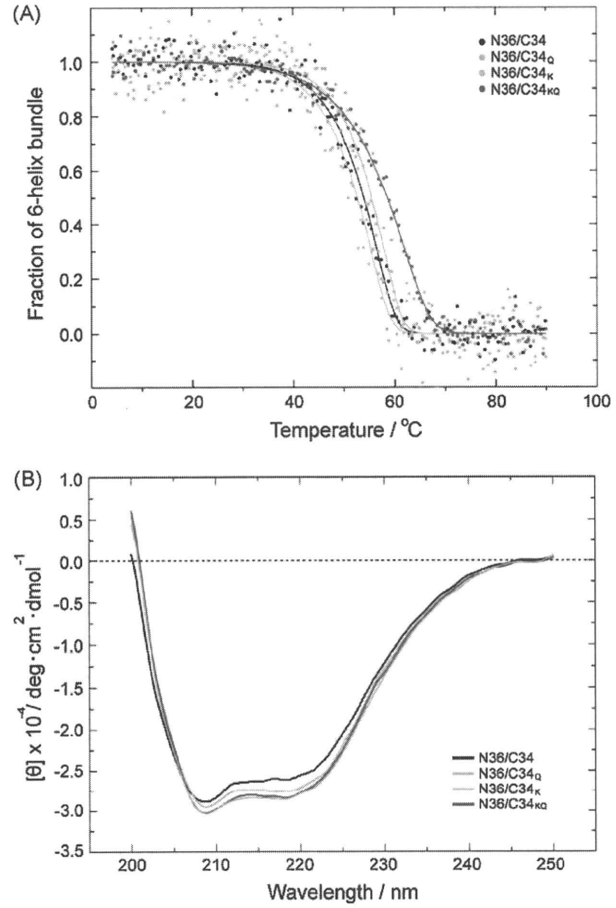

Fig. 3. Affinity of N36 and C34 with or without mutation. Melting temperature (T_m) and CD spectra of the N36/C34 (wild-type), N36/C34$_K$, N36/C34$_Q$ and N36/C34$_{KQ}$ complexes are shown (A). CD spectra profile of N36/C34 and N36/C34$_{KQ}$ complexes are shown (B).

(Fig. 2B). These results indicate that the primary mutations, N126K and E137Q, are strongly and weakly associated with a reduction in HIV-1 replication kinetics, respectively. In contrast, P183Q in gp120 solely enhanced the replication kinetics of HIV-1 (Fig. 2A) but did little to alter the replication kinetics of HIV-1$_{D36G/N126K/E137Q}$ (Fig. 2B). P183Q and D36G appear to be secondary or spontaneous mutations in HIV-1 induced by serial passage in an attempt to adapt to a cell culture environment.

3.4. *Thermal stability of the 6-helix bundle*

Synthetic peptides representing the N- and C-HRs have been shown to fold into thermally stable 6-helix bundles (Lu et al., 1999; Lu and Kim, 1997), and thus provide a model of gp41 for investigating structural and other biophysical properties of a 6-helix bundle. To determine whether N126K and E137Q contributed to the stability of the 6-helix bundle, we measured the T_m of the complexes, N36/C34 (wild-type) and N36/C34$_{KQ}$ (C34 with N126K and E137Q mutations). As shown in Fig. 3A, T_m values of the complex of N36/C34$_K$ and N36/C34$_Q$ were similar to that of N36/C34 complex, while that of N36/C34$_{KQ}$ was increased. In addition, the CD spectra observed in N36/C34$_{KQ}$ and N36/C34$_Q$ at 25 °C showed a high helix extent compared to that in N36/C34, while N36/C34$_K$ showed intermediate helicity (Fig. 3B). These results indicate that the two mutations, N126K and E137Q, co-operatively provide high thermal stability of the N36/C34$_{KQ}$ complex.

Table 1
Crystallization data, statistics and refinement parameters.

Crystallization	
Peptide solution	
N36	10 mg/mL
C34$_{KQ}$	10 mg/mL
Mother liquor	
Ammonium chloride	80 mM
2-Methylpentan-2,4-diol	16%
Isopropanol	25%
Data collection	
Resolution range/Å	35–1.7
No. of observed reflections	99,054
No. of unique reflections	27,434
Completeness	99.1%
Redundancy	3.61
R_{merge}	7.2%
Space group	C2
Unit-cell parameters	
a = 88.63, b = 50.48, c = 56.15 Å, β = 90.88°	
Refinement	
Resolution range/Å	23.8–1.7
No. of reflections in the working set	25,818
No. of protein atoms	1803
No. of water molecules	146
R/R_{free}	18.8/21.9%

3.5. *Crystal structure of the 6-helix bundle*

To reveal the structural basis of the resistance and stabilization mechanisms by the double mutations, we solved the crystal structure of the N36/C34$_{KQ}$ complex (PDB code 3AHA). The statistics for data collection and refinement are summarized in Table 1. The solved structure showed a 6-helix bundle structure, which was the same as in the wild-type N36/C34 complex (PDB code 1AIK) (Fig. 4). Their main chain structures are completely identical in helical pitch as well as in twist angle. A careful comparison between the N36/C34 and N36/C34$_{KQ}$ complexes revealed some differences in side-chain interactions. The N36/C34 complex has been solved in the trigonal *P*321 space group so that the asymmetric unit contains one of each N36 and C34 peptide, in which symmetrical side-chain interactions along the 3-fold rotation axis are formed around the bundle. We solved the N36/C34$_{KQ}$ complex in the lower symmetry *C*2 space group, and this asymmetric unit contained one helix bundle structure (Fig. 4).

The N126K mutation does not seem to induce conformational changes in the crystal structure when compared to the N36/C34 complex (Fig. 4C). It is consistent with the location of N126 that the N126K mutation does not directly interact with the binding groove formed by the N-HR trimer (Fig. 4A). In the case of E137Q, local rearrangement of a hydrogen bond was induced (Fig. 4D–G), although the hydrogen bond network formed by E/Q137-N43-R46-N42-Q142′-Q40′ is maintained. These asymmetrical and inter-helical side-chain interactions may contribute to the stability of gp41.

4. Discussion

In this study, we induced N36-resistant variants of HIV-1 *in vitro* and characterized them. The variants contained four mutations, P183Q in gp120 and D36G, N126K, and E137Q in gp41. Among these mutations, N126K and E137Q were directly associated with N36 resistance. The N126K mutation also conferred resistance in HIV-1 to C-HR-derived peptides (Baldwin et al., 2004; Eggink et al., 2008; Nameki et al., 2005) and a cell surface expressed peptide (Hermann et al., 2009), indicating that N126K is a key mutation for acquisition of resistance to all gp41-derived fusion inhibitors.

E137Q was not observed as a polymorphism (Kuiken et al., 2009) and was not identified in HIV-1 that was resistant to the N-HR-derived peptide, N13ΔTrimer, which had reasonable solubility, high helicity and thermostability (Dwyer et al., 2008). Therefore, E137Q is a novel mutation for N36 resistance. The solved crystal structure revealed that E137Q induces local rearrangement of the hydrogen bond network in gp41. A polymorphism E137K (Kuiken et al., 2009) has been identified as one of resistance associated mutations to N44 in a CCR5 tropic HIV strain, HIV-1_{JR-CSF} (Desmezieres et al., 2005). Recently, Tolstrup et al. (2007) also reported that E137K restored T-20-resistant virus infectivity impaired by the acquisition of the N43D mutation. Since E137 formed hydrogen bonds with N43, it is likely that the E137K mutation partially restores the 6-helix bundle stability (Bai et al., 2008), suggesting that E137 is an important position for stability of the 6-helix bundle and E137Q is a mutation for conferring N36-resistance in HIV-1. On the other hand, the overall structure of the 6-helix bundle with mutations was barely affected, which is consistent with our recent observations. The C34 derivative, SC34EK, which contains 12 hydrophilic and one artificial substitution (Nishikawa et al., 2009) has highly potent anti-HIV activity and maintains its structure in the 6-helix bundle even with these extensive modifications. These results indicate that the basic structure of the 6-helix bundle appears to be crucial for gp41 function, suggesting that agents which disrupt this structure will have inhibitory effects upon fusion.

Other substitutions observed in the N36 selection, P183Q in gp120 and D36G in gp41, enhanced viral replication kinetics (Fig. 2) but little influenced N36 susceptibility (Table 2). It is likely that faster entry kinetics theoretically provides resistance to fusion inhibitors through relatively short period to allow interaction of fusion inhibitors with the target, gp41. Indeed, HIV-1 with the

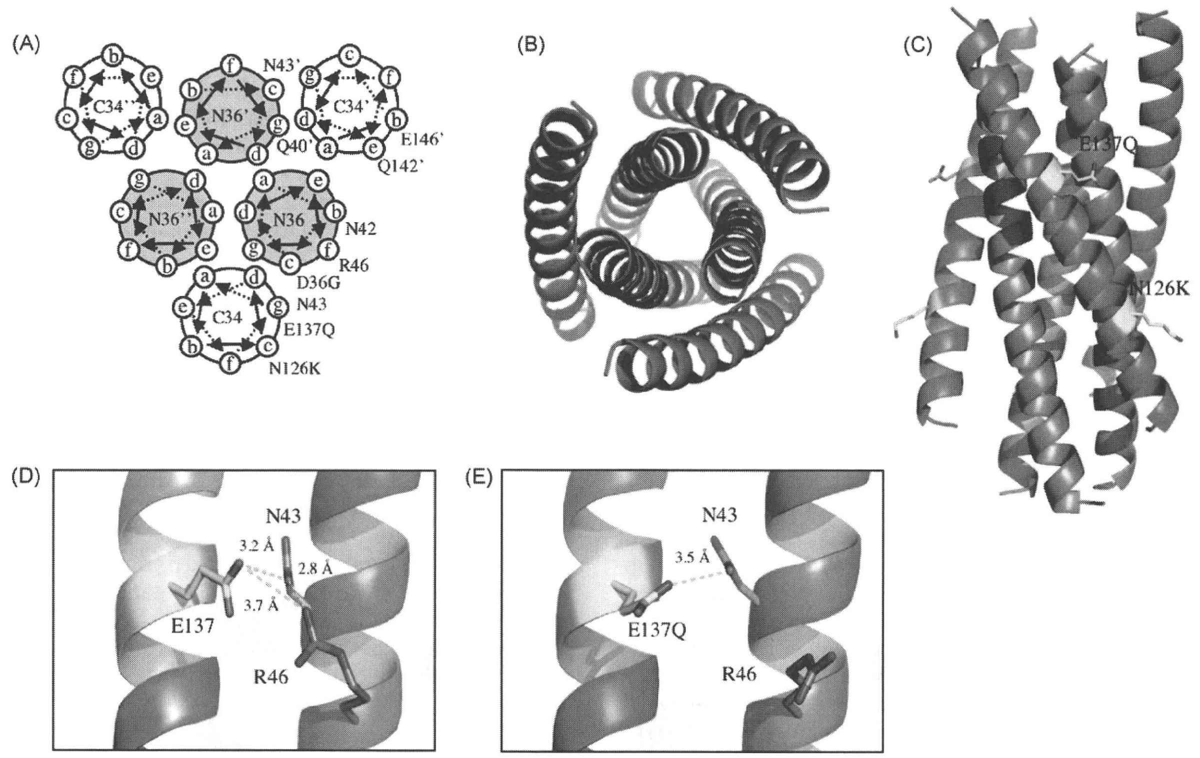

Fig. 4. Helical wheel representation of N36 and C34, and structure of N36-resistant HIV-1 gp41. The 6-helix bundle is represented as helical wheel projections (Chan et al., 1997) and the view is from the top of the complex. N36 and C34, N36′ and C34′, and N36″ and C34″ indicate each pair of a N36- and a C34-helix. The position of amino acid mutations in N36-resistant HIV-1 and the amino acids related to formation of hydrogen bonds is shown (A and B). (C) The entire structure of the 6-helix bundle with N126K and E137Q mutations. The views, focused on binding between an N36 and a C34 (D and F) and an N36 and a $C34_{KQ}$ (E and G), are shown. A hydrogen bond is depicted by the blue dashed line. (D) A hydroxyl group of the E137 side chain formed two hydrogen bonds with the amide oxygen of N43 and the amine of R46. A hydrogen bond also formed between the amide oxygen of N43 and the amine of R46. (E) The amide hydrogen of the E137Q side chain sustains a hydrogen bond with the amide oxygen of N43. The hydrogen bond formed between the amide hydrogen of E137Q and the amine of R46 was lost. The hydrogen bond formed between the amide oxygen of N43 and the amine of R46 in N36 was also lost. The views, focused on the hydrogen bond in the entire 6-helix bundle, formed with N36 and C34 (F) and N36 and $C34_{KQ}$ (G) are shown. A hydrogen bond is depicted by a blue dashed line. An apostrophe, upper right of an amino acid, means the amino acids of a neighboring helix pair as indicated in (A).

Table 2
Antiviral activity of HIV-1 gp41-derived peptides against recombinant viruses.

Viruses	EC_{50} [a] (nM)			
	ddC	DS5000	C34	N36
HIV-1_{WT}	505 ± 15[b]	56 ± 8.2	2.8 ± 1.2	200 ± 13
HIV-1_{D36G}	640 ± 76[c] (1.3)	94 ± 22 (1.7)	2.8 ± 1.2 (1)	250 ± 46 (1.3)
HIV-1_{N126K}	515 ± 86 (1)	88 ± 19 (1.5)	11 ± 3.1 (4)	550 ± 105 (2.8)
HIV-1_{E137Q}	500 ± 90 (1)	84 ± 14 (1.5)	3.0 ± 1.0 (1.1)	636 ± 159 (3.2)
HIV-$1_{N126K/E137Q}$	642 ± 84 (1.3)	74 ± 6.0 (1.3)	12 ± 4.7 (4.4)	731 ± 121 (3.7)
HIV-$1_{D36G/N126K/E137Q}$	728 ± 29 (1.4)	40 ± 4.0 (0.7)	15 ± 1.0 (5.2)	796 ± 144 (4)
HIV-$1_{gp120\text{-}P183Q}$	414 ± 50 (0.8)	58 ± 7.5 (1)	2.7 ± 0.6 (1)	343 ± 76 (1.7)
HIV-$1_{gp120\text{-}P183Q/D36G/N126K/E137Q}$	634 ± 150 (1.2)	43 ± 5.7 (0.8)	15 ± 2.4 (5.2)	573 ± 64 (2.9)

[a] Anti-HIV activity was determined with the MAGI assay.

[b] The data shown are mean value and standard deviation (SD) obtained from the results of at least three independent experiments.

[c] Values in parentheses represent fold-resistance in EC_{50} for recombinant viruses compared to HIV-1_{WT}.

faster entry kinetics shows resistance to T-20 (Reeves et al., 2002; Heredia et al., 2007; Hermann et al., 2009). However, only CCR5 tropic HIV-1s were subjected to be examined the entry kinetics and T-20 susceptibility in these studies, while CXCR4 tropic HIV-1s have been less examined yet. Interestingly, cell surface expression level of CCR5 influenced T-20 susceptibility but those of CD4 did not (Heredia et al., 2007), suggesting that entry kinetics of HIV-1 only through CCR5 influences T-20 susceptibility. In contrast, our study and others also revealed that D36G, the faster virus with CXCR4 tropism, showed high susceptibility to T-20 (Ueno et al., 2009; Mink et al., 2005; Kinomoto et al., 2005). In the present study, the faster viruses with mutations, such as P183Qand D36G little provided N36 resistance (Table 2). Thus, it is likely that there are some unknown differences in fusion inhibitor susceptibility between CXCR4 and CCR5 tropic viruses. As one of possibilities, Kahle et al. (2009) reported interesting data that asymmetric deactivation is observed in the C-HR targeted 5-helix peptide. Activity of the C-HR-derived peptide, C37 is well-correlated with binding affinity to the N-HR (K_D value), while that of 5-helix targeted the C-HR is poorly-correlated. They proposed a novel mechanism of fusion inhibition that peptides targeting N-HR and C-HR have distinctively reversible and irreversible deactivation of gp41 function, respectively.

D36G is one of the characteristic substitutions or polymorphisms for HIV-1_{NL4-3}, since only HIV-1_{NL4-3} has D36 and others originally contains G36 (Kuiken et al., 2009). Kinomoto et al. (2009) analyzed D36G effect in a structure modeling with SIV gp41 and revealed D36 could induce distortion or incorrect positioning of the N and C helices by misdirected salt bridge with K144. However, less difference between the N36 conformations of wild-type and D36G mutant was expected, since the D36G, the second amino acid from the N-terminal end of N36, is located in the flexible region of N36 sequence. Experiments with the N-HR-derived peptide including expanded N-terminal region of N36 will be needed to reveal structural feature of D36G in the N-HR. In addition to a role in the viral entry, D36G has another role in viral replication kinetics as nucleotides ($D_{GAU}36G_{GGU}$) in the stem II_C of Rev responsive element (Ueno et al., 2009; Nameki et al., 2005). Nameki et al. (2005) demonstrated that $A_{GC\underline{C}}30V_{G\underline{UC}}$ located outside of the N-HR but complementally with $D_{\underline{GAU}}36$ in the stem II_C, also enhanced replication kinetics of HIV-1_{NL4-3} (underlined nucleotides; complementally located in the stem II_C) through compensation of the RNA structure. As such, D36G has at least 2 advantages, fusion and RNA stability for viral replication. In the present study, we, therefore, focused mainly on mutations in the C-HR region. In the CD analysis, enhanced 6-helix bundle stability of N36/$C34_{KQ}$ was observed, while structural alteration of the 6-helix bundle was not apparent. We hypothesize that the mechanism of resistance to N36 has little association with the binding ability of C-HR. Although the resistance of HIV-1 to C-HR-derived peptides accounts for a correlation with the binding affinity of N-HR, an important factor in the resistance to N36 may exist before completion of 6-helix bundle formation. Steger et al. reported that the 5-helix fusion inhibitor, containing three N-HR and two C-HR segments, targeting the C-HR region in gp41 as well as N36, demonstrated poor correlation between inhibitory potency and interaction affinity with C-HR (Steger and Root, 2006). The IC_{50} values of 5-helix variants with some mutations in the sequence were inversely proportional to their association rate constants. It is consistent with observations by Kahle et al. (2009) mentioned above that mechanism of inhibition of peptides targeting the N- and C-HRs seems to be different, deactivation of gp41 function and dominant negative/decoy effect, respectively. The anti-HIV activity of N36, which has the same target as the 5-helix variants, might be also correlated with association rate constants to C-HR and finally irreversibly deactivate the gp41 function.

In conclusion, non-aggregating and trimeric coiled-coil N-HR-derived peptides such as $(CCIZN17)_3$ have been developed and exhibit more potent anti-HIV-1 activity (Bianchi et al., 2005; Eckert and Kim, 2001a), as well as exerting a strong synergistic effect with T-20 (Bianchi et al., 2005). Therefore, N-HR-derived peptides with appropriate modifications are promising because of possible co-administration with T-20 and modulation of the resistance profile. The mechanism of resistance to N36, described in this paper, provides a role for the N126K and E137Q mutations in 6-helix bundle stability, although N126 does not directly associate with the N36 surface. Further experiments are needed to clarify the role of the common N126K mutation in HIV-1 fusion.

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References

Aquaro, S., D'Arrigo, R., Svicher, V., Di Perri, G., Lo Caputo, S., Visco-Comandini, U., Santoro, M., Bertoli, A., Mazzotta, F., Bonora, S., Tozzi, V., Bellagamba, R., Zaccarelli, M., Narciso, P., Antinori, A., Perno, C.F., 2006. Specific mutations in HIV-1 gp41 are associated with immunological success in HIV-1-infected patients receiving enfuvirtide treatment. J. Antimicrob. Chemother..

Armand-Ugon, M., Gutierrez, A., Clotet, B., Este, J.A., 2003. HIV-1 resistance to the gp41-dependent fusion inhibitor C-34. Antiviral Res. 59 (2), 137–142.

Bai, X., Wilson, K.L., Seedorff, J.E., Ahrens, D., Green, J., Davison, D.K., Jin, L., Stanfield-Oakley, S.A., Mosier, S.M., Melby, T.E., Cammack, N., Wang, Z., Greenberg, M.L., Dwyer, J.J., 2008. Impact of the enfuvirtide resistance mutation N43D and the associated baseline polymorphism E137K on peptide sensitivity and six-helix bundle structure. Biochemistry.

Baldwin, C.E., Sanders, R.W., Deng, Y., Jurriaans, S., Lange, J.M., Lu, M., Berkhout, B., 2004. Emergence of a drug-dependent human immunodeficiency virus type 1 variant during therapy with the T20 fusion inhibitor. J. Virol. 78 (22), 12428–12437.

Bianchi, E., Finotto, M., Ingallinella, P., Hrin, R., Carella, A.V., Hou, X.S., Schleif, W.A., Miller, M.D., Geleziunas, R., Pessi, A., 2005. Covalent stabilization of coiled coils of the HIV gp41 N region yields extremely potent and broad inhibitors of viral infection. Proc. Natl. Acad. Sci. U.S.A. 102 (36), 12903–12908.

Bienvenu, B., Krivine, A., Rollot, F., Pietri, M.P., Lebault, V., Meritet, J.F., Guerin, C., Spiridon, G., Salmon, D., Guillevin, L., Lebon, P., Launay, O., 2006. A cohort study of enfuvirtide immunological and virological efficacy in clinical practice. J. Med. Virol. 78 (10), 1312–1317.

Brunger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.S., Kuszewski, J., Nilges, M., Pannu, N.S., Read, R.J., Rice, L.M., Simonson, T., Warren, G.L., 1998. Crystallography & NMR system: a new software suite for macromolecular structure determination. Acta Crystallogr. D: Biol. Crystallogr. 54 (Pt 5), 905–921.

Cabrera, C., Marfil, S., Garcia, E., Martinez-Picado, J., Bonjoch, A., Bofill, M., Moreno, S., Ribera, E., Domingo, P., Clotet, B., Ruiz, L., 2006. Genetic evolution of gp41 reveals a highly exclusive relationship between codons 36, 38 and 43 in gp41 under long-term enfuvirtide-containing salvage regimen. AIDS 20 (16), 2075–2080.

Chan, D.C., Chutkowski, C.T., Kim, P.S., 1998. Evidence that a prominent cavity in the coiled coil of HIV type 1 gp41 is an attractive drug target. Proc. Natl. Acad. Sci. U.S.A. 95 (26), 15613–15617.

Chan, D.C., Fass, D., Berger, J.M., Kim, P.S., 1997. Core structure of gp41 from the HIV envelope glycoprotein. Cell 89 (2), 263–273.

Desmezieres, E., Gupta, N., Vassell, R., He, Y., Peden, K., Sirota, L., Yang, Z., Wingfield, P., Weiss, C.D., 2005. Human immunodeficiency virus (HIV) gp41 escape mutants: cross-resistance to peptide inhibitors of HIV fusion and altered receptor activation of gp120. J. Virol. 79 (8), 4774–4781.

Dubay, J.W., Roberts, S.J., Brody, B., Hunter, E., 1992. Mutations in the leucine zipper of the human immunodeficiency virus type 1 transmembrane glycoprotein affect fusion and infectivity. J. Virol. 66 (8), 4748–4756.

Dwyer, J.J., Wilson, K.L., Martin, K., Seedorff, J.E., Hasan, A., Medinas, R.J., Davison, D.K., Feese, M.D., Richter, H.T., Kim, H., Matthews, T.J., Delmedico, M.K., 2008. Design of an engineered N-terminal HIV-1 gp41 trimer with enhanced stability and potency. Protein Sci. 17 (4), 633–643.

Dwyer, J.J., Wilson, K.L., Davison, D.K., Freel, S.A., Seedorff, J.E., Wring, S.A., Tvermoes, N.A., Matthews, T.J., Greenberg, M.L., Delmedico, M.K., 2007. Design of helical, oligomeric HIV-1 fusion inhibitor peptides with potent activity against enfuvirtide-resistant virus. Proc. Natl. Acad. Sci. U.S.A. 104 (31), 12772–12777.

Eckert, D.M., Kim, P.S., 2001a. Design of potent inhibitors of HIV-1 entry from the gp41 N-peptide region. Proc. Natl. Acad. Sci. U.S.A. 98 (20), 11187–11192.

Eckert, D.M., Kim, P.S., 2001b. Mechanisms of viral membrane fusion and its inhibition. Annu. Rev. Biochem. 70, 777–810.

Eggink, D., Baldwin, C.E., Deng, Y., Langedijk, J.P., Lu, M., Sanders, R.W., Berkhout, B., 2008. Selection of T1249-resistant human immunodeficiency virus type 1 variants. J. Virol. 82 (13), 6678–6688.

Eron, J.J., Gulick, R.M., Bartlett, J.A., Merigan, T., Arduino, R., Kilby, J.M., Yangco, B., Diers, A., Drobnes, C., DeMasi, R., Greenberg, M., Melby, T., Raskino, C., Rusnak, P., Zhang, Y., Spence, R., Miralles, G.D., 2004. Short-term safety and antiretroviral activity of T-1249, a second-generation fusion inhibitor of HIV. J. Infect. Dis. 189 (6), 1075–1083.

Fikkert, V., Cherepanov, P., Van Laethem, K., Hantson, A., Van Remoortel, B., Pannecouque, C., De Clercq, E., Debyser, Z., Vandamme, A.M., Witvrouw, M., 2002. Env chimeric virus technology for evaluating human immunodeficiency virus susceptibility to entry inhibitors. Antimicrob. Agents Chemother. 46 (12), 3954–3962.

Heredia, A., Gilliam, B., DeVico, A., Le, N., Bamba, D., Flinko, R., Lewis, G., Gallo, R.C., Redfield, R.R., 2007. CCR5 density levels on primary CD4 T cells impact the replication and Enfuvirtide susceptibility of R5 HIV-1. AIDS 21 (10), 1317–1322.

Hermann, F.G., Egerer, L., Brauer, F., Gerum, C., Schwalbe, H., Dietrich, U., von Laer, D., 2009. Mutations in gp120 contribute to the resistance of human immunodeficiency virus type 1 to membrane-anchored C-peptide maC46. J. Virol. 83 (10), 4844–4853.

Izumi, K., Kodama, E., Shimura, K., Sakagami, Y., Watanabe, K., Ito, S., Watabe, T., Terakawa, Y., Nishikawa, H., Sarafianos, S.G., Kitaura, K., Oishi, S., Fujii, N., Matsuoka, M., 2009. Design of peptide-based inhibitors for human immunodeficiency virus type 1 strains resistant to T-20. J. Biol. Chem. 284 (8), 4914–49120.

Kahle, K.M., Steger, H.K., Root, M.J., 2009. Asymmetric deactivation of HIV-1 gp41 following fusion inhibitor binding. PLoS Pathog. 5 (11), e1000674.

Kilby, J.M., Hopkins, S., Venetta, T.M., DiMassimo, B., Cloud, G.A., Lee, J.Y., Alldredge, L., Hunter, E., Lambert, D., Bolognesi, D., Matthews, T., Johnson, M.R., Nowak, M.A., Shaw, G.M., Saag, M.S., 1998. Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. Nat. Med. 4 (11), 1302–1307.

Kinomoto, M., Yokoyama, M., Sato, H., Kojima, A., Kurata, T., Ikuta, K., Sata, T., Tokunaga, K., 2005. Amino acid 36 in the human immunodeficiency virus type 1 gp41 ectodomain controls fusogenic activity: implications for the molecular mechanism of viral escape from a fusion inhibitor. J. Virol. 79 (10), 5996–6004.

Kuiken, C., Leitner, T., Foley, B., Hahn, B., Marx, McCutchan, F., Wolinsky, S., Korber, B., 2009. HIV sequence compendium, 2009. Theoretical Biology and Biophysics Group. Los Alamos National Laboratory, Los Alamos, NM.

Labrosse, B., Morand-Joubert, L., Goubard, A., Rochas, S., Labernardiere, J.L., Pacanowski, J., Meynard, J.L., Hance, A.J., Clavel, F., Mammano, F., 2006. Role of the envelope genetic context in the development of enfuvirtide resistance in human immunodeficiency virus type 1-infected patients. J. Virol. 80 (17), 8807–8819.

Lalezari, J.P., Henry, K., O'Hearn, M., Montaner, J.S., Piliero, P.J., Trottier, B., Walmsley, S., Cohen, C., Kuritzkes, D.R., Eron Jr., J.J., Chung, J., DeMasi, R., Donatacci, L., Drobnes, C., Delehanty, J., Salgo, M., 2003. Enfuvirtide, an HIV-1 fusion inhibitor, for drug-resistant HIV infection in North and South America. N. Engl. J. Med. 348 (22), 2175–2185.

Lazzarin, A., Clotet, B., Cooper, D., Reynes, J., Arasteh, K., Nelson, M., Katlama, C., Stellbrink, H.J., Delfraissy, J.F., Lange, J., Huson, L., DeMasi, R., Wat, C., Delehanty, J., Drobnes, C., Salgo, M., 2003. Efficacy of enfuvirtide in patients infected with drug-resistant HIV-1 in Europe and Australia. N. Engl. J. Med. 348 (22), 2186–2195.

Lohrengel, S., Hermann, F., Hagmann, I., Oberwinkler, H., Scrivano, L., Hoffmann, C., von Laer, D., Dittmar, M.T., 2005. Determinants of human immunodeficiency virus type 1 resistance to membrane-anchored gp41-derived peptides. J. Virol. 79 (16), 10237–10246.

Lu, M., Blacklow, S.C., Kim, P.S., 1995. A trimeric structural domain of the HIV-1 transmembrane glycoprotein. Nat. Struct. Biol. 2 (12), 1075–1082.

Lu, M., Ji, H., Shen, S., 1999. Subdomain folding and biological activity of the core structure from human immunodeficiency virus type 1 gp41: implications for viral membrane fusion. J. Virol. 73 (5), 4433–4438.

Lu, M., Kim, P.S., 1997. A trimeric structural subdomain of the HIV-1 transmembrane glycoprotein. J. Biomol. Struct. Dyn. 15 (3), 465–471.

McRee, D.E., 1999. XtalView/Xfit—a versatile program for manipulating atomic coordinates and electron density. J. Struct. Biol. 125 (2–3), 156–165.

Menzo, S., Castagna, A., Monachetti, A., Hasson, H., Danise, A., Carini, E., Bagnarelli, P., Lazzarin, A., Clementi, M., 2004. Genotype and phenotype patterns of human immunodeficiency virus type 1 resistance to enfuvirtide during long-term treatment. Antimicrob. Agents Chemother. 48 (9), 3253–3259.

Mink, M., Mosier, S.M., Janumpalli, S., Davison, D., Jin, L., Melby, T., Sista, P., Erickson, J., Lambert, D., Stanfield-Oakley, S.A., Salgo, M., Cammack, N., Matthews, T., Greenberg, M.L., 2005. Impact of human immunodeficiency virus type 1 gp41 amino acid substitutions selected during enfuvirtide treatment on gp41 binding and antiviral potency of enfuvirtide in vitro. J. Virol. 79 (19), 12447–12454.

Nameki, D., Kodama, E., Ikeuchi, M., Mabuchi, N., Otaka, A., Tamamura, H., Ohno, M., Fujii, N., Matsuoka, M., 2005. Mutations conferring resistance to human immunodeficiency virus type 1 fusion inhibitors are restricted by gp41 and Rev-responsive element functions. J. Virol. 79 (2), 764–770.

Nishikawa, H., Nakamura, S., Kodama, E., Ito, S., Kajiwara, K., Izumi, K., Sakagami, Y., Oishi, S., Ohkubo, T., Kobayashi, Y., Otaka, A., Fujii, N., Matsuoka, M., 2009. Electrostatically constrained alpha-helical peptide inhibits replication of HIV-1 resistant to enfuvirtide. Int. J. Biochem. Cell Biol. 41 (4), 891–899.

Otaka, A., Nakamura, M., Nameki, D., Kodama, E., Uchiyama, S., Nakamura, S., Nakano, H., Tamamura, H., Kobayashi, Y., Matsuoka, M., Fujii, N., 2002. Remodeling of gp41-C34 peptide leads to highly effective inhibitors of the fusion of HIV-1 with target cells. Angew. Chem. Int. Ed. Engl. 41 (16), 2937–2940.

Perez-Alvarez, L., Carmona, R., Ocampo, A., Asorey, A., Miralles, C., Perez de Castro, S., Pinilla, M., Contreras, G., Taboada, J.A., Najera, R., 2006. Long-term monitoring of genotypic and phenotypic resistance to T20 in treated patients infected with HIV-1. J. Med. Virol. 78 (2), 141–147.

Ray, N., Harrison, J.E., Blackburn, L.A., Martin, J.N., Deeks, S.G., Doms, R.W., 2007. Clinical resistance to enfuvirtide does not affect susceptibility of human immunodeficiency virus type 1 to other classes of entry inhibitors. J. Virol. 81 (7), 3240–3250.

Rimsky, L.T., Shugars, D.C., Matthews, T.J., 1998. Determinants of human immunodeficiency virus type 1 resistance to gp41-derived inhibitory peptides. J. Virol. 72 (2), 986–993.

Reeves, J.D., Gallo, S.A., Ahmad, N., Miamidian, J.L., Harvey, P.E., Sharron, M., Pohlmann, S., Sfakianos, J.N., Derdeyn, C.A., Blumenthal, R., Hunter, E., Doms, R.W., 2002. Sensitivity of HIV-1 to entry inhibitors correlates with envelope/coreceptor affinity, receptor density, and fusion kinetics. Proc. Natl. Acad. Sci. U.S.A. 99 (25), 16249–16254.

Steger, H.K., Root, M.J., 2006. Kinetic dependence to HIV-1 entry inhibition. J. Biol. Chem. 281 (35), 25813–25821.

Tolstrup, M., Selzer-Plon, J., Laursen, A.L., Bertelsen, L., Gerstoft, J., Duch, M., Pedersen, F.S., Ostergaard, L., 2007. Full fusion competence rescue of the enfuvirtide resistant HIV-1 gp41 genotype (43D) by a prevalent polymorphism (137K). AIDS 21 (4), 519–521.

Ueno, M., Kodama, E.N., Shimura, K., Sakurai, Y., Kajiwara, K., Sakagami, Y., Oishi, S., Fujii, N., Matsuoka, M., 2009. Synonymous mutations in stem-loop III of Rev responsive elements enhance HIV-1 replication impaired by primary mutations for resistance to enfuvirtide. Antiviral Res. 82 (1), 67–72.

Vagin, A., Teplyakov, A., 1997. MOLREP: an Automated Program for Molecular Replacement. J. Appl. Crystallogr. 30 (Pt 6), 1022–1025.

Wei, X., Decker, J.M., Liu, H., Zhang, Z., Arani, R.B., Kilby, J.M., Saag, M.S., Wu, X., Shaw, G.M., Kappes, J.C., 2002. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. Antimicrob. Agents Chemother. 46 (6), 1896–1905.

Wild, C., Dubay, J.W., Greenwell, T., Baird Jr., T., Oas, T.G., McDanal, C., Hunter, E., Matthews, T., 1994. Propensity for a leucine zipper-like domain of human immunodeficiency virus type 1 gp41 to form oligomers correlates with a role in virus-induced fusion rather than assembly of the glycoprotein complex. Proc. Natl. Acad. Sci. U.S.A. 91 (26), 12676–12680.

Wild, C., Greenwell, T., Matthews, T., 1993. A synthetic peptide from HIV-1 gp41 is a potent inhibitor of virus-mediated cell–cell fusion. AIDS Res. Hum. Retroviruses 9 (11), 1051–1053.

Wild, C., Oas, T., McDanal, C., Bolognesi, D., Matthews, T., 1992. A synthetic peptide inhibitor of human immunodeficiency virus replication: correlation between solution structure and viral inhibition. Proc. Natl. Acad. Sci. U.S.A. 89 (21), 10537–10541.

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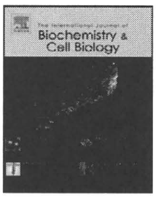

Rev-derived peptides inhibit HIV-1 replication by antagonism of Rev and a co-receptor, CXCR4

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ABSTRACT

Rev, a viral regulatory protein of HIV-1, binds through its arginine-rich domain to the Rev-responsive element (RRE), a secondary structure in transcribed HIV-1 RNA. Binding of Rev to RRE mediates export of singly spliced or unspliced mRNAs from the nucleus to the cytoplasm. It has been previously shown that a certain arginine-rich peptide exhibits not only RRE-binding ability but also cell permeability and antagonism of CXCR4, one of the major coreceptors of HIV-1. Here we designed and synthesized arginine-rich peptides derived from the RNA-binding domain of Rev (Rev$_{34-50}$) and evaluated their anti-HIV-1 activities. Rev$_{34-50}$-A$_4$C, comprising Rev$_{34-50}$ with AAAAC at the C-terminus to increase the α-helicity, inhibited HIV-1 entry by CXCR4 antagonism and virus production in persistently HIV-1-infected PM1-CCR5 cells. Interestingly, similar motif of human lymphotropic virus type I Rex (Rex$_{1-21}$) also exerted moderate anti-HIV-1 activity. These results indicate that arginine-rich peptide, Rev$_{34-50}$-A$_4$C exerts dual antagonism against CXCR4 and Rev.

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1. Introduction

HIV-1 attaches to its target cells through a major receptor, CD4, and then interacts with coreceptors such as chemokine receptors to enter the cells (Este and Telenti, 2007). The T-cell-line-tropic (X4) and macrophage-tropic (R5) HIV-1 strains mainly use CXCR4 and CCR5 as coreceptors, respectively. HIV-1 completes its infection by integration of reverse-transcribed double-stranded cDNA into the host genome. Expression of HIV-1 genes is enhanced by the viral transactivator protein Tat, while regulation of the expressed genes through RNA splicing is tightly controlled by the viral regulatory protein Rev. Some expressed RNAs are protected against RNA splicing by binding of Rev to a Rev-responsive element (RRE), comprising an RNA secondary structure co-encoded with gp41, resulting in singly spliced (e.g., for *env* mRNA) or unspliced (e.g., for *gag-pol* mRNA or viral progeny genomes) RNAs, which are exported to the cytoplasm by Rev and used for viral protein synthesis and/or progeny genomes (Felber et al., 1989; Malim et al., 1989b).

Rev contains several functional signals, such as a nuclear localization signal (NLS; aa 35–50), which also serves as an RNA-binding domain (Malim and Cullen, 1991; Olsen et al., 1990), and a nuclear export signal (NES; aa 75–84) as shown in Fig. 1 (Fischer et al., 1995; Szilvay et al., 1995). The RNA structure of the RRE in these singly and unspliced RNAs plays an important role in the interaction with Rev (Charpentier et al., 1997; Dayton et al., 1989). Rev mainly binds to stem-loop IIB of the RRE and oligomerization of Rev on the RRE is required for sufficient RNA export (Daugherty et al., 2008; Kjems et al., 1992; Mann et al., 1994; Zapp et al., 1991). Another human retrovirus, human T-cell leukemia virus type I (HTLV-I), also has a regulatory protein, Rex, which is the counterpart of HIV-1 Rev. Rex interacts with not only a Rex-responsive element (RxRE) but also with the HIV-1 RRE via its RNA-binding domain (aa 1–16), which also acts as an NLS (Bogerd et al., 1991; Hammes and Greene, 1993; Siomi et al., 1988). Functionally, Rex is also involved in the nuclear export of RNAs containing an RxRE or RRE (Rimsky et al., 1988). However, Rex and Rev interact with the RRE in different manners, since Rev mainly binds to stem-loop IIB while Rex mainly binds to the stem-loop III/IV/V region (Ahmed et al., 1990; Bogerd et al., 1991; Charpentier et al., 1997; Kjems et al., 1992).

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