View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

ELSEVIER

Available online at www.sciencedirect.com

SCIENCE DIRECT®

Virology 344 (2006) 354 – 362

VIROLOGY

www.elsevier.com/locate/yviro

Mutations other than 103N in human immunodeficiency virus type 1 reverse transcriptase (RT) emerge from K103R polymorphism under non-nucleoside RT inhibitor pressure

Hiroyuki Gatanaga *, Atsuko Hachiya, Satoshi Kimura, Shinichi Oka

AIDS Clinical Center, International Medical Center of Japan, 1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655, Japan

Received 19 June 2005; returned to author for revision 29 July 2005; accepted 7 September 2005
Available online 10 October 2005

Abstract

K103N mutation in human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) confers high-level resistance against non-nucleoside RT inhibitors (NNRTIs) and it easily occurs partly because it arises by a single nucleotide substitution from wild-type K103. There are polymorphisms at codon 103 of HIV-1 RT. We found K103R polymorphic mutation in 3.3% of treatment-naive HIV-1-infected patients. R103N does not seem to occur as easily as K103N because R103N requires two nucleotide substitutions. To induce NNRTI resistance-associated mutations, HIV-1_{K103R} was propagated in the presence of increasing concentrations of efavirez (EFV) or nevirapine (NVP). V179D emerged in all three EFV cultures and in two of four NVP cultures. R103G emerged by a single nucleotide substitution in one of three EFV cultures. R103N did not emerge in any of 7 NNRTI cultures. Analysis of recombinant HIV-1s showed that HIV-$1_{K103R/V179D}$ was significantly resistant and HIV-1_{K103G} was moderately resistant against EFV and NVP.
© 2005 Elsevier Inc. All rights reserved.

Keywords: K103R; Polymorphic; Mutation; Non-nucleoside reverse transcriptase inhibitor; Resistance; HIV-1

Introduction

The emergence of human immunodeficiency virus type 1 (HIV-1) variants resistant to antiretroviral agents is one of the most common causes for therapeutic failure in infected individuals. Fortunately, the availability of drug resistance testing has substantially improved the ability of clinicians to deal knowledgeably with drug-resistant HIV-1 strains (Vandamme et al., 2004). Recent studies, however, have revealed that certain polymorphic amino acid residues of HIV-1 contribute to drug resistance (Gatanaga et al., 2002; Harrigan et al., 2005; Tanaka et al., 1997). Even if polymorphic mutations themselves do not alter drug susceptibility, their combination with other unreported mutations might confer significant drug resistance. Such combinations are probably not included in the current reference tables of drug resistance-associated mutations and the effect of preexisting polymorphic mutations on the development of drug resistance is not well taken into consideration (Johnson et al., 2005).

Non-nucleoside reverse transcriptase inhibitor (NNRTI)-containing combination of antiretroviral agents are recommended as the first-line regimens for HIV-1-infected individuals (Yeni et al., 2004). One of the most critical problems, however, is the easy emergence of NNRTI resistance-associated mutations, among which the K103N mutation in reverse transcriptase (RT) is the most common and important (Bacheler, 1999; De Clercq, 1998; Harrigan et al., 2005; Nunberg et al., 1991). However, there are polymorphic mutations at codon 103 of HIV-1 RT (Harrigan et al., 2005), and their effect on emergence of drug resistance is not well studied. In this study, we analyzed the polymorphic frequencies at codon 103 of HIV-1 RT in treatment-naive patients and determined the effect of such polymorphism on the development of NNRTI resistance.

* Corresponding author. Fax: +81 3 5273 5193.
E-mail address: higatana@imcj.acc.go.jp (H. Gatanaga).

0042-6822/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2005.09.005

Results

Frequency of K103R in treatment-naïve patients

We analyzed HIV-1 sequences in plasma samples of 211 antiretroviral treatment-naive infected individuals who visited our clinic from January, 2000 to December, 2003, and found wild-type K103 in 204 patients (96.7%; nucleotide triplet: AAA in 202 samples, AAG in 2 samples) and K103R in six (2.8%; nucleotide triplet: all AGA) and a mixture of these in one (0.5%; nucleotide triplet: AAA and AGA) (Table 1). Taken together, K103R was detected in 3.3% of our treatment-naive patients by direct sequencing.

Induction of EFV-resistant HIV-1 variants

The K103N mutation can arise by a single nucleotide substitution (AAA to AAC or AAT, AAG to AAC or AAT) from wild-type K103, while the amino acid substitution from arginine (R) to asparagine (N) at codon 103, R103N, necessitates at least two nucleotide substitutions (AGA to AAC or AAT). Therefore, R103N does not seem to occur as easily as K103N. In order to find the types of mutations that can be induced by NNRTI from K103R, the infectious HIV-1 clones harboring wild-type K103 (HIV-1_{WT}) and K103R (HIV-1_{K103R}) in RT were propagated in MT-2 cells in the presence of increasing concentrations of EFV, respectively. The culture supernatant was harvested on day 7 of culture and used to infect fresh MT-2 cells for the next round of culture. When the virus began to propagate in the presence of the drug, the drug concentration was increased. HIV-1_{WT} harboring nucleotide triplet AAA at codon 103 of RT, was propagated in two independent cultures (Figs. 1A and B). In one culture, I178M mutation first emerged at 11 weeks of passages and K103N was added to it at 22 weeks (Fig. 1A). In the other culture, Y181C first emerged at 6 weeks, and K101E and V179D were combined to it at 16 weeks (Fig. 1B). Furthermore, V106I and V108I were added to them at 20 weeks, but V106I could not be detected by direct sequencing at 25 weeks (Fig. 1B). In order to determine the effect of synonymous nucleotide substitution on the development of EFV resistance, HIV-1_{WT} harboring nucleotide triplet AAG at codon 103 of RT, was also propagated in the same way (Fig. 1C). K103N emerged at 12 weeks, and L100I was combined to it at 25 weeks. Considered together, these results indicated it was easy for K103N to emerge as it arose in two of three cultures of HIV-1_{WT} under the selective pressure of EFV.

HIV-1_{K103R} was propagated in three independent cultures (Figs. 1D–F). In one culture, G190A emerged at 11 passages, and V179D was added at 25 weeks (Fig. 1D). In another culture, R103G, which has not been reported previously, arose by a single nucleotide mutation (nucleotide triplet: AGA to GGA) at 8 weeks, and V106I emerged at 11 weeks (Fig. 1E). At 22 weeks, V106I could not be detected by direct sequencing and V108I, V179D, and G190A emerged. Finally, R103G and V108I could not be detected and V106I re-emerged at 25 weeks (Fig. 1E). In the last culture, V179D emerged at 14 weeks and no other mutations were added to it until the EFV concentration reached 1000 nM at 25 weeks, suggesting that the combination of K103R and V179D conferred high-level resistance to EFV (Fig. 1F). In conclusion, R103N mutation was not detected in any of the three culture of HIV-1_{K103R}, although it often arose in cultures of HIV-1_{WT}. Instead of R103N, R103G occurred by a single nucleotide mutation in one culture. V179D emerged in all of three cultures of HIV-1_{K103R} and the combination of K103R and V179D seemed to confer high-level resistance to EFV.

Clonal determination of amino acid mutations in EFV-resistant HIV-1 variants

In one culture of HIV-1_{K103R} described above (Fig. 1E), 103G mutation, which has not been reported previously, and V106I mutation, which is polymorphic (Rhee et al., 2003), were detected at early passages and the mutation pattern was complicated with the appearance and disappearance of various mutations at late passages. Therefore, we decided to analyze the mutations by cloning at several passages (Table 2). At 14 weeks when EFV concentration had not yet increased, R103G and V106I emerged in independent clones. However, the V106I clone without any other acquired mutation was not detected and every analyzed clone had R103G at 18 weeks when EFV concentration was increased to 30 nM. These results indicate that V106I itself conferred little resistance to EFV to HIV-1_{K103R} and that R103G conferred moderate resistance. At 20 weeks when EFV concentration was further increased to 100 nM, 19 of 20 clones had R103G, and one clone had V106I and V179D. The latter clone still existed at 22 weeks (EFV: 500 nM) at a frequency of 2 of 21, which suggests that the addition of V106I and V179D to HIV-1_{K103R} conferred high-level resistance to EFV. At 25 weeks (EFV: 1000 nM), the clone with V106I, V179D, and G190A predominated other clones. Considered together, the results indicate that V106I clone without any other acquired mutation existed only at the initial EFV concentration (3 nM), and once EFV concentration was increased, every analyzed clone had R103G or the combination of K103R, V106I, and V179D, suggesting that V106I itself conferred little EFV resistance to HIV-1_{K103R} and that R103G and the combination K103R, V106I, and V179D conferred significant resistance to EFV.

Table 1
Frequency of mutations at codon 103 of HIV-1 RT in treatment-naive patients

Amino acid (nucleotides)	Number of patients
K (AAA)	202
K (AAG)	2
R (AGA)	6
K (AAA)/R (AGA)[a]	1
Total	211

Deduced amino acids and nucleotide triplets at codon 103 of HIV-1 RT are shown.
[a] Mixture of K (AAA) and R (AGA).

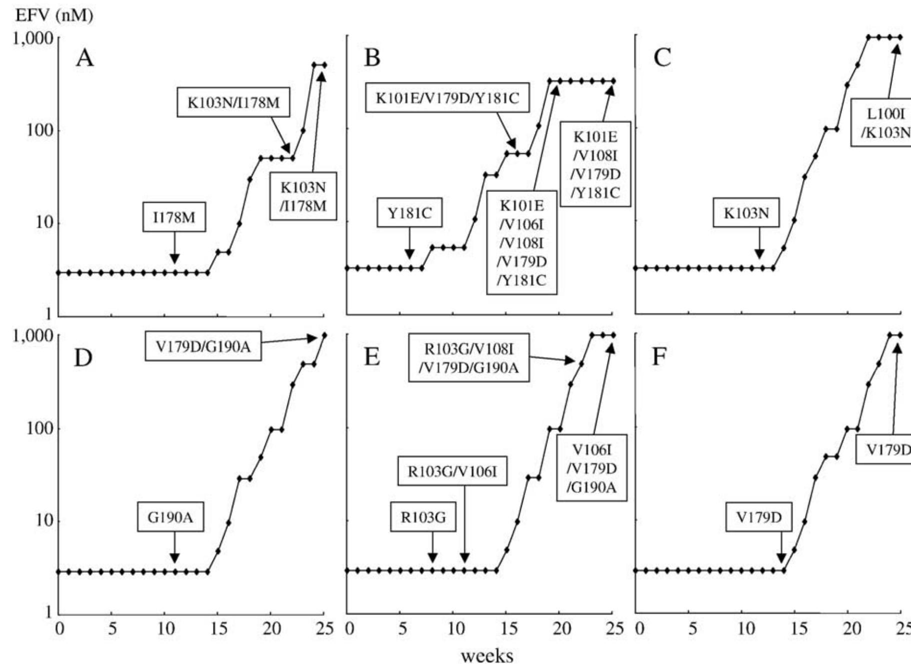

Fig. 1. Induced amino acid substitutions under the selective pressure of EFV. HIV-1_{WT} harboring nucleotide triplet AAA (panels A and B), and AAG (panel C) at codon 103 of RT, and HIV-1_{K103R} (panels D–F) were propagated in MT-2 cells in the presence of increasing concentrations of EFV. Induced amino acid substitutions were analyzed at several passages by sequencing proviral HIV-1 RT in MT-2 cells.

Induction of NVP-resistant HIV-1 variants

Under the selective pressure of EFV, R103N did not emerge but the unreported R103G appeared instead, and V179D developed in every culture of HIV-1_{K103R}. To obtain further insight on the development of NNRTI resistance from HIV-1_{K103R}, we passaged HIV-1_{K103R} in the presence of increasing concentrations of NVP. HIV-1_{K103R} was propagated in four independent cultures (Figs. 2A–D). In one culture, V179D emerged first at 10 weeks and V106A, which is well known to confer significant resistance to NVP (Johnson et al., 2005), was added to it at 19 weeks (Fig. 2A). In the other three cultures (Figs. 2B–D), V106A emerged first at 8–10 weeks, and V179D was combined to it at 16 weeks in one culture (Fig. 2C). In summary, R103N did not emerge in any of the four cultures of HIV-1_{K103R} as expected, and V106A appeared in every culture. V179D, which was observed in all three cultures with EFV, emerged in two of four cultures with NVP, suggesting that the combination of K103R and V179D conferred significant resistance not only to EFV but also to NVP and increased the viral replication cooperated with V106A in the presence of NVP.

Table 2
Clonal sequence analysis of RT gene of HIV-1 passaged in the presence of EFV (Fig. 1E)

Weeks (EFV)	Amino acid						Clones/total
	103 (R)	106 (V)	108 (V)	135 (I)	179 (V)	190 (G)	
14 (3 nM)	–	–	–	–	–	–	7
	G	–	–	–	–	–	7
	–	I	–	–	–	–	3/17
18 (30 nM)	G	–	–	–	–	–	21
	G	I	–	–	–	–	3/24
20 (100 nM)	G	I	–	–	–	–	10
	G	–	–	–	–	–	9
	–	I	–	–	D	–	1/20
22 (500 nM)	G	–	I	–	–	A	14
	G	–	–	–	D	–	2
	–	I	–	–	D	–	2
	G	–	–	–	–	A	1
	G	–	I	–	D	A	1
	G	–	I	V	D	–	1/21
23 (1000 nM)	–	I	–	–	D	A	7
	G	–	–	V	D	–	6
	G	–	I	–	–	A	5
	G	–	I	–	D	A	1
	G	–	–	V	D	A	1/20
25 (1000 nM)	–	I	–	–	D	A	20/20

Data are deduced amino acids at the positions where mutations were detected. Identity with original amino acids shown at the top is indicated by the dash sign.

NNRTI resistance conferred by K103R/V179D and K103G

In order to analyze the effect of each mutation and their combinations on drug resistance, we constructed a panel of recombinant HIV-1 clones and determined their IC_{50} for four NRTIs, including zidovudine (AZT), lamivudine (3TC), stavudine (d4T), and tenofovir disoproxil fumarate (TDF), and two NNRTIs, including EFV and NVP. Drug resistance assay was performed in triplicate using MAGIC-5 cells (Table 3) (Hachiya et al., 2001, 2003, 2004). As reported previously, K103R and V106I alone did not confer significant drug resistance (Harrigan et al., 2005; Rhee et al., 2003). Furthermore, their combination, K103R/V106I, did not exhibit significant drug resistance, although it was observed

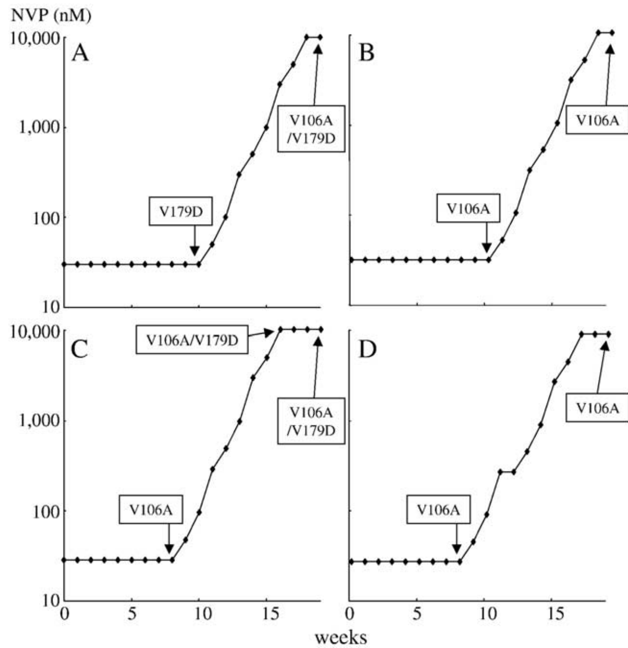

Fig. 2. Induced amino acid substitutions under the selective pressure of NVP. HIV-1_{K103R} was propagated in four cultures (panels A–D) of MT-2 cells in the presence of increasing concentrations of NVP. Induced amino acid substitutions were analyzed at several passages by sequencing proviral HIV-1 RT in MT-2 cells.

during passages in one culture under EFV selective pressure (Fig. 1E). The previously unreported mutation K103G, which emerged in one culture of HIV-1_{K103R} under EFV pressure, conferred 5.60- and 4.71-fold resistance to EFV and NVP, respectively. The combination of K103R and V179D, which was observed in all three cultures of HIV-1_{K103R} under EFV pressure and in two of four cultures under NVP pressure, conferred 10.4- and 9.41-fold resistance to EFV and NVP, respectively, though V179D alone had little effect on EFV resistance (1.60-fold) but moderate resistance only to NVP (5.10-fold). Therefore, as expected from the data of emergence patterns of induced mutations under NNRTI selective pressure, K103G conferred moderate resistance to EFV and NVP, and the combination of K103R and V179D conferred high-level resistance to these NNRTIs.

Decreased replication kinetics of HIV-$1_{K103R/V179D}$

Finally, we analyzed the effect of single mutations and their combinations on HIV-1 replication kinetics in MT-2 cells in the absence and presence of NNRTI (Fig. 3 and Table 4). Each replication assay was performed in triplicate and repeated three times. In the absence of NNRTI, K103R and V106I did not significantly alter HIV-1 replication. Their combination (K103R/V106I), however, significantly reduced viral replication (p24 at day 8 of K103R/V106I vs. wild-type: $P < 0.05$, Fig. 3A and Table 4). K103G also resulted in reduced replication (K103G vs. wild-type: $P < 0.05$). V179D tended to reduce HIV-1 replication but the effect was not statistically significant in our assay. The combination of K103R and V179D severely compromised HIV-1 replication (K103R/V179D vs. wild-type: $P < 0.01$, Fig. 3A and Table 4). As reported previously, K103N did not significantly decrease HIV-1 replication (Gianotti et al., 2004; Schmit et al., 1996).

In the presence of 10 nM EFV, HIV-1 clones harboring wild-type K103 (HIV-1_{WT}), K103R (HIV-1_{K103R}), V106I (HIV-1_{V106I}), and K103R/V106I (HIV-$1_{K103R/V106I}$), failed to propagate and HIV-1 harboring V179D (HIV-1_{V179D}) exhibited reduced replication compared with that in the absence of NNRTI ($P < 0.05$, Fig. 3B and Table 4). HIV-1 clones harboring K103N (HIV-1_{K103N}), K103G (HIV-1_{K103G}), and the combination of K103R and V179D (HIV-$1_{K103R/V179D}$) showed efficient replication though their replication was slightly reduced compared with those in the absence of NNRTI. In the presence of 100 nM NVP, HIV-1_{K103N}, HIV-1_{K103G}, HIV-1_{V179D}, and HIV-$1_{K103R/V179D}$ exhibited efficient replications (Fig. 3C). The replication of the other HIV-1 clones was severely compromised. These data of the replication kinetics were well compatible with the IC_{50} data (Table 3).

Competitive HIV-1 replication assay using H9 cells

In order to confirm some of the results shown above, competitive HIV-1 replication assay was performed using H9 cells (Hachiya et al., 2004; Kosalaraksa et al., 1999) (Fig. 4). Two infectious HIV-1 clones to be compared for their fitness were mixed and used to infect H9 cells, and their population changes through weekly passages were determined by the

Table 3
Drug susceptibilities of recombinant HIV-1s

HIV-1	Mean IC_{50} (nM) ± SD (fold resistance)					
	AZT	3TC	d4T	TDF	EFV	NVP
Wild-type	30.0 ± 3.5	775 ± 14	1510 ± 200	27.0 ± 2.8	2.50 ± 0.25	25.5 ± 3.6
K103R	17.0 ± 1.2 (0.567)	340 ± 32 (0.439)	1500 ± 120 (0.993)	23.0 ± 1.9 (0.852)	2.40 ± 0.34 (0.960)	19.0 ± 2.2 (0.745)
K103N	14.0 ± 1.3 (0.467)	380 ± 27 (0.490)	1400 ± 130 (0.927)	29.0 ± 2.2 (1.07)	31.0 ± 2.3 (12.4)	1100 ± 130 (43.1)
V106I	19.0 ± 2.4 (0.633)	860 ± 33 (1.11)	1090 ± 70 (0.722)	31.0 ± 2.0 (1.15)	3.00 ± 0.33 (1.20)	20.0 ± 1.2 (0.784)
K103G	24.0 ± 1.0 (0.800)	1160 ± 30 (1.50)	660 ± 100 (0.437)	24.0 ± 2.0 (0.889)	14.0 ± 1.3 (5.60)	120 ± 10 (4.71)
K103R/V106I	29.0 ± 1.3 (0.967)	1420 ± 80 (1.83)	1200 ± 330 (0.795)	31.0 ± 1.3 (1.15)	3.00 ± 0.33 (1.20)	30.0 ± 11 (1.18)
V179D	16.0 ± 1.5 (0.533)	830 ± 23 (1.07)	3600 ± 230 (2.38)	36.0 ± 3.0 (1.33)	4.00 ± 0.17 (1.60)	130 ± 20 (5.10)
K103R/V179D	34.0 ± 2.0 (1.13)	1980 ± 12 (2.55)	1430 ± 140 (0.947)	29.0 ± 2.7 (1.07)	26.0 ± 2.0 (10.4)	240 ± 12 (9.41)

Drug resistance assay was performed in triplicate using MAGIC-5 cells (Hachiya et al., 2001, 2003, 2004). Fold resistance was calculated by dividing IC_{50} of recombinant HIV-1 by that of wild-type HIV-1.

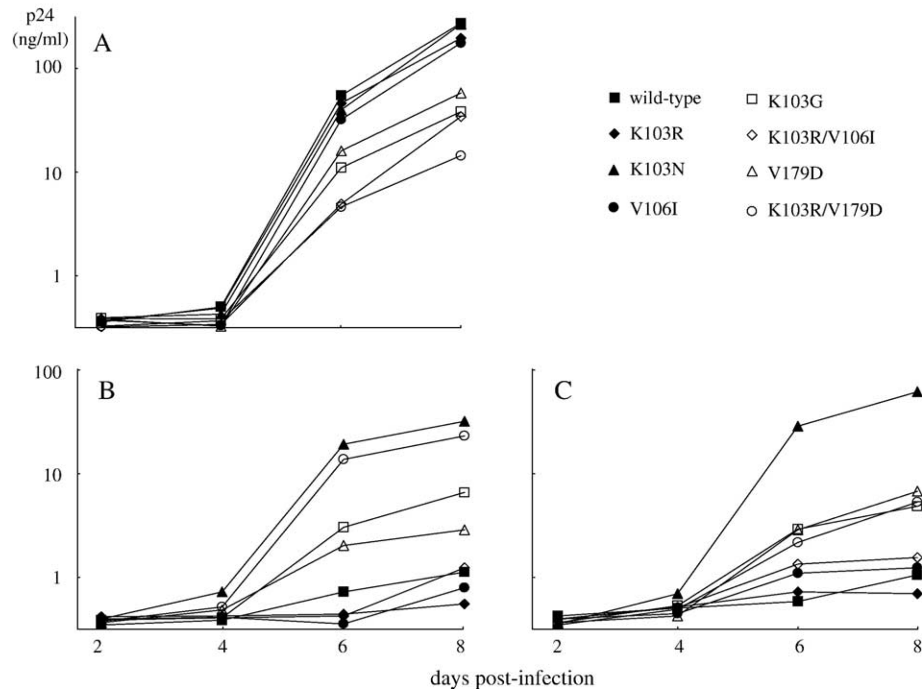

Fig. 3. Replication kinetics of recombinant HIV-1 clones in the absence and presence of NNRTIs. Recombinant HIV-1 clones were propagated in MT-2 cells in the absence (panel A) and presence of 10 nM EFV (panel B) or 100 nM NVP (panel C). The concentration of p24 in the culture medium was measured every other day. The assay was performed in triplicate and repeated three times and the data represent the logarithmic mean values of p24 concentrations.

relative peak height on sequencing electrogram. In the absence of NNRTI (Fig. 4A), and in the presence of EFV (3 nM) (Fig. 4B) and NVP (30 nM) (Fig. 4C), HIV-1_{WT} and HIV-1_{K103R} showed comparable replication efficiency, though the replication of HIV-1_{K103R} may be a little smaller than that of HIV-1_{WT} in the absence of NNRTI and in the presence of NVP. HIV-1_{K103N} was slowly outgrown by HIV-1_{WT} in the absence of NNRTI and it was still detectable at 3 weeks of competitive culture (wild-type:K103N = 91%:9%) (Fig. 4D). In the presence of EFV (10 nM) (Fig. 4E) and NVP (100 nM) (Fig. 4F), however, HIV-1_{K103N} readily outgrew HIV-1_{WT}. HIV-$1_{K103R/V179D}$ was predominated by HIV-1_{WT} in the absence of NNRTI (Fig. 4G), though it readily outgrew HIV-1_{WT} in the presence of EFV (10 nM) (Fig. 4H) and NVP (100 nM) (Fig. 4I). These data of competitive replication assay were well compatible with replication kinetic data (Fig. 3) and confirmed that the combination of K103R and V179D reduced viral replication in the absence of NNRTI though K103R alone had little impact on viral replication ability.

Table 4
Replication ability of recombinant HIV-1s

HIV-1	Logarithmic mean ± SD of p24 (ng/ml) at day 8		
	No drug	10 nM EFV	100 nM NVP
Wild-type	2.437 ± 0.309	0.021 ± 0.134	−0.003 ± 0.257
K103R	2.289 ± 0.343	−0.284 ± 0.102	−0.188 ± 0.266
K103N	2.422 ± 0.273	1.477 ± 0.287	1.759 ± 0.257
V106I	2.243 ± 0.777	−0.128 ± 0.137	0.063 ± 0.105
K103G	1.579 ± 0.285	0.789 ± 0.571	0.658 ± 0.399
K103R/V106I	1.534 ± 0.245	0.064 ± 0.096	0.164 ± 0.096
V179D	1.764 ± 0.450	0.431 ± 0.347	0.805 ± 0.288
K103R/V179D	1.159 ± 0.330	1.339 ± 0.208	0.697 ± 0.315

Recombinant HIV-1 clones were propagated in MT-2 cells in the absence and presence of 10 nM EFV or 100 nM NVP (Fig. 3). The assay was performed in triplicate and repeated three times. The logarithmic mean values and SD of p24 concentrations in the culture media at day 8 were shown.

Discussion

K103R mutation in RT was detected in 3.3% of our treatment-naive patients and was not associated with NNRTI resistance in our recombinant HIV-1 experiments. It was also reported that K103R was most often observed in individuals not receiving NNRTI among patients with known treatment history (Harrigan et al., 2005). Taken together, K103R can be considered a naturally occurring polymorphism in HIV-1 RT and is not associated with NNRTI exposure or resistance. K103N is the most commonly observed NNRTI resistance-associated mutation, which is considered to arise by a single nucleotide mutation from wild-type K103. However, R103N, the same amino acid substitution from K103R, which necessitates two nucleotide substitutions, did not occur in our in vitro induction of NNRTI resistance. Instead, a previously unreported mutation, R103G, which consisted of a single nucleotide substitution, was observed and shown to confer moderate resistance to EFV and NVP. It is worth noting that 103S, a rare mutation, was reported to follow K103R in three cases in the British Columbia database and was shown to confer high-level resistance to NNRTIs by analyzing recombinant HIV-1s (Harrigan et al., 2005). R103S mutation can also arise by a single nucleotide substitution from K103R, though

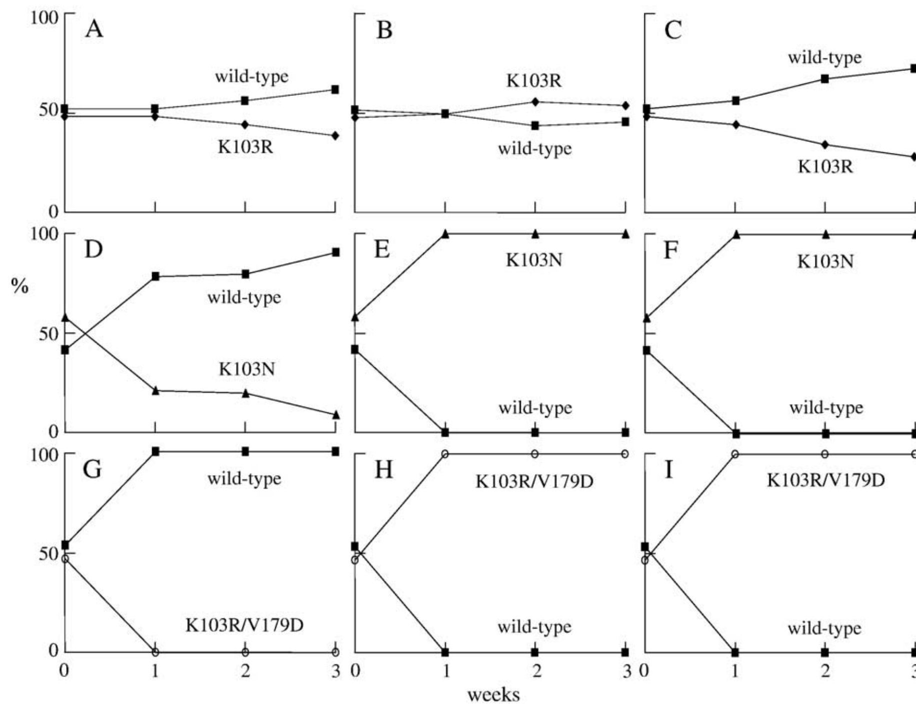

Fig. 4. Competitive HIV-1 replication assay in H9 cells. Two infectious HIV-1 clones to be compared for their fitness (HIV-1_{WT} vs. HIV-1_{K103R} in panels A–C, HIV-1_{WT} vs. HIV-1_{K103N} in panels D–F, and HIV-1_{WT} vs. HIV-$1_{K103R/V179D}$ in panels G–I) were mixed and used to infect H9 cells in the absence (panels A, D, and G) and presence of EFV (3 nM in panel B, 10 nM in panels E and H) or NVP (30 nM in panel C, 100 nM in panels F and I). The cell-free supernatant was transferred to fresh H9 cells every 7 days. High-molecular-weight DNAs extracted from infected cells on day 1 of the culture (0 week) and at the end of each passage (1, 2, and 3 weeks) were subjected to nucleotide sequencing, and proportion of Lys and Arg at position 103 (panels A–C and G–I), those of Lys and Asn at position 103 (panels D–F), and those of Val and Asp at position 179 (panels G–I) were determined.

K103S necessitates at least two nucleotide substitutions. Virological analysis of such polymorphism-specific mutations seems important, considering that the prevalence of 103S increased over fourfold in both Virco and British Columbia databases from 1998 to 2002 (Harrigan et al., 2005).

V179D is not included in the current table of drug resistance mutations by the International AIDS Society-USA Drug Resistance Mutations Group (Johnson et al., 2005), though it was reported to confer a small resistance to NNRTIs (Byrnes et al., 1993; Kleim et al., 1996; Winslow et al., 1996; Young et al., 1995). In the present study, V179D was often induced by NNRTIs in HIV-1 harboring K103R. In Stanford University HIV Drug Resistance Database (http://hivdb.stanford.edu/index.html), there are 57 registered clinical isolates harboring K103R and V179D mutations, and most of them are derived from the patients with a history of NNRTI treatment, which indicates that our in vitro observation actually often occurs in the patients. The combination of K103R and V179D conferred high-level resistance to EFV and NVP in our assay, suggesting that certain polymorphic mutation can alter the frequency of emergence of resistance-associated mutations and that the combination of a certain polymorphic mutation and a minor resistance-associated mutation can confer high-level resistance even if the polymorphism itself is not associated with drug resistance. The combination of K103R and V179D complicated viral replication though K103R alone did not significantly alter replication kinetics (Figs. 3A and 4A, G). These results suggest that HIV-$1_{K103R/V179D}$ might disappear rapidly and be replaced by HIV-1_{K103R} or HIV-1_{WT} after the discontinuation of NNRTI treatment, and that routine genotypic assay might not detect this combination in patients during NNRTI-free periods even if V179D emerged in patients during NNRTI-based therapy. On the other hand, K103N did not severely impair HIV-1 replication (Figs. 3A and 4D), in agreement with the recent studies reporting that HIV-1_{K103N} replicates comparably with HIV-1_{WT} and that K103N mutation remains stably detectable for a long time after the cessation of the NNRTI administration (Collins et al., 2004; Gianotti et al., 2004; Schmit et al., 1996).

Certain polymorphic amino acid residues in HIV-1 strains are associated with HIV-1 drug resistance (Gatanaga et al., 2002; Harrigan et al., 2005; Tanaka et al., 1997). It is also known that certain drug resistance-conferring amino acid substitutions found in one subtype HIV-1 isolated from patients under therapy may be detected in HIV-1 of other subtypes from untreated individuals (Cornelissen et al., 1997; Quinones-Mateu et al., 1998). Moreover, a recent study revealed that HIV-2 strains harbor specific patterns of natural polymorphism and resistance (Colson et al., 2004). More attention should be given to resistance-associated mutations that are specifically derived from certain polymorphisms and certain subtypes. In vitro induction of drug resistance to HIV strains harboring such polymorphisms and subtypes is a reasonable strategy for analyzing such mutations.

Materials and methods

HIV-1 sequences in treatment-naive patients

We analyzed HIV-1 sequences in plasma samples of 211 antiretroviral treatment-naive infected individuals who visited the outpatient clinic of the AIDS Clinical Center, International Medical Center of Japan from January, 2000 to December, 2003. The Institutional Review Board approved this study (IMCJ-H13-80) and a written informed consent was obtained from all the participants. Viral RNA was extracted from plasma samples with Smi-test nucleic acid extraction kit (Genome Science, Fukushima, Japan). The HIV-1 RT gene was amplified by RT-PCR using One Step RNA PCR Kit (Takara Shuzo, Otsu, Japan). Nested PCR was conducted subsequently to amplify enough DNA for direct sequencing. The primer sets were T1 and T4 for the first PCR, and T12 and T15 for the second PCR (Hachiya et al., 2004). Specific precautions, including physical separation of processing areas, were taken to avoid template and amplified product carryover. Stringent quality control to prevent PCR contamination was employed to protect against cross-contamination of product DNA. Direct sequencing was performed using dye terminators (BigDye Terminator Cycle Sequencing Ready Reaction Kit; Applied Biosystems, Foster, CA) and model 3700 automated DNA sequencer (Applied Biosystems). Amino acid sequences were deduced with Genetyx-Win program version 6.1 (Software Development, Tokyo).

Antiviral agents and cells

EFV, NVP, and 3TC were generously provided by Merck Co. Inc. (Rahway, NJ), Boehringer Ingelheim Pharmaceutics Inc. (Ridgefield, CT), and Nippon Glaxo-SmithKline (Tokyo, Japan), respectively. AZT and d4T were purchased from Sigma Co. (Tokyo, Japan). TDF was purchased from Moravek Biochemicals (Brea, CA). COS-7 cells and MT-2 cells were grown in Dulbecco's modified eagle medium (DMEM) and RPMI 1640, respectively, supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (0.1 mg/ml). MAGIC-5 cells (CCR5 and CD4 expressing HeLa-LTR-β-gal cells) (Hachiya et al., 2001, 2003, 2004) were grown in DMEM supplemented with 10% FCS, hygromycin B (0.1 mg/ml) (Invitrogen Co., San Diego, CA) and blasticidin (1000 ng/ml) (Funakoshi, Tokyo).

Generation of recombinant HIV-1 clones

The desired mutations were introduced into the *Xma*I–*Nhe*I region of pTZNX, which encodes Gly-15 to Ala-267 of HIV-1 RT (strain BH 10), by the oligonucleotide-based mutagenesis method (Hachiya et al., 2004; Kodama et al., 2001). The *Xma*I–*Nhe*I fragment was inserted into pNL_{H219Q}, which was modified from pNL101 and encoded the full genome of HIV-1. pNL_{H219Q} harbors H219Q mutation in HIV-1 Gag region, which facilitated HIV-1 replication in MT-2 cells (Gatanaga et al., 2002). Determination of the nucleotide sequences of plasmids confirmed that each clone had the desired mutations but no unintended mutations. Each molecular clone was transfected into COS-7 cells with GenePORTER Transfection Reagent (Gene Therapy Systems, San Diego, CA), and thus obtained virions were harvested 48 h after transfection and stored at −80 °C until use.

Induction of HIV-1 resistant to NNRTIs

The infectious HIV-1 clones harboring wild-type K103 ($HIV\text{-}1_{WT}$) or K103R ($HIV\text{-}1_{K103R}$) in RT were propagated in MT-2 cells in the presence of increasing concentrations of EFV and NVP, respectively (Gatanaga et al., 2002; Nunberg et al., 1991; Tanaka et al., 1997; Yoshimura et al., 1999). Briefly, MT-2 cells (1×10^5) were exposed to $HIV\text{-}1_{WT}$ or $HIV\text{-}1_{K103R}$ (500 blue cell-forming units [BFU] in MAGIC-5 cells) (Hachiya et al., 2001, 2003, 2004) and cultured in the presence of EFV and NVP at the initial concentrations of 3 nM and 30 nM, respectively. Viral replication was monitored by observation of the cytopathic effect in MT-2 cells. The culture supernatant was harvested on day 7 of culture and used to infect fresh MT-2 cells for the next round of culture. When the virus began to propagate in the presence of the drug, the drug concentration was increased. This selection was carried out for a total of 25 and 19 passages for cultures with EFV and NVP, respectively. Proviral DNAs from lysates of infected MT-2 cells from several passages were sequenced as indicated. Proviral HIV-1 RT gene was amplified and sequenced by using the primers T1 and T4. When necessary, molecular cloning was performed by using pT7 Blue T-Vector (Novagen, CA) and their sequences were analyzed.

Drug susceptibility assay with MAGIC-5 cells

Recombinant HIV-1 susceptibility to various RT inhibitors (RTIs) was determined in triplicate by using MAGIC-5 cells as described previously (Hachiya et al., 2001, 2003, 2004). Briefly, MAGIC-5 cells were infected with adjusted virus stock (300 BFU) in various concentrations of RTIs, cultured for 48 h, fixed, and stained with 5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside (Takara Shuzo, Ohtsu, Japan). The blue-stained cells were counted under a light microscope. Drug concentrations that inhibited 50% of the stained cells of drug-free control (IC_{50}) were determined by referring to the dose–response curve.

Replication kinetic assay

MT-2 cells (10^5) were exposed to each infectious virus preparation (500 BFU) for 2 h, washed twice with PBS, and cultured in 1 ml of complete medium in the presence or absence of EFV or NVP. The culture supernatants were harvested every other day, and p24 Gag amounts were determined by chemiluminescence enzyme immunoassay (CLEIA) kit (Fuji-Rebio, Tokyo). Replication assays were performed in triplicate and repeated three times using independently generated virus preparations.

Competitive HIV-1 replication assay

Freshly prepared H9 cells (3×10^5) were exposed to the mixtures of paired virus preparations (300 BFU each) (HIV-1_{WT} vs. HIV-1_{K103R} in Figs. 4A–C, HIV-1_{WT} vs. HIV-1_{K103N} in Figs. 4D–F, and HIV-1_{WT} vs. HIV-$1_{K103R/V179D}$ in Figs. 4G–I) to be examined for their replication ability for 2 h, washed twice with PBS, and cultured in the absence (Figs. 4A, D, and G) or presence of EFV (3 nM for Fig. 4B, 10 nM for Figs. 4E and H) or NVP (30 nM for Fig. 4C, 100 nM for Figs. 4F and I) as describe previously (Hachiya et al., 2004; Kosalaraksa et al., 1999). On day 1, one-third of infected H9 cells were harvested and washed twice with PBS, and proviral DNAs were sequenced (0 week). Every 7 days, the supernatant of the virus culture was transmitted to new uninfected H9 cells, the cells harvested at the end of each passage (1, 2, and 3 weeks) were subjected to direct DNA sequencing of HIV-1 RT gene, and the viral population change was determined by the relative peak height on sequencing electrogram. The persistence of the original amino acid substitution was confirmed for all infectious clones used in this assay.

Acknowledgments

This work was supported in part by a Grant-in-Aid for AIDS research from the Ministry of Health, Labor, and Welfare of Japan (H15-AIDS-001), by the Program for the Promotion of Fundamental Studies in Health Sciences of the Pharmaceuticals and Medical Devices Agency (01–4), and by the Japan Antibiotics Research Association.

References

Bacheler, L.T., 1999. Resistance to non-nucleoside inhibitors of HIV-1. Drug Resist. Updates 2, 56–67.

Byrnes, V.W., Sardana, V.V., Schleif, W.A., Condra, J.H., Waterbury, J.A., Wolfgang, J.A., Long, W.J., Schneider, C.L., Schlabach, A.J., Wolanski, B.S., Graham, D.J., Gotlib, L., Rhodes, A., Titus, D.L., Roth, E., Blahy, O.M., Quintero, J.C., Staszewski, S., Emini, E.A., 1993. Comprehensive mutant enzyme and viral variant assessment of human immunodeficiency virus type 1 reverse transcriptase resistance to nonnucleoside inhibitors. Antimicrob. Agents Chemother. 37, 1576–1579.

Collins, J.A., Thompson, M.G., Paintsil, E., Ricketts, M., Gedzior, J., Alexander, L., 2004. Competitive fitness of nevirapine-resistant human immunodeficiency virus type 1 mutants. J. Virol. 78, 603–611.

Colson, P., Henry, M., Tourres, C., Lozachmeur, D., Gallais, H., Gastaut, J.A., Moreau, J., Tamalet, C., 2004. Polymorphism and drug-selected mutations in the protease gene of human immunodeficiency virus type 2 from patients living in southern France. J. Clin. Microbiol. 42, 570–577.

Cornelissen, M., van den Burg, R., Zorgdrager, F., Lukashov, V., Goudsmit, J., 1997. pol gene diversity of five human immunodeficiency virus type 1 subtypes: evidence for naturally occurring mutations that contribute to drug resistance, limited recombination patterns, and common ancestry for subtypes B and D. J. Virol. 71, 6348–6358.

De Clercq, E., 1998. The role of non-nucleoside reverse transcriptase inhibitors (NNRTIs) in the therapy of HIV-1 infection. Antiviral Res. 38, 153–179.

Gatanaga, H., Suzuki, Y., Tsang, H., Yoshimura, K., Kavlick, M.F., Nagashima, K., Gorelick, R.J., Mardy, S., Tang, C., Summers, M.F., Mitsuya, H., 2002. Amino acid substitutions in Gag protein at non-cleavage sites are indispensable for the development of a high multitude of HIV-1 resistance against protease inhibitors. J. Biol. Chem. 277, 5952–5961.

Gianotti, N., Boeri, E., Maillard, M., Serra, G., Ratti, D., Gallotta, G., Vacchini, D., Tremolada, Y., Castagna, A., 2004. In vivo dynamics of the 103N mutation following the withdrawal of non-nucleoside reverse transcriptase inhibitors in HIV-infected patients: preliminary results. New Microbiol. 27, 111–117.

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

Hachiya, A., Matsuoka-Aizawa, S., Tsuchiya, K., Gatanaga, H., Kimura, S., Tatsumi, M., Oka, S., 2003. "All-in-One Assay", a direct phenotypic anti-human immunodeficiency virus type 1 drug resistance assay for three-drug combination therapies that takes into consideration in vivo drug concentrations. J. Virol. Methods 111, 43–53.

Hachiya, A., Gatanaga, H., Kodama, E., Ikeuchi, M., Matsuoka, M., Harada, S., Mitsuya, H., Kimura, S., Oka, S., 2004. Novel patterns of nevirapine resistance-associated mutations of human immunodeficiency virus type 1 in treatment-naïve patients. Virology 327, 215–224.

Harrigan, P.R., Mo, T., Wynhoven, B., Hirsch, J., Brumme, Z., McKenna, P., Pattery, T., Vingerhoets, J., Bacheler, L.T., 2005. Rare mutations at codon 103 of HIV-1 reverse transcriptase can confer resistance to non-nucleoside reverse transcriptase inhibitors. AIDS 19, 549–554.

Johnson, V.A., Brun-Vezinet, F., Clotet, B., Conway, B., Kuritzkes, D.R., Pillay, D., Schapiro, J., Telenti, A., Richman, D., 2005. Update of the drug resistance mutations in HIV-1: 2005. Top. HIV Med. 13, 51–57.

Kleim, J.P., Rosner, M., Winkler, I., Paessens, A., Kirsch, R., Hsiou, Y., Arnold, E., Riess, G., 1996. Selective pressure of a quinoxaline nonnucleoside inhibitor of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) on HIV-1 replication results in the emergence of nucleoside RT-inhibitor specific (RT Leu-74→Val or Ile and Val-75→Leu or Ile) HIV-1 mutants. Proc. Natl. Acad. Sci. U.S.A. 93, 34–48.

Kodama, E.I., Kohgo, S., Kitano, K., Machida, H., Gatanaga, H., Shigeta, S., Matsuoka, M., Ohrui, H., Mitsuya, H., 2001. 4′-ethynyl nucleoside analogs: potent inhibitors of multidrug-resistant human immunodeficiency virus variants in vitro. Antimicrob. Agents Chemother. 45, 1539–1546.

Kosalaraksa, P., Kavlick, M.F., Maroun, V., Le, R., Mitsuya, H., 1999. Comparative fitness of multi-dideoxynucleoside-resistant human immunodeficiency virus type 1 (HIV-1) in an in vitro competitive HIV-1 replication assay. J. Virol. 73, 5356–5363.

Nunberg, J.H., Schleif, W.A., Boots, E.J., O'Brien, J.A., Quintero, J.C., Hoffman, J.M., Emini, E.A., Goldman, M.E., 1991. Viral resistance to human immunodeficiency virus type 1-specific pyridinone reverse transcriptase inhibitors. J. Virol. 65, 4887–4892.

Quinones-Mateu, M.E., Albright, J.L., Mas, A., Soriano, V., Arts, E.J., 1998. Analysis of pol gene heterogeneity, viral quasispecies, and drug resistance in individuals infected with group O strains of human immunodeficiency virus type 1. J. Virol. 72, 9002–9015.

Rhee, S.Y., Gonzales, M.J., Kantor, R., Betts, B.J., Ravela, J., Shafer, R.W., 2003. Human immunodeficiency virus reverse transcriptase and protease sequence database. Nucleic Acids Res. 31, 298–303.

Schmit, J.C., Cogniaux, J., Hermans, P., Van Vaeck, C., Sprecher, S., Van Remoortel, B., Witvrouw, W., Balzarini, J., Desmyter, J., De Clercq, E., Vandamme, A.M., 1996. Multiple drug resistance to nucleoside analogues and nonnucleoside reverse transcriptase inhibitors in an efficiently replicating human immunodeficiency virus type 1 patient strain. J. Infect. Dis. 174, 962–968.

Tanaka, M., Srinivas, R.V., Ueno, T., Kavlick, M.F., Hui, F.K., Fridland, A., Driscoll, J.S., Mitsuya, H., 1997. In vitro induction of human immunodeficiency virus type 1 variants resistant to 2′-beta-fluoro-2′,3′-dideoxyadenosine. Antimicrob. Agents Chemother. 41, 1313–1318.

Vandamme, A.M., Sonnerborg, A., Ait-Khaled, M., Albert, J., Asjo, B., Bacheler, L., Banhegyi, D., Boucher, C., Brun-Vezinet, F., Camacho, R., Clevenbergh, P., Clumeck, N., Dedes, N., De Luca, A., Doerr, H.W., Faudon, J.L., Gatti, G., Gerstoft, J., Hall, W.W., Hatzakis, A., Hellmann, N., Horban, A., Lundgren, J.D., Kempf, D., Miller, M., Miller, V., Myers, T.W., Nielsen, C., Opravil, M., Palmisano, L., Perno, C.F., Phillips, A., Pillay, D., Pumarola, T., Ruiz, L., Salminen, M., Schapiro, J., Schmidt, B., Schmit, J.C., Schuurman, R., Shulse, E., Soriano, V., Staszewski, S., Vella, S., Youle,

M., Ziermann, R., Perrin, L., 2004. Updated European recommendations for the clinical use of HIV drug resistance testing. Antiviral Ther. 9, 829–848.

Winslow, D.L., Garber, S., Reid, C., Scarnati, H., Baker, D., Rayner, M.M., Anton, E.D., 1996. Selection conditions affect the evolution of specific mutations in the reverse transcriptase gene associated with resistance to DMP 266. AIDS 10, 1205–1209.

Yeni, P.G., Hammer, S.M., Hirsch, M.S., Saag, M.S., Schechter, M., Carpenter, C.C., Fischl, M.A., Gatell, J.M., Gazzard, B.G., Jacobsen, D.M., Katzenstein, D.A., Montaner, J.S., Richman, D.D., Schooley, R.T., Thompson, M.A., Vella, S., Volberding, P.A., 2004. Treatment for adult HIV infection: 2004 recommendations of the International AIDS Society-USA Panel. JAMA 292, 251–265.

Yoshimura, K., Feldman, R., Kodama, E., Kavlick, M.F., Qiu, Y.L., Zemlicka, J., Mitsuya, H., 1999. In vitro induction of human immunodeficiency virus type 1 variants resistant to phosphoralaninate prodrugs of Z-methylenecyclopropane nucleoside analogues. Antimicrob. Agents Chemother. 43, 2479–2483.

Young, S.D., Britcher, S.F., Tran, L.O., Payne, L.S., Lumma, W.C., Lyle, T.A., Huff, J.R., Anderson, P.S., Olsen, D.B., Carroll, S.S., Pettibone, D.J., O'Brien, J.A., Ball, R.G., Balani, S.K., Lin, J.H., Chen, I.W., Schleif, W.A., Sardana, V.V., Long, W.J., Byrnes, V.W., Emini, E.A., 1995. L-743, 726 (DMP-266): a novel, highly potent nonnucleoside inhibitor of the human immunodeficiency virus type 1 reverse transcriptase. Antimicrob. Agents Chemother. 39, 2602–2605.