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Distinct Mutation Pathways of Non-Subtype B HIV-1 during *In Vitro* Resistance Selection with Nonnucleoside Reverse Transcriptase Inhibitors▽†

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Studies were conducted to investigate mutation pathways among subtypes A, B, and C of human immunodeficiency virus type 1 (HIV-1) during resistance selection with nonnucleoside reverse transcriptase inhibitors (NNRTIs) in cell culture under low-multiplicity of infection (MOI) conditions. The results showed that distinct pathways were selected by different virus subtypes under increasing selective pressure of NNRTIs. F227C and Y181C were the major mutations selected by MK-4965 in subtype A and C viruses during resistance selection. With efavirenz (EFV), F227C and V106M were the major mutations responsible for viral breakthrough in subtype A viruses, whereas a single pathway (G190A/V106M) accounted for mutation development in subtype C viruses. Y181C was the dominant mutation in the resistance selection with etravirine (ETV) in subtype A, and E138K/H221Y were the mutations detected in the breakthrough viruses from subtype C viruses with ETV. In subtype B viruses, on the other hand, known NNRTI-associated mutations (e.g., Y181C, P236L, L100I, V179D, and K103N) were selected by the NNRTIs. The susceptibility of the subtype A and B mutant viruses to NNRTIs was determined in order to gain insight into the potential mechanisms of mutation development. Collectively, these results suggest that minor differences may exist in conformation of the residues within the NNRTI binding pocket (NNRTIBP) of reverse transcriptase (RT) among the three subtypes of viruses. Thus, the interactions between NNRTIs and the residues in the NNRTIBPs of different subtypes may not be identical, leading to distinct mutation pathways during resistance selection in cell culture.

Human immunodeficiency virus type 1 (HIV-1) infection has become a global epidemic, as there are more than 33 million people worldwide who are infected with HIV-1, and approximately 2.7 million people were newly infected in 2008 (11). This epidemic resulted from the cross-species transmission of a lentivirus that began spreading among humans during the first half of the previous century (14, 17, 22). HIV-1 strains can be classified into three groups: the "major" group M, the "outlier" group O, and the "new" group N. These three groups may represent three separate introductions of simian immunodeficiency virus into humans. Group O appears to be restricted to west and central Africa, and group N (discovered in 1998 in Cameroon) is extremely rare (34). More than 90% of HIV-1 infections belong to HIV-1 group M. During their spread among humans, group M viruses have developed an extraordinary degree of genetic diversity. There are at least nine genetically distinct subtypes (or clades) of HIV-1 within group M: subtypes A, B, C, D, F, G, H, J, and K. In addition, recombination between genomes of two viruses of different subtypes can occur in the cells of an infected person to create a new hybrid virus. Many of these new strains do not survive for long, but those that infect more than one person are known as circulating recombinant forms (CRFs). For example, CRF A/B is a mixture of subtypes A and B (2).

The different subtypes are not distributed evenly throughout the world. Subtype B predominates in North America and Europe, subtype A is most prevalent in west and central Africa, and subtype C is the major subtype in sub-Saharan Africa (31, 43). Analysis of the current worldwide distribution of HIV-1 subtypes indicates that HIV-1 subtypes A and C are the most prevalent subtypes globally (37). At this stage of the HIV infection pandemic, these subtypes are expanding faster and are of greater global significance than subtype B (29).

Despite the diversity of the HIV-1 subtypes, many of the data on the genetic mechanisms of HIV-1 drug resistance are based on the study of subtype B viruses. However, HIV-1 subtype B viruses account for only ~12% of global HIV-1 infections (37). Although it was reported, both *in vitro* and *in vivo*, that the currently available protease and reverse transcriptase (RT) inhibitors are as active against non-subtype B viruses as they are against subtype B viruses (1, 21, 30), fewer data are available on the genetic mechanisms of drug resistance in non-B viruses. Furthermore, some *in vitro* and *in vivo* observations suggest that the various subtypes may respond

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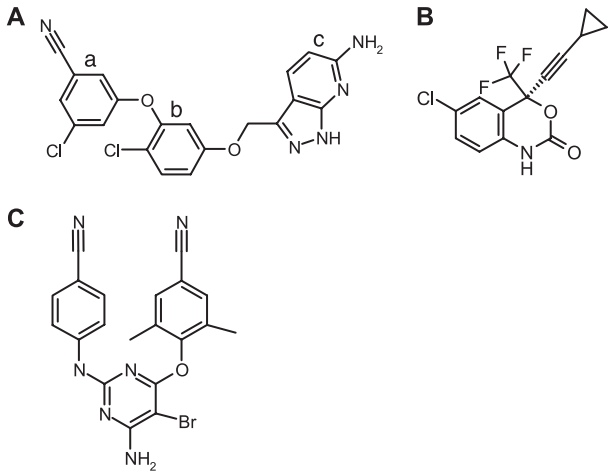

FIG. 1. Structures of MK-4965 (A), EFV (B), and ETV (C). Letters a, b, and c represent respective ring moieties in MK-4965.

differently to certain antiretroviral drugs (8–10, 15, 21). The frequency and pattern of mutations conferring resistance to these drugs differ among HIV-1 subtypes and can influence therapeutic outcome (20). In addition, differences in replication capacity or fitness exist among various HIV-1 subtypes, and these may become magnified under conditions of high selective pressure (36). As a result, it is important to understand the mutation pathways among non-subtype B viruses during resistance selection in cell culture with antiviral agents. This information will aid in designing initial treatment strategies for persons infected with non-B viruses and interpreting genetic resistance among non-B virus patients for whom antiretroviral therapy fails.

To this end, we conducted *in vitro* resistance selection with MK-4965, a novel nonnucleoside RT inhibitor (NNRTI) containing diaryl ether and indazole moieties (Fig. 1) (38). MK-4965 displays excellent activities against not only the wild-type (WT) virus but also a broader panel of NNRTI-resistant viruses, including viruses containing a K103N and/or a Y181C mutation (24). In this study, we performed resistance selection with MK-4965 in HIV-1 subtypes A, B, and C under low-multiplicity of infection (MOI) conditions in the presence of 10% fetal bovine serum (FBS). In addition, two licensed NNRTIs, efavirenz (EFV) and etravirine (ETV), were included in the studies. Our results suggest that distinct mutation pathways occur in different virus subtypes in the presence of escalating concentrations of the inhibitors. Emergence of different mutation pathways among the different subtypes may be ascribed to the fact that interactions between the NNRTI and residues in the NNRTI-binding pocket (NNRTIBP) may not be identical or that the genetic backgrounds of residues in the pocket are different among different HIV-1 subtypes. In addition, resistance selection with subtype B virus was conducted under high-MOI conditions at fixed concentrations of the inhibitors in the presence of 10% FBS or 50% human serum (HS) (39). Although the development of MK-4965 was terminated due to toxicity found in an animal study, these data should provide important insight into the factors that impact the development of mutations in different HIV-1 subtypes in the presence of NNRTIs.

MATERIALS AND METHODS

Materials. A QiaAmp kit was purchased from Qiagen (Valencia, CA). A Big Dye termination kit and an ABI 3100 analyzer were purchased from Applied Biosystems (Foster City, CA). A Victor luminometer was obtained from Perkin Elmer (Waltham, MA). EDTA was ordered from BioWhittaker (Walkersville, MD). Purified WT HIV-1 strain R8 was custom-made by Advanced Biotechnologies Inc. (Columbia, MD). ViroMag R/L magnetic beads were purchased from Boca Scientific (Boca Raton, FL). A Dynal MPC-S magnetic-particle concentrator, RPMI 1640 medium, SOC medium, and plasmid pcCDNA 3.1^+ were purchased from Invitrogen (Carlsbad, CA). BioStor vials (1.5 ml) were ordered from National Scientific Supply (Claremont, CA). JM109 competent cells, an Access RT-PCR system, and a pGEM-T Easy vector system II were obtained from Promega (Madison, WI). A Guava instrument was obtained from Millipore (Billerica, MA). A QuikChange site-directed mutagenesis (SDM) kit was ordered from Stratagene (La Jolla, CA).

Resistance selection with subtype B virus under low-MOI conditions. Since CXCR4 HIV variants replicate efficiently in SupT1 cells, the cell line was employed in this study. As shown in Results, with the SupT1 cell line, the mutations selected by the viruses in the presence of NNRTIs appear to reflect those that have been observed in clinical trials with EFV and ETV. SupT1 cells were grown to log phase, spun down, and resuspended in growth medium to a density of 2×10^6/ml. The cells (0.5 ml) were infected with 1 μl of R8 virus, with a 50% tissue culture infective dose ($TCID_{50}$) of $10^{5.67}$/ml. After 2 to 4 h of infection, the cell culture was diluted into 5 ml of growth medium and incubated at 37°C in a 5% CO_2 incubator overnight. The cells were subsequently treated with NNRTIs at an initial concentration of 1× EC_{95} (concentration of drug required to inhibit 95% of viral replication). Cultures were split every 3 or 4 days, and viral breakthrough was monitored by syncytium formation. Breakthrough viruses (BTV) were harvested and stored at −80°C or immediately subjected to genetic analysis. BTV (10 μl) from the previous round were employed for the subsequent round of selection at a higher concentration of inhibitor, up to 50× EC_{95}.

Resistance selection with subtype A and C viruses under low-MOI conditions. The resistance selection procedure with subtype A and C viruses was similar to that used for resistance selection with subtype B virus, except that SupT1 cells containing CCR5 receptors were employed for the viral infection. Subtype A (patient identifier [ID] RW/92/026) and subtype C (patient ID 93MW 959) viruses were used for the resistance selection study. Since WT subtype A and C viruses exhibit similar susceptibilities to NNRTIs (4), the EC_{95} for subtype A and C viruses was based on the EC_{95} determined with subtype B virus.

Determination of the degree of infection under high-MOI conditions. SupT1 cells were grown to log phase, spun down, and resuspended in growth medium to a density of 2×10^6/ml. The resulting culture was distributed into each well of a 6-well plate. R8 viruses (200 μl; $TCID_{50}$ of $10^{5.67}$/ml) were introduced into each well. After 4 h of infection at 37°C in a 5% CO_2 incubator, the cell culture was diluted into 3 ml of growth medium, and the entry inhibitor T20 (100 nM) was then added to the mixture to block reinfection. The cells were fixed on the plate 27 h postinfection. The infected cells were stained with monoclonal fluorescein isothiocyanate (FITC)–anti-p24, and the resulting fluorescent cells were quantified with a Guava instrument. The results showed that the percentage of infection was greater than 50% under these conditions.

Resistance selection under high-MOI conditions. SupT1 cells were infected with 200 μl of R8 virus and diluted into 5 ml of growth medium as described above. The resulting infected cells were treated with the NNRTIs at three different concentrations (40 nM, 200 nM, and 1 μM). Depending on the degree of confluence of the cells, the cells were split at a 1:6 or 1:8 ratio with growth medium containing the same concentration of inhibitor after 3 to 4 days of incubation. When the majority of the cells (>50%) formed syncytia, the medium containing the BTV was harvested and stored at −80°C or subjected to immediate genetic analysis.

Identification of resistance mutation(s) in BTV from resistance selection studies. Viral RNA of BTV was isolated using a QiaAmp kit, following the manufacturer's instructions. The entire RT region of the cDNA sequence was generated via an Access RT-PCR system using primers AGGTACAGTGTTG GTAGGACCTAC/TGTTCAGCTTGGTCTCTTACCTG for subtype A virus, TTAAAGCCCGGGATGGATGGCCCAAAAGTTAAAC/GTACTTTTCGA ATGCCAGCACTGACCAATTTATC for subtype B virus, and ACCTGTCAA CATAATTGGAAGAAAT/ATACTTCTCATGCTCTTCTTGAG for subtype C virus. The synthesized cDNA was purified by agarose gel electrophoresis. Population sequencing was performed with the purified cDNA by use of a Big Dye termination kit, and the sequences were resolved on an ABI 3100 analyzer. Several sequencing primers for each virus subtype were designed and employed

TABLE 1. Resistance selection with MK-4965 in HIV-1 A, B, and C subtypes in the presence of 10% FBS under low-MOI conditions[d]

Subtype	Expt	1× EC_{95}[a]		2× EC_{95}		5× EC_{95}		10× EC_{95}		50× EC_{95}	
		T[b]	Mutation(s)	*T*	Mutation(s)	*T*	Mutation(s)	*T*	Mutation(s)	*T*	Mutation(s)
A	1	24	NM[c]	20	NM	23	T165K	14	T165K F227C	9	T165K F227C
	2	27	NM	20	NM	15	Y181(Y/N)	12	Y181C	9	Y181C
	3	34	A200E	10	A200E F227(F/C)	7	A200E F227C	8	A200E F227C	7	A200E F227C
B	1	13	P236(P/L)	10	P236(P/L)	9	P236L	10	P236L E138(E/K)	10	P236L[e] V106A[e]
	2	13	NM	7	Y181(Y/C)	6	Y181(Y/C) V106(V/A)	10	Y181(Y/C) V106A R172(R/K)	13	Y181C[e] V106A[e] R172K[e]
	3	13	K219E D256E	7	K219E D256E E138(E/K)	6	K219E D256E E138(E/K)	10	K219E D256E E138K V106(V/I)	10	K219E D256E E138K V106(I/A) P236L
C	1	42	NM	11	F227C	12	F227C	12	F227C	6	F227C Y181C
	2	42	NM	11	F227(F/C)	12	F227C	12	Y181(Y/C) F227C	12	Y181C F227C

[a] The EC_{95} of MK-4965 with subtype B virus is 4.4 ± 2.0 nM (24).
[b] *T*, time (in days).
[c] NM, no mutation was detected.
[d] Amino acids in parentheses indicate a mixture of the wild-type residue and the emerging mutation.
[e] Contained a mixture of mutant viruses. Clonal sequencing was performed, and the results are shown in Table 2.

for the identification of mutations in the BTV obtained after resistance selection in cell culture.

Isolation of HIV-1 subtype A viral particles by ViroMag R/L beads. BTV resulting from resistance selection with subtype A viruses in the presence of NNRTIs were isolated as described previously (40). Briefly, 100 μl of the medium containing BTV was diluted with 900 μl of serum-free RPMI in a 1.5-ml BioStor vial, followed by the addition of 100 μl of ViroMag R/L beads. The resulting mixture was gently inverted back and forth, and the tube was placed into a Dynal MPC-S magnetic-particle concentrator, allowing all of the beads to attach to the inner wall of the tube. After the solution was removed, the beads were resuspended with fresh serum-free RPMI, and the beads were washed one more time via the same procedure. The beads were finally resuspended in the starting volume of serum-free RPMI and were either used immediately for infection experiments or stored at −70°C for future use.

HIV-1 single-cycle replication assay. P4/R5 cells containing the β-galactosidase gene with the long terminal repeat (LTR) as the promoter were detached from the plate using EDTA and placed in a 384-well plate at a density of 1,000 cells per well. Cells were plated in the presence of medium (40 μl) containing Dulbecco's modified Eagle's medium (DMEM), 10% FBS, and 1% penicillin-streptomycin. After incubation at 37°C and 5% CO_2 overnight, 20 μl medium was removed from the cells and discarded. Medium containing viruses (the number of viruses added was dependent on the infectivity of the individual virus) was added to a 384-well plate containing the inhibitor to generate a mixture containing a 2× concentration of viruses and inhibitor. The resulting mixture (20 μl) was added back to the cells as described above (in 20 μl medium), resulting in a 1× concentration of both virus and inhibitor. The mixture was then incubated for an additional 48 h. Viral replication gave rise to the expression of β-galactosidase in infected cells. RT inhibitor activity was evaluated using 10 serial drug concentrations. The level of viral replication was assessed by addition of β-galactosidase substrate at a 1:50 ratio. After incubation for 60 to 90 min, the mixtures were read on a Victor luminometer.

Drug susceptibility was measured by comparing the amount of β-galactosidase activity produced in the presence of the RT inhibitor to the amount of β-galactosidase activity produced in the absence of the RT inhibitor. Susceptible viruses produce low levels of β-galactosidase activity in the presence of antiviral drugs, whereas viruses with reduced susceptibility produce higher levels of β-galactosidase activity.

Data were analyzed by plotting the percent inhibition of β-galactosidase activity versus $\log_{10}$ drug concentration. Inhibition curves were fitted to the data by a 4-parameter fitting algorithm and were used to calculate the concentration of drug required to inhibit viral replication by 50% (EC_{50}). The extent of drug resistance was estimated using the fold change (FC) in EC_{50}, defined as the EC_{50} of the resistant virus divided by the EC_{50} of the wild-type virus.

Clonal sequencing of the RT DNA sequences of the BTV obtained from resistance selection in cell culture. The cDNA samples purified from the RT-PCRs of RNA samples were digested with restriction enzymes. The resulting cDNA (1 μl; 30 ng/μl) was ligated with 1 μl pGEM-T Easy vector (50 ng/μl) at room temperature for 1 h. The ligation mixture (2 μl) was introduced into 100 μl competent cells and mixed gently on ice. After heat shock at 42°C for 30 s, the mixture was incubated on ice for an additional 2 min. SOC medium (1 ml) was added into the mixture, which was incubated at 37°C for 1 h with shaking. Different volumes of the mixtures (50 μl and 200 μl) were spread onto LB ampicillin plates and incubated at 37°C overnight. More than 20 white colonies were selected, and each colony was cultured in 3 ml growth medium overnight. DNA was purified from the resulting culture and subjected to genetic analysis.

SDM. Mutant viruses that contained the desired mutation(s) were generated by introducing the respective mutation into the WT HIV-1 strain HXB2 backbone using a QuikChange site-directed mutagenesis (SDM) kit. The resulting proviral DNA construct was used to transfect 293T cells; culture supernatants containing the SDM-derived mutant viruses were collected and concentrated, and titers were determined prior to the infection experiments.

RESULTS

***In vitro* resistance selection with MK-4965 under low-MOI conditions.** The resistance selection experiments were initiated with MK-4965 at a concentration of 1× EC_{95}, and the concentration was gradually increased to 50× EC_{95}. The BTV emerging from each round of selection were used to infect cells in the subsequent round of selection. Resistance selection was conducted in triplicate for subtypes A and B and in duplicate for subtype C.

As shown in Table 1, three pathways for the development of mutations were identified during resistance selection with MK-4965 in the subtype A virus. The first viral breakthroughs in all

three experiments with subtype A virus were observed after more than 3 weeks of incubation with MK-4965 at the 1× EC_{95} concentration. No mutations were identified in the BTV obtained from early selection from the first two experiments, and a rare mutation (A200E) was identified in the BTV from the third experiment. In the first experiment, a non-NNRTI-associated mutation (T165K) emerged during resistance selection at the 5× EC_{95} concentration of MK-4965, and an additional mutation (F227C) was acquired from subsequent selections at higher concentrations of MK-4965. The second mutation pathway was led by Y181C, which appeared in the BTV at an inhibitor concentration of 10× EC_{95}. This mutation remained the only mutation found in the BTV during selection with MK-4965 at concentrations up to 50× EC_{95}. In addition to the A200E mutation identified at 1× EC_{95}, F227C began to emerge at 2× EC_{95} of the inhibitor in experiment 3, and both mutations A200E and F227C coexisted in the BTV from the selection with increasing inhibitor concentrations up to 50× EC_{95}.

In the resistance selection study with subtype B virus, the initial breakthrough was observed within 2 weeks in the presence of 1× EC_{95} of MK-4965. Three known mutation pathways associated with NNRTIs were also selected in this study. In experiment 1, an NNRTI-resistant mutation (P236L) was identified in the BTV from selection at 1× EC_{95}. This mutation was persistent throughout the selection experiment. Additional mutations E138K and V106A emerged at concentrations of 10× and 50× EC_{95}, respectively. In experiment 2, no mutations were detected in the BTV at 1× EC_{95}, and a Y181C mutation was identified in BTV at 2× EC_{95}. A known NNRTI-resistant mutation (V106A) and an NRTI-associated mutation (R172K) both emerged at higher selective pressure with MK-4965 (10× and 50× EC_{95}) (18). Another NRTI-associated mutation (K219E) led the third pathway of mutation development, though this mutation did not result in measurable MK-4965 resistance. A mutation (D256E) that had not been previously identified was also found in the BTV along with K219E, but this change also did not confer MK-4965 resistance. Additional NNRTI-associated mutations, such as E138K, V106I, and P236L, sequentially emerged as the concentration of the inhibitor increased gradually to 50× EC_{95}.

In contrast to the selection study conducted with the subtype B virus, in which the first BTV were observed within 2 weeks, it took more than 6 weeks to detect the first viral breakthrough at 1× EC_{95} of MK-4965 in the resistance selection with subtype C virus. A single mutation development pathway was responsible for viral breakthrough in both experiments. No mutations were detected in BTV from resistance selection at 1× EC_{95}. F227C was the first mutation identified in BTV, at 2× EC_{95}, followed by the emergence of a Y181C mutation at an inhibitor concentration of 50× EC_{95}.

The BTV obtained from the resistance selection at 50× EC_{95} with subtype B virus were subjected to clonal sequencing. As shown in Table 2, sequencing results of the BTV from experiment 1 indicated that the majority of the viruses possessed a double mutation of P236L/V106A (18/21 colonies) and that a minor population of viruses harbored either a double mutation of P236L/E138K (2/21 colonies) or a triple mutation of P236L/V106A/E138K (1/21 colonies). Fifty percent of the BTV from experiment 2 had the double mutation Y181C/V106A, and the other 50% had the additional mutation R172K. The BTV from experiment 3, however, were much more heterogeneous. The mutation V106I/E138K/P236L/K219E/D256E was found in 60% of the viral population, and five different mutant variants were distributed among the remaining 40%.

TABLE 2. Clonal sequencing of the BTV from resistance selection at 50× EC_{95} MK-4965 in subtype B virus

Expt	Identified mutations	No. of colonies
1	P236L/V106A	18
	P236L/E138K	2
	P236L/V106A/E138K	1
2	Y181C/V106A	10
	Y181C/V106A/R172K	9
3	V106I/E138K/P236L/K219E/D256E	12
	V106A/E138K/P236L/K219E/D256E	2
	V106A/E138K/P236L/K219E	2
	V106I/E138K/P236L/K219E/D256E/Y318F	2
	V106I/E138K/K219E/D256E	1
	V106M/E138K/P236L/K219E/D256E	1

***In vitro* resistance selection with EFV under low-MOI conditions.** Resistance selection with EFV was carried out under the same conditions described above. A summary of the results of the selection is shown in Table 3. The time that was required to detect the first viral breakthrough at 1× EC_{95} of EFV was again more than 3 weeks with subtype A virus, which is similar to that observed with MK-4965. Three pathways contributed to the evolution of mutations to escape viral suppression by EFV. The first pathway started with Y188C at 2× EC_{95}. F227C and a novel mutation (A288T) arose along with Y188C at 5× EC_{95}. Interestingly, Y188C was not detected in the BTV obtained from subsequent selection at 10× EC_{95}. Instead, V106M began to emerge. No sequence information was available from the selection at an EFV concentration of 50× EC_{95}, due to insufficient quantities of isolated BTV. In experiment 2, two mutations that are not commonly associated with NNRTIs (D312E and Q452L) were identified in the BTV at 1× EC_{95} and were persistent throughout the experiment. These two rare mutations were also accompanied by the addition of an L234F mutation and a rare F77Y mutation at higher concentrations of EFV. A single mutation (V106M) led the third pathway of mutation development at 2× EC_{95} and remained the only mutation found in BTV with EFV concentrations of up to 50× EC_{95}.

Consistent with reports in the literature, resistance selection with subtype B virus in the presence of EFV showed three familiar mutation development pathways. The three pathways were led by L100I, V179D, and K103N, respectively. One novel mutation (N348I) in the connection region was associated with L100I in the BTV selected at 50× EC_{95} in experiment 1. Emergence of L100I with V179D at 5× EC_{95}, followed by the appearance of V108I at 50× EC_{95}, represented the second mutation development pathway. Y188C was identified in the BTV at 10× EC_{95}, along with the initial K103N mutation, in experiment 3, and no additional mutations were identified at a concentration of 50× EC_{95}.

TABLE 3. Resistance selection with EFV in HIV-1 A, B, and C subtypes in the presence of 10% FBS under low-MOI conditions[e]

Subtype	Expt	1× EC_{95}[a]		2× EC_{95}		5× EC_{95}		10× EC_{95}		50× EC_{95}	
		T[b]	Mutation(s)	*T*	Mutation(s)	*T*	Mutation(s)	*T*	Mutation(s)	*T*	Mutation(s)
A	1	34	NM[c]	12	Y188(Y/C)	10	Y188(Y/C) F227(F/C) A288(A/T)	8	V106(V/M) F227(F/C) A288(A/T)		NA[d]
	2	24	D312E Q452L	17	D312E Q452L	15	D312E Q452L L234(L/F) F77(F/Y)	16	D312E Q452L L234F F77Y	11	D312E Q452L L234F F77Y
	3	30	NM	10	V106(V/M)	10	V106(V/M)	8	V106M	9	V106M
B	1	11	L100(L/I)	9	L100I	13	L100I	11	L100I	28	L100I N348I
	2	10	V179(V/D)	5	V179D	7	V179D L100(L/I)	8	V179D L100I		V179D[f] L100I[f] V108I[f]
	3	13	NM	9	K103N	9	K103N	8	K103N Y188(Y/C)	8	K103N Y188C
C	1	34	G190A L279F	10	G190A L279F	12	G190A L279F V106(V/M)	12	G190A L279F V106M	6	G190A L279F V106M
	2	34	G190A L279F	10	G190A L279F	12	G190A L279F	12	G190A L279F V106M	12	G190A L279F V106M

[a] The EC_{95} of EFV with subtype B virus is 3.8 ± 1.4 nM (24).
[b] *T*, time (in days).
[c] NM, no mutation was detected.
[d] NA, not available.
[e] Amino acids in parentheses indicate a mixture of the wild-type residue and the emerging mutation.
[f] Based on clonal sequencing, 14 out of 20 colonies contained L100I/V179D/V108I mutations, and 6 out of 20 clones had L100I/V179D mutations.

For resistance selection in the subtype C virus, the time required for viral breakthrough was more than 4 weeks at 1× EC_{95} of EFV, and a single pathway accounted for mutation in the subtype C virus. Genotypic analysis revealed a novel L279F mutation, along with a known NNRTI mutation (G190A), in the BTV selected at 1× EC_{95} of EFV. V106M started emerging at a concentration of 5× EC_{95} in experiment 1, and the triple mutation was present in the BTV from both experiments in selection with subtype C virus at 10× and 50× EC_{95}.

***In vitro* resistance selection with ETV under low-MOI conditions.** Resistance selection studies with ETV in subtype A suggested that the selected mutations evolved from three different pathways. As shown in Table 4, at 1× EC_{95} of ETV, no mutations were found in the BTV from experiments 1 and 3, but the A200E mutation was identified in the BTV from experiment 2. The A200E mutation was also selected by MK-4965 in the subtype A virus in experiment 3, as described above. It appears that Y181C was responsible for the viral breakthrough in experiment 1, and it remained the only mutation at compound concentrations up to 50× EC_{95}. In addition to A200E, Y181C started to emerge at 2× EC_{95} in the BTV from experiment 2, and both mutations remained the only ones identified in the experiment. In experiment 3, on the other hand, a relatively rare NNRTI-associated single mutation (H221Y) was identified in the BTV at 2× EC_{95} and 5× EC_{95}. However, no viral breakthrough was observed at higher selective pressure.

During resistance selection in the subtype B virus, no mutations were identified in BTV from any of the three experiments at 1× EC_{95}. The Y181C mutation was acquired in BTV obtained in experiment 1 at 2× EC_{95} and was acquired in experiment 2 at both 2× and 5× EC_{95}. The first mutation identified in the BTV of experiment 3 was also Y181C at 5× EC_{95}. An A272T mutation was identified along with Y181C from the selection at 2× EC_{95} in experiment 1, and both mutations continued to coexist as selection escalated to higher concentrations. A known ETV-associated mutation, V179I, subsequently emerged with Y181C at concentrations of 10× EC_{95} and 50× EC_{95} for both experiment 2 and experiment 3. An NNRTI-resistant mutation, L100I, was identified in the BTV from experiments 1 and 2 at 50× EC_{95}. As a result, a single mutation pathway converged from experiments 2 and 3, where Y181C led the mutation pathway followed by the emergence of V179I, with an additional L100I mutation selected in experiment 2 at 50× EC_{95}.

Resistance selection with subtype C virus, however, showed a distinct pattern of mutation evolution. More than 9 weeks of cell culture passage was required to detect the first viral breakthrough. For both experiments, V314I was the first mutation identified in the BTV obtained at 1× EC_{95} of ETV, followed by the acquisition of the E138K mutation at a concentration of 2× EC_{95}. V241A and H221Y emerged at a concentration of 10× EC_{95}, and two additional mutations (V106M and V90I) arose in the BTV at 50× EC_{95} of ETV. Together, there was a total of six mutations in the BTV at a concentration of 50× EC_{95}.

Assessment of the susceptibility of subtype A BTV to NNRTIs. To investigate the extent of resistance conferred by the BTV selected at high concentrations of NNRTIs with subtype A virus, a novel method using ViroMag R/L magnetic

TABLE 4. Resistance selection with ETV in HIV-1 A, B, and C subtypes in the presence of 10% FBS under low-MOI conditions[e]

Subtype	Expt	1× EC_{95}[a]		2× EC_{95}		5× EC_{95}		10× EC_{95}		50× EC_{95}	
		T[b]	Mutation	*T*	Mutation(s)	*T*	Mutation(s)	*T*	Mutation(s)	*T*	Mutation(s)
A	1	30	NM[c]	10	Y181(Y/C)	9	Y181C	8	Y181C	7	Y181C
	2	24	A200(A/E)	14	A200E Y181(Y/C)	9	A200E Y181C	8	A200E Y181C	7	A200E Y181C
	3	18	NM	12	H221(H/Y)	15	H221Y		NB[d]		NB
B	1	11	NM	34	Y181(Y/C) A272(A/T)	11	Y181C A272T	21	Y181C A272T	28	Y181C A272T L100I
	2	10	NM	9	Y181C	7	Y181C	10	Y181C V179(V/I)	15	Y181C[f] V179I[f] L100I[f]
	3	10	NM	9	NM	11	Y181C	10	Y181C V179(V/I)	20	Y181C V179I
C	1	65	V314I	12	V314I E138K	15	V314I E138K	11	V314I E138K V241A H221(H/Y)	14	V314I E138K V241A H221(H/Y) V106(V/M) V90(V/I)
	2	65	V314I	12	V314I E138K	15	V314I E138K V241(V/A)	11	V314I E138K V241A	14	V314I E138K V241A H221(H/Y) V106(V/M) V90(V/I)

[a] The EC_{95} of ETV with subtype B virus is 4.0 ± 1.1 nM (24).
[b] *T*, time (in days).
[c] NM, no mutation was detected.
[d] NB, no breakthrough.
[e] Amino acids in parentheses indicate a mixture of the wild-type residue and the emerging mutation.
[f] Based on clonal sequencing, 15 out of 23 colonies contained Y181C/V179I/L100I mutations, 7 out of 23 displayed Y181C/V179I mutations, and 1 out of 23 clones showed Y181C/V179I/L100I/V189I mutations.

beads was employed to isolate viral particles. BTV isolated using this method were employed to infect cells to assess the susceptibility of the BTV to NNRTIs (18). The results are summarized in Table 5. Viruses containing the A200E/F227C mutation, which was selected by MK-4965, displayed FCs in EC_{50} of 31, 15, and 7.1 against MK-4965, EFV, and ETV, respectively. During resistance selection with EFV, the emergence of the V106M/F227C double mutation significantly reduced the susceptibility of the virus to EFV, with an FC of 113. The BTV also displayed a high level of resistance to MK-4965 and a low level of resistance to ETV, with FCs of 36 and 2.6, respectively. The single V106M mutation selected by EFV conferred a 16-fold resistance to EFV, yet viruses containing this mutation were highly sensitive to MK-4965 and ETV, with FCs of 0.5 and 0.7, respectively. This hypersensitivity may be derived from the greater extent of interactions between the NNRTIs and M106 gained by the longer side chain of methionine. The Y181C and Y181C/A200E mutations were selected by ETV. A200E appeared to have no impact on the susceptibility of the mutant viruses to the NNRTIs, because mutant viruses with and without A200E showed similar levels of resistance to MK-4965, EFV, and ETV. Notably, subtype A BTV containing the Y181C mutation showed significant resistance to ETV, with an FC of ~30, compared to an FC of 5.5 seen in subtype B viruses containing the same mutation, as described below. It should be noted that the mutations described here were based on population sequencing, so a minor population of mutant viruses was also present, and these viruses may also have contributed to the reduced susceptibility of the BTV to NNRTIs.

TABLE 5. Susceptibility of the subtype A BTV selected by MK-4965, EFV, and ETV to the NNRTIs[a]

Selection compound	Selected mutation(s)	Fold change in EC_{50} against:		
		MK-4965	EFV	ETV
MK-4965	A200E/F227C	31 ± 4.2	15 ± 4.4	7.1 ± 1.7
EFV	V106M	0.55 ± 0.25	16 ± 6.7	0.74 ± 0.31
	V106(V/M)/F227(F/C)	36 ± 8.9	113 ± 18	2.6 ± 1.5
ETV	Y181C	7.7 ± 1.6	2.3 ± 0.3	32 ± 10
	Y181C/A200E	9.3 ± 2.0	1.9 ± 0.46	27 ± 6

[a] Experiments were performed at least in quadruplicate.

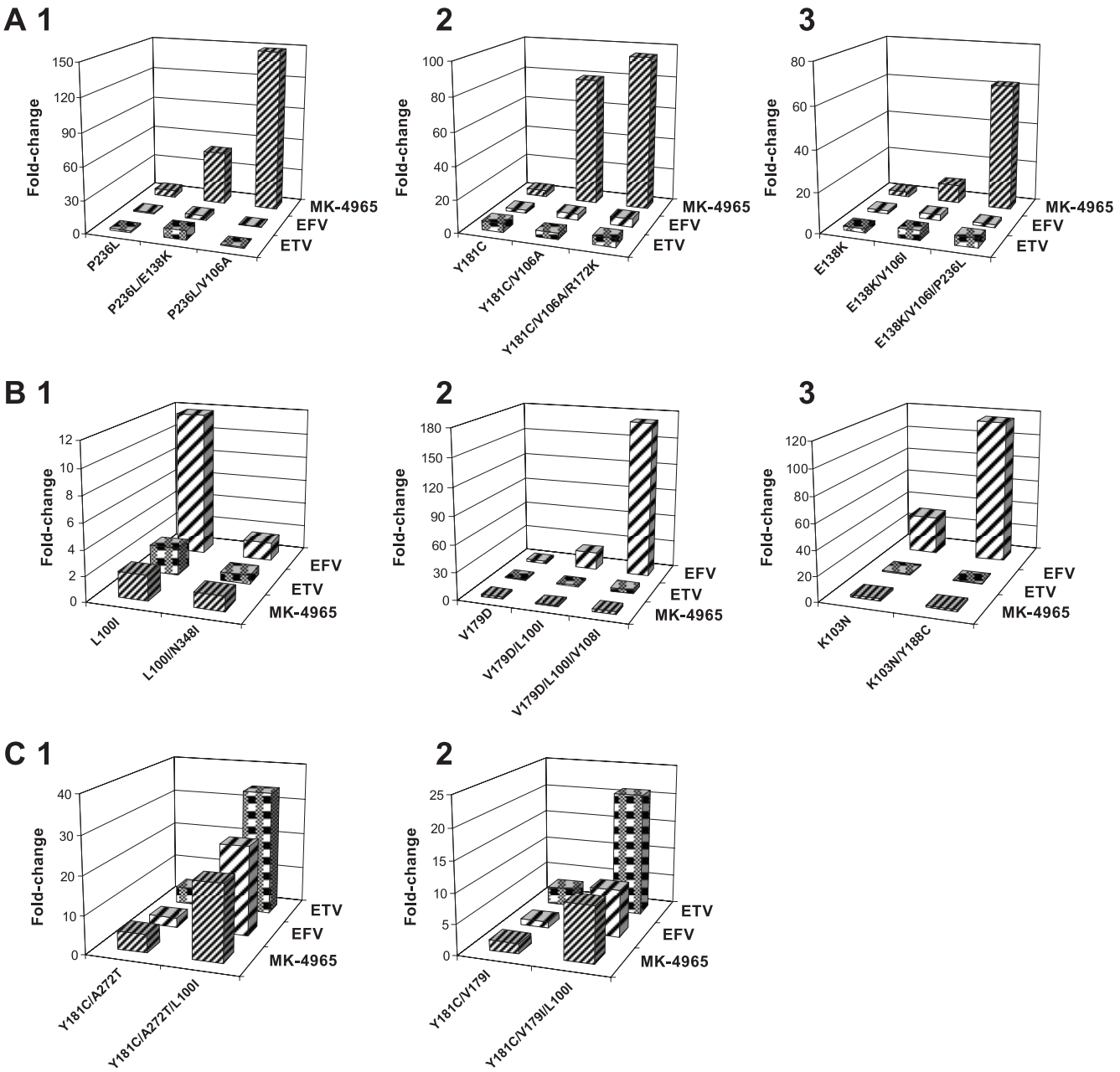

FIG. 2. Susceptibility of subtype B mutant viruses derived from SDM to MK-4965, EFV, and ETV. (A) Mutant viruses derived from mutations selected by MK-4965 in experiments 1 to 3. (B) Mutant viruses derived from mutations selected by EFV in experiments 1 to 3. (C) Mutant viruses derived from mutations selected by ETV in experiments 1 to 3. The values presented in the figure represent means of quadruplicate results.

Attempts to assess the susceptibility of subtype C BTV obtained from resistance selection to NNRTIs by using the same approach were futile because the magnetic beads were not able to efficiently capture the virus for subsequent cell infection.

Assessment of the susceptibility of subtype B mutant viruses selected by MK-4965 to NNRTIs. To evaluate the degree of NNRTI resistance conferred by the mutations identified in BTV of subtype B virus during resistance selection, a traditional SDM method was utilized to generate the desired mutant viruses. An optimal viral titer that gave a signal-to-background ratio of 10 was chosen for drug titration. All experiments were performed in quadruplicate, except two of the mutant viruses.

As shown in Fig. 2A (a table with all FC values, including standard deviations, is provided in the supplemental material), mutations selected by MK-4965 all conferred a higher level of resistance to MK-4965 than to EFV or ETV. More importantly, viruses containing mutations that evolved at early stages conferred a lower degree of resistance to MK-4965 than those containing mutations that developed at later stages. For instance, in experiment 1 (Fig. 2A1), P236L (selected at 1×

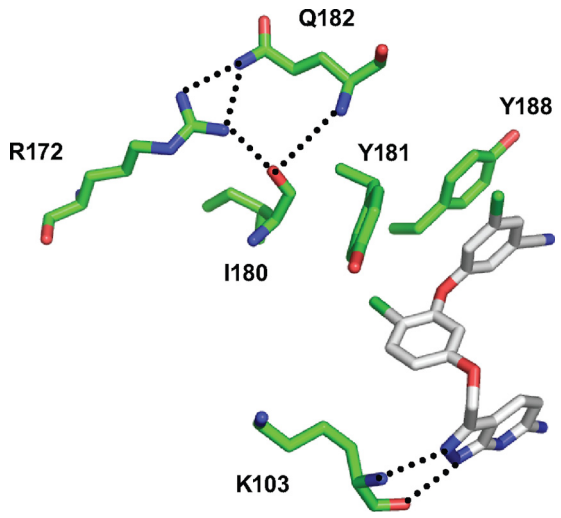

FIG. 3. Hydrogen bonding network of R172, based on the X-ray structure of the RT/MK-4965 complex.

EC_{95}) exhibited an FC of 6, P236L/E138K (evolved at 10× EC_{95}) showed an FC of 49, and P236L/V106A (identified at 50× EC_{95}) displayed an FC of 148. P236L mutant viruses were sensitive to EFV and ETV, and P236L/E138K-containing viruses had modest resistance to EFV and ETV, with FCs of 4 and 9, respectively. In contrast, P236L/V106A mutant viruses appeared to be hypersensitive to both EFV and ETV, with FCs of 0.5 and 0.6, respectively. Since the susceptibility of the P236L virus to both EFV and ETV was comparable to that of the WT virus, this hypersensitivity may be ascribed to the additional room gained by changing the valine to alanine at residue 106 in the NNRTIBP, thus enabling the binding pocket to better accommodate alternative conformations that may be exerted by either compound.

The Y181C mutation selected in experiment 2 displayed similar levels of resistance to all three NNRTIs (Fig. 2A2); FCs were around 2, 4, and 6 against EFV, MK-4965, and ETV, respectively. The presence of the V106A mutation along with Y181C, however, dramatically reduced the susceptibility of the mutant virus to MK-4965, with an increase in FC from 4 to 79. The inclusion of an additional novel mutation, R172K, further enhanced the resistance of the mutant virus to MK-4965, with an FC of 95. The R172K mutation has been identified in resistance selection with the NRTI 2,3-didehydro-2,3-dideoxy-5-fluorocytidine (d4FC) (18, 19). The mutant virus containing R172K, K70N, and V90I mutations confers a 3.9-fold resistance to d4FC. Based on the X-ray structure of MK-4965 RT (Fig. 3), R172 forms a network of hydrogen bonds with the backbone carbonyl group of I180 and the side chain of Q182. Because both residues are adjacent to Y181, the R172K mutation may change the hydrogen bonding network and, in turn, alter the conformation of the NNRTIBP, thereby reducing the affinity of MK-4965 for the pocket. However, the addition of V106A followed by the R172K mutation did not alter the susceptibility of the mutant virus to EFV and ETV.

The acquisition of the E138K mutation slightly increased the level of resistance to all three NNRTIs approximately 2-fold (Fig. 2A3). The inclusion of the V106I mutation at a higher selective pressure conferred a greater degree of resistance to MK-4965, with an FC of 9. However, susceptibility to the inhibitor was significantly reduced by the coexistence of the P236L mutation, with an FC of 62. In contrast, the presence of additional V106I and P236L mutations did not further reduce the susceptibility of the viruses to EFV and ETV.

Assessment of the susceptibility of subtype B mutant viruses selected by EFV to NNRTIs. Several common NNRTI resistance mutations, such as L100I, V179D, and K103N, were selected by EFV during resistance selection with subtype B viruses. As shown in Fig. 2B1, mutant viruses containing the L100I mutation had a 12-fold resistance to EFV, whereas the variant displayed only an ~2-fold resistance to both MK-4965 and ETV. Interestingly, the addition of a mutation in the connection region (N348I), selected at 50× EC_{95} EFV, restored the sensitivity of the virus to EFV, with an FC of 1.5, but did not have any impact on the susceptibility of the virus to MK-4965 or ETV. Although this mutation was observed frequently in zidovudine (AZT)- and/or didanosine (ddI)-containing therapies and was accompanied by thymidine analog mutations (TAMs), such as T215Y (12), EFV treatment is thought to be associated with the emergence of the N348I mutation (16). Viruses harboring the mutation exhibit resistance to NNRTIs, such as nevirapine (NVP) and delavirdine, and to NRTIs, such as AZT and ddI (16, 41). Viruses containing the L100I or N348I mutation have been shown to have a lower replication capacity than the WT (16, 23). It is unknown whether the combination of both mutations could enable the virus to regain or even enhance replication capacity to compensate for the increase in susceptibility. It is worth noting that a mutation outside the NNRTI binding pocket (M16R) was also identified in the BTV. It remains to be elucidated whether this mutation, in combination with the L100I/N348I mutations, plays a role in enhancing the resistance of the mutant virus to EFV.

Mutant viruses containing the V179D mutation that were selected in experiment 2 were somewhat sensitive to inhibition by NNRTIs, with an FC of <3 (Fig. 2B2). Inclusion of an additional L100I mutation reduced the susceptibility of the mutant virus to EFV, with an FC of 20. Viruses with V179D/L100I mutations were still highly sensitive to MK-4965 and ETV, with an FC of <2. The additional emergence of the V108I mutation significantly enhanced the resistance of the mutant virus to EFV, with an FC of 172. However, only a minor reduction in susceptibility was observed when mutant viruses containing the triple mutation were tested against MK-4965 and ETV, with FCs of 3.4 and 4.1, respectively.

It is known that K103N is the most prevalent NNRTI-resistant mutation in viruses from patients who have failed an EFV-containing regimen. As expected, K103N mutant viruses showed a 31-fold resistance to EFV, whereas the mutant virus did not show resistance to MK-4965 or ETV (Fig. 2B3), which is consistent with previous reports (24, 39). Y188C appears to work synergistically with K103N in enhancing the resistance to EFV, as viruses with the double mutation have a >100-fold resistance and those with the Y188C mutation alone showed only an approximately 2-fold resistance (data not shown). These two mutations, however, did not affect the potency of MK-4965 in the inhibition of the mutant viruses, with an FC lower than 2. Furthermore, the presence of the Y188C mutation ap-

TABLE 6. Resistance selection with NNRTIs in HIV-1 subtype B virus in the presence of 10% FBS or 50% HS under high-MOI conditions[e]

Serum	Inhibitor	Concn (nM)	Expt 1		Expt 2		Expt 3	
			T[a]	Mutation(s)	*T*	Mutation(s)	*T*	Mutation
10% FBS	MK-4965	40	13	V106A	13	V106A	15	V106A
		200	NB[b]	NA[d]	NB	NA	36	V106A
		1,000	NB*[c]	NA	NB*	NA	NB*	NA
	EFV	40	12	L100I	13	L100I	12	K103N
		200	NB	NA	NB	NA	NB	NA
		1,000	NB	NA	NB	NA	34	G190V
	ETV	40	NB	NA	28	Y181C	NB	NA
		200	NB	NA	NB	NA	NB	NA
		1,000	NB	NA	NB	NA	NB	NA
50% HS	MK-4965	200	13	V106A	10	V106A		
		500	15	V106A	15	V106A		
		1,250	20	V106A/V189I/V108I	20	V106A		
	EFV	200	13	Q197(Q/K)	13	L100I		
		500	15	L100(L/I)/Y188(Y/L/F)/R463(R/K)	15	K103N/S191(S/F)/L283(L/F)		
		1,250	15	K103N	NB	NA		
	ETV	200	15	Y181C	15	Y181C		
		500	NB	NA	NB	NA		
		1,250	NB	NA	NB	NA		

[a] *T*, time (in days).
[b] NB, no breakthrough.
[c] NB*, minor cell lysis was observed.
[d] NA, not applicable.
[e] Amino acids in parentheses indicate a mixture of the wild-type residue and the emerging mutation.

peared to increase the susceptibility of the mutant virus to ETV, with an FC of 0.36.

Assessment of the susceptibility of subtype B mutant viruses selected by ETV to NNRTIs. As described above, only two mutation development pathways were observed in the resistance selection with ETV. Y181C was identified in all three rounds of selection. As shown in Fig. 2C1, the inclusion of a novel A272T mutation in experiment 1 seemed to have no impact on the susceptibility of the mutant virus to all three NNRTIs, comparing the FC to that of the Y181C mutant virus (Fig. 2A2). Therefore, this mutation could be derived from a linked viral polymorphism. However, the addition of the L100I mutation significantly reduced the susceptibility of the triple mutant virus to MK-4965, EFV, and ETV, with FCs of 20, 24, and 34, respectively. Viruses with the Y181C/V179I double mutation exhibited a 3-fold resistance to ETV (Fig. 2C2). The mutant virus also displayed similar susceptibilities to MK-4965 and EFV. At a higher selective pressure with ETV (50× EC_{95}), L100I began to emerge with Y181C/V179I. All three mutations (Y181C, V179I, and L100I) are known to be associated with ETV resistance (39). Mutant viruses containing the triple mutation exhibited a high level of resistance to ETV, with an FC of 21. The mutant virus also displayed modest resistance to MK-4965 and EFV, with FCs of 9 and 8, respectively.

***In vitro* resistance selection of subtype B viruses with NNRTIs under high-MOI conditions.** As described above, resistance selection of subtype B viruses with MK-4965, EFV, and ETV under low-MOI conditions did identify NNRTI-associated mutations, such as P236L, V106A, K103N, L100I, V179D, V179I, Y181C, etc. To investigate the resistance barrier of the NNRTIs further, resistance selection was also conducted under high-MOI conditions at fixed high concentrations of inhibitors in the presence of 10% FBS (39). Because all three NNRTIs exhibit comparable EC_{95}s (~4 nM), identical concentrations of each inhibitor were used for resistance selection study (40 nM, 200 nM, and 1 μM). As shown in Table 6, during the resistance selection with MK-4965, only the V106A mutation was identified in the BTV from all three experiments at 40 nM and one experiment at 200 nM. No breakthrough was observed in the other two experiments at 200 nM MK-4965. Similarly, no breakthrough was observed in any of the three experiments at 1 μM, and the infected cells displayed a minor degree of lysis at this concentration. During resistance selection with EFV at 40 nM, the L100I mutation was identified in two experiments, and K103N was identified in the third experiment. No breakthrough was observed at 200 nM. Surprisingly, viral breakthrough was found in one of the experiments conducted with 1 μM EFV, and a rare mutation (G190V) was identified in the BTV. For ETV, on the other hand, only one of the experiments at 40 nM showed breakthrough, at week 4; Y181C was the mutation responsible for the viral escape of the selective pressure.

Resistance selection under high-MOI conditions was also conducted (in duplicate) with 50% HS to account for the higher protein concentration in the human bloodstream (Table 6). The EC_{95} shifted ~5-fold in the presence of 50% HS compared to that in the presence of 10% FBS. Consequently, the lowest concentration used for the study with 50% HS was 200 nM instead of 40 nM. Two additional concentrations, 500 nM and 1.25 μM, were also included in the resistance selection. Viral breakthroughs were observed with all three concentrations of MK-4965. Consistent with the selection at 10% FBS, V106A was identified in BTV from five out of six experiments with MK-4965. A triple mutation, V106A/V189I/V108I, was found in one of the experiments at 1.25 μM. The BTV with the triple mutation were subjected to clonal analysis, and the results indicated that the majority of the BTV (22/23) possessed all three mutations. Under the same conditions, EFV

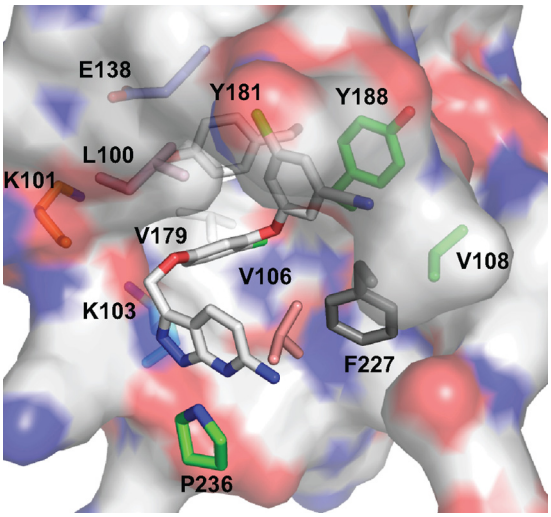

FIG. 4. Residues surrounding the NNRTIBP, based on the X-ray structure of the RT/MK-4965 complex.

selected Q197K and L100I at a concentration of 200 nM for experiments 1 and 2, respectively. At 500 nM EFV, BTV from experiments 1 and 2 all contained a mixture of three mutations: L100I, Y188L, and R463K for experiment 1 and K103N, S191F, and L283F for experiment 2. Viral breakthrough was observed only in experiment 1 at 1.25 μM; the mutation was identified to be K103N. During resistance selection with ETV under the same conditions, Y181C was again the only mutation responsible for the viral breakthrough at 200 nM, and no breakthrough was found at concentrations of 500 nM and 1.25 μM.

These results indicate that, under high-MOI conditions, V106A is the dominant pathway in the development of mutations under the selective pressure of MK-4965. The V106A mutation is known to be associated with nevirapine (NVP) (33). Mutant viruses containing the V106A mutation conferred a 15-fold resistance to MK-4965 but were susceptible to EFV and ETV, with FCs of 1.5 and 0.7, respectively. These results suggest that V106 plays an important role in the interactions between MK-4965 and WT RT. Based on the X-ray structure of the RT/MK-4965 complex (Fig. 4), one of the methyl groups of the V106 side chain is in close proximity to the bicyclic indazole ring (c ring). The van der Waals interactions between the methyl group and the indazole ring are apparently crucial for the binding of MK-4965 to the NNRTIBP. L100I and K103N were the two major mutations selected by EFV, and Y181C was the dominant mutation selected by ETV under either high- or low-MOI conditions.

DISCUSSION

On a global scale, the most prevalent HIV-1 subtypes in descending order are C, A, and B, accounting for 47, 27, and 12% of HIV-1-infected patients, respectively. Therefore, in addition to subtype B, subtype A and C viruses were included in our resistance selection study with NNRTIs using SupT1 cells under low-MOI conditions to investigate the mutation pathways evolved from each subtype.

In spite of the fact that K103N is the most prevalent mutation found in patients who have failed clinically with an EFV-containing regimen, the K103N mutation has not been reported as the sole mutation resulting from resistance selection with EFV in cell culture (39, 42). In this study, however, in addition to common NNRTI-associated mutations identified in the BTV from EFV resistance selection with subtype B viruses, a single K103N mutation was selected by EFV under both high- and low-MOI conditions. Moreover, Y181C, V179I, and L100I, which were involved in the mutation development pathways in this resistance selection study with ETV, were also identified in other selection studies, as well as in clinical trials with ETV (25, 27, 39). Therefore, mutations identified in this study are, to some extent, consistent with the resistance mutations detected in viruses from patients who failed EFV- or ETV-containing regimens.

In the resistance selection study with MK-4965 in subtype B viruses, P236L/E138(E/K) was selected at a concentration of 10× EC_{95}. Interestingly, E138(E/K) was replaced by V106A at a concentration of 50× EC_{95}. This replacement may be due to the fact that viruses with the P236L/V106A mutation exhibit greater resistance to MK-4965 than viruses with P236L/E138K mutations (FCs of 148 versus 49, respectively). It appears that E138K and P236L work synergistically to reduce the susceptibility of the mutant virus to MK-4965, as viruses containing either the E138K or the P236L mutation alone conferred a 3.6- or 6-fold resistance, respectively, to MK-4965, while mutant viruses with the double mutation had a 49-fold resistance to MK-4965. The synergistic effect between P236L and E138K is also shown, to a lesser extent, by the resistance of the mutant viruses to EFV and ETV. Residues E138 of the p51 subunit and K101 of the p66 subunit form the upper part of the entrance to the NNRTIBP. Based on the X-ray structure of WT HIV-1, E138 interacts with K101 by forming a salt bridge via a water molecule (13). The two residues can also form a salt bridge directly in RT mutants in the presence of an NNRTI (32). Therefore, the E138K mutation may prevent salt bridge formation, resulting in a weaker association between the two residues, which would facilitate the NNRTI dissociation from the NNRTIBP through the gap between the residues. In addition, P236 interacts with the C ring of MK-4965 via hydrogen bonds, thereby playing an important role in the compound binding to the pocket (Fig. 4). It is conceivable that the P236L mutation abolishes the hydrogen bond formation, causing a 6-fold reduction in affinity. The P236L mutation may work synergistically with E138K by reducing the interactions between the NNRTIBP and MK-4965 and facilitating the dissociation of the compound through the disrupted salt bridge. The K101E mutation has been observed in mutant viruses isolated from patients who were treated with NVP or EFV (5, 26). In addition, both E138K and K101E have been identified in resistance selection with TMC278 (4). However, both mutations did not exist in one virus at the same time, as the salt bridge remains intact with the double mutation (E138K/K101E), thus maintaining the same conformation as the WT.

Based on the results from resistance selection under low-MOI conditions, it is not immediately obvious which NNRTI (EFV, ETV, or MK-4965) possesses the highest *in vitro* resistance barrier, because all three NNRTIs selected similar numbers of NNRTI-associated mutations in comparable time

frames. However, under high-MOI conditions, ETV appears to have the highest resistance barrier among the three compounds. No viral breakthrough was observed at high concentrations of ETV in the presence of 10% FBS or 50% HS, but breakthrough viruses were obtained for all concentrations of MK-4965 and five out of six concentrations of EFV in the presence of 50% HS. It is important to point out that many other factors likely influence the resistance barrier *in vivo*, but based on the results shown in Table 6, the rank order of *in vitro* resistance barrier was ETV > EFV ≥ MK-4965. These results are, in general, consistent with the mutant profile reported in the literature. Therefore, *in vitro* resistance selection under high-MOI conditions may be a better assessment of resistance barrier for different NNRTIs.

Interestingly, the V106A mutation was acquired only at high concentrations of MK-4965 under low-MOI conditions, despite the fact that viruses containing the mutation exhibit greater resistance to MK-4965 than viruses containing mutations selected at early stages of selection (e.g., P236L or E138K). This result may be due to the fact that viruses with the V106A mutation are less fit than some of the other mutant viruses (3, 28). As a result, mutant viruses with better replication capacity dominate the viral population at early stages of selection under low-MOI conditions, even though they have a lower degree of resistance. Under high-MOI conditions, however, the lowest concentration of the antiviral agents was approximately 10× EC_{95}, which is much higher than the starting concentration of 1× EC_{95} under low-MOI conditions. Consequently, mutant viruses that evolved with a low degree of resistance during selection under high-MOI conditions would not be able to replicate efficiently at MK-4965 concentrations of 10× EC_{95} or higher. These results suggest that the interplay between the level of fitness and the resistance of mutant viruses plays a crucial role in the development of mutations. This contention is also supported by the emergence of the G190V mutation at high EFV concentrations, in which G190V mutant viruses displayed an FC of >100. Under high selective pressure, mutations that confer greater resistance would be preferentially selected at the expense of the lower replication capacity of the mutant virus.

V106M was selected by EFV in subtype A and C viruses. V106M is a known clinical mutation specifically associated with subtype C viruses under an EFV-containing regimen (8, 15). Interestingly, although subtype B viruses with the V106A mutation are highly susceptible to EFV, with an FC of 1.5, the V106M mutation of subtype B viruses results in an approximately 10-fold resistance to EFV (data not shown). As mentioned above, the V106A mutation may generate additional room to accommodate EFV, owing to the lack of the dimethyl group in the alanine. V106M, on the other hand, may interfere with the binding of EFV to the NNRTIBP, as the side chain of methionine is longer than that of valine. Since the V106M mutation significantly reduces the susceptibility of subtype B viruses to EFV, it is expected that the V106M mutation should arise more often during resistance selection. Instead, V106M is listed as a rare mutation in subtype B viruses under EFV treatment. This finding may be attributed to the fact that the V106M mutation requires two base changes (from GTA to ATG) in subtype B viruses, while only one base change (from GTG to ATG) is required for subtype C and A viruses (8). Moreover, the X-ray structure (Fig. 3 and 4) reveals that residue Y188 engages in a π-π interaction with the chloro/cyano ring (a ring) of MK-4965. The abolition of the aromatic π-π interaction via the Y188L mutation resulted in a significant reduction of the affinity of MK-4965 to the binding pocket, with an FC of >100 (24). It is also known that viruses with the Y188L mutation are highly resistant to EFV. However, even though the replication capacity of viruses with the Y188L mutation is reportedly comparable to that of viruses containing the K103N mutation (6), Y188L was not selected by MK-4965 and is present in only ~5% of patients who experience virologic failure in therapy with EFV-containing regimens (35). The potential reason for the rare emergence of the Y188L mutation may be the need for two base changes in order to convert tyrosine to leucine (7). The Y188L mutation is derived from an intermediate mutant, possessing either the Y188H or the Y188F mutation. Both mutants (with the Y188H or the Y188F mutation) are highly susceptible to NNRTIs (data not shown), so the opportunity to further mutate to the Y188L mutation may be limited. The assertion that the Y188-to-L188 mutation is mediated by an intermediate mutation of Y188F is supported by the finding that a mixture of Y188Y/F/L mutations were identified in the BTV obtained from resistance selection under high-MOI conditions at 500 nM EFV (Table 6). Taken together, these results suggest that the number of base changes required to acquire a mutation also plays an important role in the mutation development pathway.

The Y181C mutation reduced virus susceptibility to ETV 5.5-fold in subtype B viruses, whereas Y181C subtype A mutant viruses showed significant resistance to ETV, with an FC of 32. Therefore, the Y181C mutation in the RT derived from subtype A viruses may perturb the conformation of the NNR TIBP to a greater extent than that derived from subtype B virus with respect to ETV binding, thus causing a higher level of resistance. Furthermore, the fact that most of the common NNRTI-associated mutations identified in subtype B viruses were not selected by NNRTIs in subtype A and C viruses in this study indicates that minor differences may exist in the relative residue conformations within the NNRTIBPs among viruses of different subtypes. As a result, the interactions between NNRTIs and the NNRTIBP are not identical among the different subtypes, leading to distinct pathways of mutation. The other possible explanation for this discrepancy is that different polymorphisms may exist among HIV-1 subtypes.

F227C was the dominant mutation selected by MK-4965 in subtype A and C viruses but was not found in the BTV from subtype B viruses during resistance selection with the three compounds, despite the fact that the mutation confers significant resistance to EFV and ETV in subtype B viruses (7, 26). Although the phenylalanine codon (TTC) at position 227 in the RT from subtype B viruses is different from that of subtype A and C viruses (TTT), the mutation from phenylalanine to cysteine (TGT or TGC) requires only a single base change for all three subtype viruses. Therefore, the lack of selection of the F227C mutation in subtype B viruses cannot be ascribed to the difference in number of base changes required for the mutation. One possible explanation for this finding is that the mutation significantly impairs the replication capacity of subtype B viruses and that the impact on replication capacity is not as significant for the subtype A and C viruses. Another potential

TABLE 7. Pathways of mutation development selected by NNRTIs in HIV-1 A, B, and C subtypes

Inhibitor	Subtype	Pathway	Mutation(s)
MK-4965	A	a	F227C
		b	Y181C
		c	F227C
	B	a	P236L→P236L/E138K→P236L/V106A
		b	Y181C→Y181C/V106A→Y181C/V106A/R172K
		c	E138K→E138K/V106I→E138K/V106I/P238L
	C	a	F227C→F227C/Y181C
EFV	A	a	F227C→F227C/V106M
		b	L234F
		c	V106M
	B	a	L100I→L100I/V179D/N348I
		b	V179D→V179D/L100I→V179D/L100I/V108I
		c	K103N→K103N/Y188C
	C	a	G190A→G190A/V106M
ETV	A	a	Y181C
		b	Y181C
		c	H221Y
	B	a	Y181C→Y181C/L100I
		b	Y181C→Y181C/V179I→Y181C/V179I/L100I
		c	Y181C→Y181C/V179I
	C	a	E138K→E138K/H221Y→E138K/H221Y/V90I

reason for different selection pathways could be that the proportions of different minor variants could differ in the starting virus stocks; for example, if F227C were present at 1% in the subtype A and C stocks but not in the subtype B stock, then F227C would be preferentially selected in subtype A and C viruses during resistance selection.

The mutation development pathways selected by NNRTIs among the different subtypes of viruses, after discounting non-NNRTI-associated mutations, are summarized in Table 7. It appears that subtype C viruses have a more convergent mutation development pathway, since only a single pathway was identified from two independent experiments for all three NNRTIs. In contrast, subtype B viruses show the most diverse pathways of mutation development; three different pathways were selected by NNRTIs in three different experiments. Subtype A viruses seem to lie in between with respect to the diversity of mutation pathways; two different pathways were selected with MK-4965 and ETV.

In summary, this study demonstrates that different virus subtypes exhibit distinct mutation development pathways. These results suggest that minor differences may be present within the NNRTIBPs of the various subtypes and thus that the interactions between the residues in the NNRTIBP and the NNRTIs may not be identical, rendering distinct patterns of mutation development. In addition, the interplay between the degree of resistance and the fitness of the mutant viruses, as well as the number of base changes required to generate a resistance mutation, plays an important role in the development of mutations during *in vitro* resistance selection with NNRTIs.

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