

Impact of Drug Resistance-Associated Amino Acid Changes in HIV-1 Subtype C on Susceptibility to Newer Nucleoside Reverse Transcriptase Inhibitors

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The objective of this study was to assess the phenotypic susceptibility of HIV-1 subtype C isolates, with nonnucleoside reverse transcriptase inhibitor (NNRTI) resistance-associated amino acid changes, to newer NNRTIs. A panel of 52 site-directed mutants and 38 clinically derived HIV-1 subtype C clones was created, and the isolates were assessed for phenotypic susceptibility to etravirine (ETR), rilpivirine (RPV), efavirenz (EFV), and nevirapine (NVP) in an *in vitro* single-cycle phenotypic assay. The amino acid substitutions E138Q/R, Y181I/V, and M230L conferred high-level resistance to ETR, while K101P and Y181I/V conferred high-level resistance to RPV. Y181C, a major NNRTI resistance-associated amino acid substitution, caused decreased susceptibility to ETR and, to a lesser extent, RPV when combined with other mutations. These included N348I and T369I, amino acid changes in the connection domain that are not generally assessed during resistance testing. However, the prevalence of these genotypes among subtype C sequences was, in most cases, <1%. The more common EFV/NVP resistance-associated substitutions, such as K103N, V106M, and G190A, had no major impact on ETR or RPV susceptibility. The low-level resistance to RPV and ETR conferred by E138K was not significantly enhanced in the presence of M184V/I, unlike for EFV and NVP. Among patient samples, 97% were resistant to EFV and/or NVP, while only 24% and 16% were resistant to ETR and RPV, respectively. Overall, only a few, relatively rare NNRTI resistance-associated amino acid substitutions caused resistance to ETR and/or RPV in an HIV-1 subtype C background, suggesting that these newer NNRTIs would be effective in NVP/EFV-experienced HIV-1 subtype C-infected patients.

Highly active antiretroviral therapy (HAART), which comprises the concomitant use of multiple potent antiretroviral drugs, has contributed to a significant decrease in the morbidity and mortality of people infected with HIV-1 (1). The failure of HAART through the acquisition of HIV drug resistance-associated substitutions that cause a decrease in viral susceptibility is usually due to poor adherence and insufficient drug concentrations. Although more than two-thirds of the global HIV-1 infections occur in sub-Saharan African countries, where HIV-1 infections are dominated by non-B subtypes, HAART regimens have largely been developed and tested against HIV-1 subtype B isolates (2). Subtype C accounts for almost half of all global infections and dominates the epidemic in southern Africa (3). While HAART agents are effective against all subtypes (4), greatly aiding the global response to HIV infection, specific resistance mutations and disparities in drug susceptibilities can differ by subtype (5). Examples include the K65R (6) and V106M (7) resistance-associated amino acid substitutions, which develop more frequently under drug pressure in HIV-1 subtype C than subtype B.

Nonnucleoside reverse transcriptase (RT) inhibitors (NNRTIs) are a component of most first-line HAART regimens. For efavirenz (EFV) and nevirapine (NVP), the genetic barrier to the development of resistance-associated mutations and a loss of potency is low. The accumulation of EFV and NVP resistance-associated mutations is rapid, often occurring within 3 months of virologic failure (8). Recent studies in South Africa have shown that up to 80% of patients who fail EFV or NVP therapy develop NNRTI resistance-associated muta-

tions (9–12). Furthermore, the substantial cross-resistance between these two drugs makes their sequential use after virologic failure inappropriate.

Etravirine (ETR; TMC125) (13–15) and rilpivirine (RPV; TMC278) (16, 17) are diarylpyrimidine (DAPY) NNRTIs with resistance profiles that only partially overlap those of EFV and NVP (18, 19). The efficacy of ETR was assessed in the DUET-1 and DUET-2 trials with treatment-experienced patients, while RPV was assessed in treatment-naïve patients in the ECHO and THRIVE trials (13–17, 20). Both ETR and RPV suppress viral replication irrespective of HIV-1 subtype and have shown activity against clinically relevant mutants (19, 21–23). However, data on the sequential use of these agents and cross-resistance have mostly

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TABLE 1 HIV-1 subtype C sequences with NNRTI resistance-associated mutations used in this study

NNRTI treatment(s)	No. (%) of patients		
	SATuRN database	Stanford database	Total
EFV	630 (82.2)	278 (41.6)	908 (63.3)
NVP	124 (16.1)	316 (47.4)	440 (30.7)
NVP, EFV	12 (1.6)	31 (4.6)	43 (3.0)
Unknown		39 (5.8)	39 (2.7)
Other ^a		3 (0.4)	3 (0.2)
Total	766	667	1,433

^a The patients received delavirdine (DLV) or MKC422.

been derived from subtype B-infected cohorts and often as part of clinical trials, with little information on how HIV-1 subtype C isolates from first-line NNRTI treatment failures might respond (22, 24). In this study, using clinical samples and site-directed mutants, we investigated the phenotypic impact of NNRTI resistance-associated amino acid substitutions on ETR and RPV susceptibility in an HIV-1 subtype C background since these drugs are likely to be increasingly used in South Africa.

MATERIALS AND METHODS

HIV-1 subtype C sequences. A total of 1,433 HIV-1 subtype C sequences from patients exposed to EFV or NVP were used to determine the prevalence of different NNRTI resistance-associated mutations. Sequences were obtained from the South African Treatment and Resistance Network (SATuRN) database (<http://www.bioafrica.net/regadb/>) ($n = 766$) (9) and the Stanford HIV Drug Resistance Database (HIVdb) (<http://hivdb.stanford.edu/>) ($n = 667$). Only sequences with NNRTI resistance-associated mutations were included. A covariation analysis was performed to identify significantly associated pairs of NNRTI resistance-associated mutations as previously described (25). Briefly, a Jaccard similarity coefficient was employed to identify pairwise correlations among NNRTI resistance-associated mutations. Holm's correction was used to control for the familywise error rate of multiple pairwise comparisons.

Clinical samples for phenotyping. Patient samples ($n = 38$) from the South African Virological Evaluation (SAVE) study (9) and a workplace HIV program within the mining industry (26) and samples submitted for routine genotyping were selected for resistance phenotyping (27). Samples were selected on the basis of availability and the presence of one or more NNRTI resistance-associated mutations. Ethical approval for drug resistance testing on these samples was provided by the Committee for Research on Human Subjects (University of the Witwatersrand, Johannesburg, South Africa) and the Regional Medical Ethics Board (Karolinska Institutet, Stockholm, Sweden).

HIV-1 expression vectors. A retroviral vector-based, single-round infection system was used for *in vitro* phenotyping (28–32). For this, a 3,500-bp fragment spanning *gag* and part of the integrase gene (bp 737 to 4,403 of strain HXB2) of the HIV-1 subtype B p8.9NSX vector (30) was replaced with the subtype C MJ4 proviral genome (GenBank accession number AF321523.1) (31, 33). NNRTI resistance-associated mutations were introduced into the p8.MJ4 plasmid using a QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) in conjunction with mutagenesis primers synthesized by Integrated DNA Technologies (Coralville, IA, USA). Plasmids were transformed into *Escherichia coli* XL10-Gold ultracompetent cells (Stratagene, La Jolla, CA, USA) by a standard heat shock method. Individual clones were sequenced by population-based sequencing using a BigDye Terminator (v3.1) cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3130xl genetic analyzer (Applied Biosystems, Foster City, CA, USA). The Stanford HIV drug resistance algorithm was used for quality assessment of

sequences and mutation recognition (34). Mutants with double NNRTI resistance-associated mutations were created by successive rounds of site-directed mutagenesis in p8.MJ4.

For the phenotypic assessment of patient samples, the p8.MJ4 expression vector was modified by the introduction of an HpaI restriction site toward the end of the protease (PR) gene (bp 2496 of HXB2) by site-directed mutagenesis. In combination with an intrinsic HpaI restriction site in the reverse transcriptase gene of MJ4 (bp 3650 in HXB2), this allowed the excision of an ~1,154-bp fragment from p8.MJ4 with HpaI endonuclease (Roche, Basel, Switzerland) and the religation of the blunt vector ends with a Rapid DNA Dephos & Ligation kit (Roche, Basel, Switzerland) to produce p8.MJ4ΔRT.

Construction of patient-derived plasmids. The viral RNA from patient plasma samples was reverse transcribed with a ThermoScript RT-PCR system (Invitrogen, Life Technologies Corporation, Carlsbad, CA, USA) and an ~1,154-kb reverse transcriptase fragment amplified by nested PCR using an Expand High-Fidelity^{plus} PCR system (Roche, Basel, Switzerland). The second-round PCR primers (primers HpaI-F [5'-CCTA CACCTGTTAACATAAATGGGAAGRAATATGTTGAC-3'] and HpaI-R [5'-CAGCCTCTGTAACTGTTTACATCATTAGTGTG-3'] introduced an HpaI restriction site (the underlined GTAAAC sequence) at the 5' and 3' ends of the PCR amplicons. The p8.MJ4ΔRT vector and amplicons were restricted with the HpaI endonuclease (Roche, Basel, Switzerland) and ligated using the Rapid DNA Dephos & Ligation kit (Roche, Basel, Switzerland) to introduce patient-derived amplicons into p8.MJ4ΔRT. Ligation products were transformed into *E. coli* XL10-Gold ultracompetent cells (Stratagene, La Jolla, CA, USA) by a standard heat shock procedure. The plasmid inserts were sequenced, and the sequences were compared to the sequence of the original amplicon.

Single-cycle nonreplicating phenotypic assay. Three plasmids were utilized for the production of virus-like particles: pMD.G (29) to facilitate

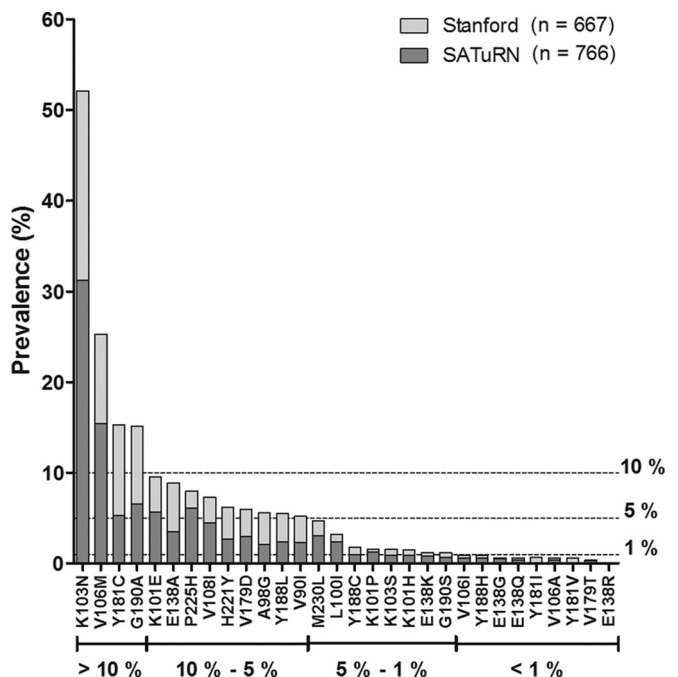


FIG 1 Prevalence of NNRTI resistance-associated amino acid substitutions among first-line treatment failures. Sequences from 1,433 HIV-1 subtype C-infected patients failing first-line therapy with NNRTI resistance-associated mutations were obtained from the SATuRN ($n = 766$; light gray bars) and Stanford ($n = 667$; dark gray bars) databases. Amino acid substitutions are grouped according to prevalence: >10%, 5% to 10%, 1% to 5%, and <1%.

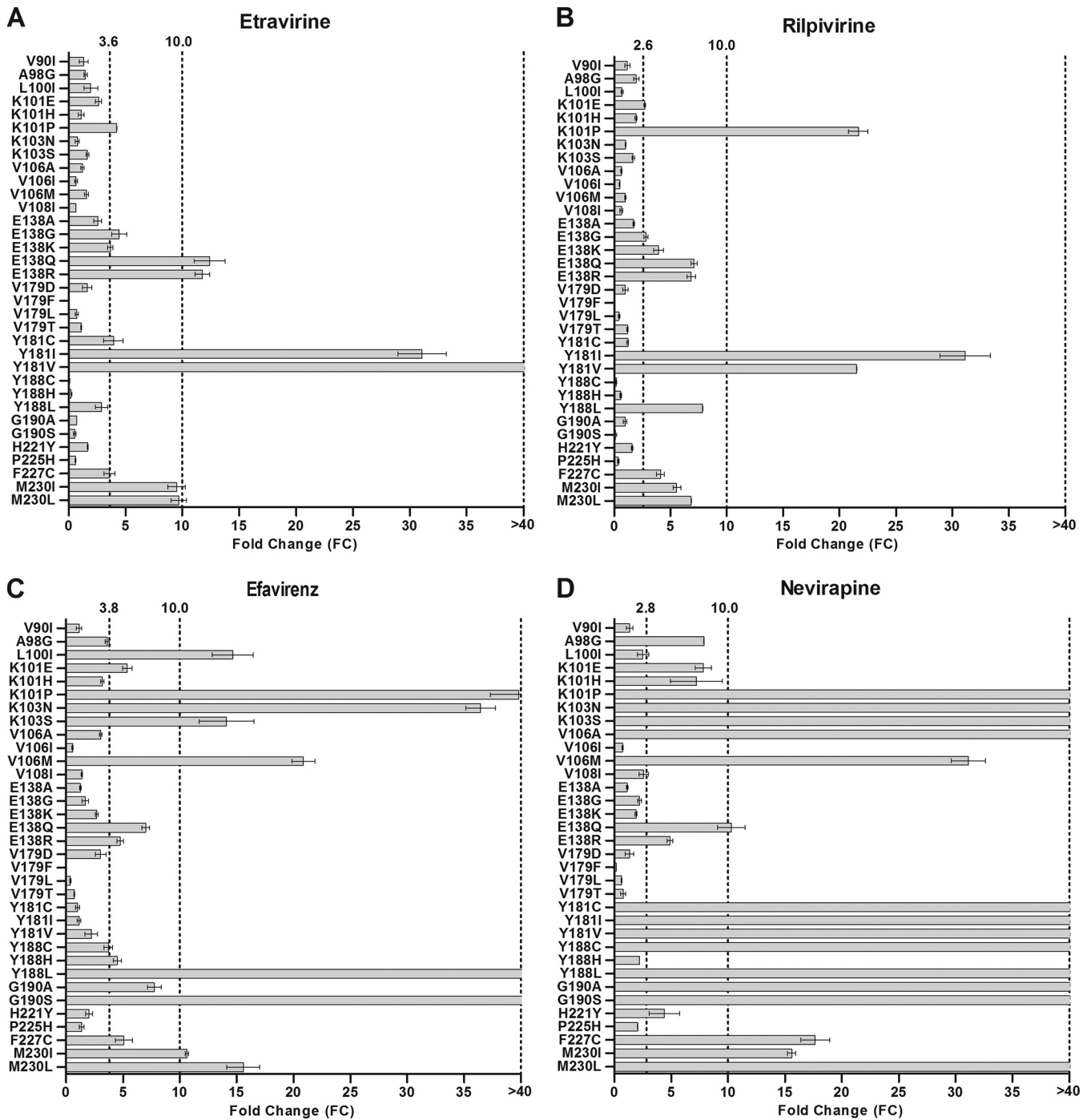


FIG 2 Phenotypic resistance of viruses with single amino acid substitutions associated with resistance to the NNRTIs ETR, RPV, EFV, and NVP. Single NNRTI resistance-associated mutations ($n = 34$) were introduced into the p8.MJ4 subtype C HIV-1 expression plasmid by site-directed mutagenesis. Phenotypic susceptibilities to ETR (A), RPV (B), EFV (C), and NVP (D) were determined using a single-cycle nonreplicative phenotypic assay. The lower cutoff values determined for this assay are indicated for each drug. Columns indicate mean values, while error bars indicate the standard errors of the means (SEMs). Each mutant was tested in duplicate in at least two independent experiments.

viral entry, pCSFLW (35) for the detection of viral integration by bioluminescence, and p8.MJ4 (or p8.MJ4 Δ RT) for the expression of HIV-1 subtype C PR-RT. Plasmids were cotransfected into HEK293T cells, in which cotransfection was facilitated with either the Fugene6 transfection reagent (Roche, Basel, Switzerland) (30) or polyethylenimine (PEI; Polysciences, Inc., Warrington, PA, USA). Virus-containing supernatants

were harvested at 48 and 72 h posttransfection. Viral titers were determined by infecting HEK293T cells with serial dilutions of virus-containing supernatants. After 48 h, the expression of firefly luciferase was determined with the Bright-Glo luciferase assay substrate (Promega, Fitchburg, WI, USA) on a Victor³ multilabel reader (PerkinElmer, Waltham, MA, USA). Virus inocula were standardized to produce a luminescence of $1 \times$

TABLE 2 Pairs of positively correlated NNRTI resistance-associated amino acid substitutions

Combination	<i>P</i> value for association	Prevalence (%)
Subtype C data set ^a		
K101E-G190A	<0.0001	5.6
K103N-P225H	<0.0001	7.7
V106M-V179D	<0.0001	4.4
V106M-F227L	<0.0001	4.0
Y181C-H221Y	<0.0001	3.5
Subtype B data set ^b		
Y181C-G190A	<0.0001	6.5
K101E-G190A	<0.0001	3.4
K101E-Y181C	<0.0001	2.7
K101E-G190S	<0.0001	1.0
H101H-G190A	<0.0001	0.9
A98G-Y181C	<0.0001	2.4
K101H-Y181C	<0.0001	0.7
A98G-G190A	<0.0001	1.6
Y181C-G190S	<0.0001	0.9
V179F-Y181C	<0.0001	0.2

^a From 1,433 subtype C sequences with NNRTI resistance-associated mutations from the SATuRN and Stanford databases.

^b From 13,039 published subtype B sequences (25).

10^4 to 1×10^5 relative light units (RLU) after 48 h of incubation with HEK293T cells in drug-free medium.

For drug susceptibility assays, duplicate serial dilutions of all four NNRTIs (ETR, RPV, EFV, and NVP) were prepared in 96-well cell culture plates to which standardized virus inocula and HEK293T cells were added. The MJ4 virus was included in each assay run as a wild-type control. After incubation for 48 h, the expression of firefly luciferase was determined as described above. Each virus was screened in at least two independent assays for each drug, and the 50% effective concentration (EC_{50}) values were calculated. Fold change (FC) values were determined by reference to the EC_{50} s for the MJ4 wild-type control virus for each drug. The lower FC cutoff value for each drug was determined using the 99th percentile of the average EC_{50} for MJ4 wild-type virus, assessed in multiple repeat screens of each drug. Samples were classified as susceptible if the FC value fell below the FC cutoff values for ETR (FC cutoff value, 3.6), RPV (FC cutoff value, 2.6), EFV (FC cutoff value, 3.8), or NVP (FC cutoff value, 2.8). For resistance, an FC of ≥ 10 was used as the upper cutoff for all 4 drugs, although in many cases these levels exceeded >40 . Samples with FC values between the lower and upper assay cutoff values were considered to show reduced susceptibility. It is important to note that the cutoff values used in this analysis are not linked to clinical correlates or outcomes. Furthermore, the classifications of susceptible, reduced susceptibility, and resistant are used merely to rank the responses for the NNRTIs used in our assay.

RESULTS

Prevalence of NNRTI resistance-associated mutations in the subtype C data set. Sequence data from 1,433 HIV-1 subtype C-infected patients who failed first-line NNRTI treatment and developed NNRTI resistance-associated mutations were used in this study. The majority of these patients were treated with EFV (63.3%) or NVP (30.7%), with 3% being exposed to both drugs (Table 1). Less than 1% of patients were treated with other NNRTIs (delavirdine and MKC422), and for 2.7% the NNRTI treatment was not known. Most patients from the SATuRN database were treated with EFV, while those from the Stanford database were more biased toward NVP use.

A total of 30 published NNRTI resistance-associated muta-

tions (36) were identified in this data set. The EFV/NVP-selected amino acid substitutions K103N, V106M, Y181C, and G190A had the highest overall prevalence: 52%, 25%, 15%, and 15% of samples, respectively (Fig. 1). V90I, A98G, L100I, K101E/H/P, K103S, V108I, E138A/K, V179D, Y188C/L, G190S, H221Y, P225H, and M230L were identified in 1% to 10% of sequences. The remaining 9 substitutions, V106A/I, E138G/Q/R, V179T, Y181I/V, and Y188H, had prevalences of $<1\%$. Overall, the frequencies of resistance-associated amino acid substitutions were similar between the Stanford and SATuRN data sets, despite the differences in treatment histories.

Single site-directed mutations and NNRTI phenotypic susceptibility. Thirty-four NNRTI resistance-associated mutations were introduced by site-directed mutagenesis into an HIV-1 subtype C expression vector, and the activities of the NNRTIs against the mutants were tested in a single-cycle nonreplicating phenotypic assay. The mutations included the 30 amino acid substitutions identified in our data set plus an additional 4 changes (V179F, V179L, F227C, and M230I) shown by others to be associated with ETR/RPV resistance (36).

Of these 34 single amino acid substitutions, only Y181I and Y181V caused high levels of resistance ($FC > 40$) to both ETR (Fig. 2A) and RPV (Fig. 2B). E138Q/R and M230I/L caused ~ 10 -fold resistance to ETR but had a lesser effect on susceptibility to RPV, whereas K101P and Y188L caused resistance to RPV but not to ETR. None of the remaining substitutions, including those frequently associated with EFV and/or NVP resistance, caused a significant reduction in susceptibility to ETR or RPV.

For EFV and NVP, the K101P, K103N/S, V106M, Y188L, G190S, and M230I/L amino acid substitutions caused resistance to both drugs (Fig. 2C and D). In addition, V106A, E138Q, Y181C/I/V, Y188C, G190A, and F227C caused resistance to NVP. Susceptibility to EFV was not affected by the V106A, Y181C/I/V, or Y188C substitution, but E138Q, G190A, and F227C caused reduced susceptibility. L100I caused resistance to EFV but did not affect the susceptibility to NVP. For the remainder, only A98G, K101E/H, E138R, and H221Y reduced the susceptibility to NVP, while only K101E and E138R reduced the susceptibility to EFV.

Effect of NNRTI resistance-associated combinations on NNRTI susceptibility. Five pairs of significantly ($P < 0.0001$) associated amino acid substitutions were identified in the subtype C data set: K101E-G190A, K103N-P225H, V106M-V179D, V106M-F227L, and Y181C-H221Y (Table 2). These 5 combinations occurred in 4 to 8% of the subtype C sequences from patients treated with EFV and/or NVP. In addition, 10 positively correlated pairs of ETR resistance-associated substitutions from a similar analysis of subtype B were selected (25) (Table 2). The K101E-G190A combination, identified in our subtype C analysis, was also identified in the subtype B analysis, with a similar prevalence being detected in both subtypes (5.6% and 3.4%, respectively). None of the remaining 9 combinations identified in the subtype B data set, all of which had a low prevalence (0.2% to 6.5%), were observed in our subtype C data set.

All 14 significantly associated amino acid substitution pairs (5 subtype C and 9 subtype B amino acid substitution pairs) were generated, and the isolates were tested for phenotypic NNRTI resistance (Fig. 3). The seven NNRTI resistance-associated combinations that lacked Y181C had no major impact on ETR or RPV susceptibility (Fig. 3A and B). Combinations containing Y181C had a greater effect on ETR susceptibility than RPV susceptibility,

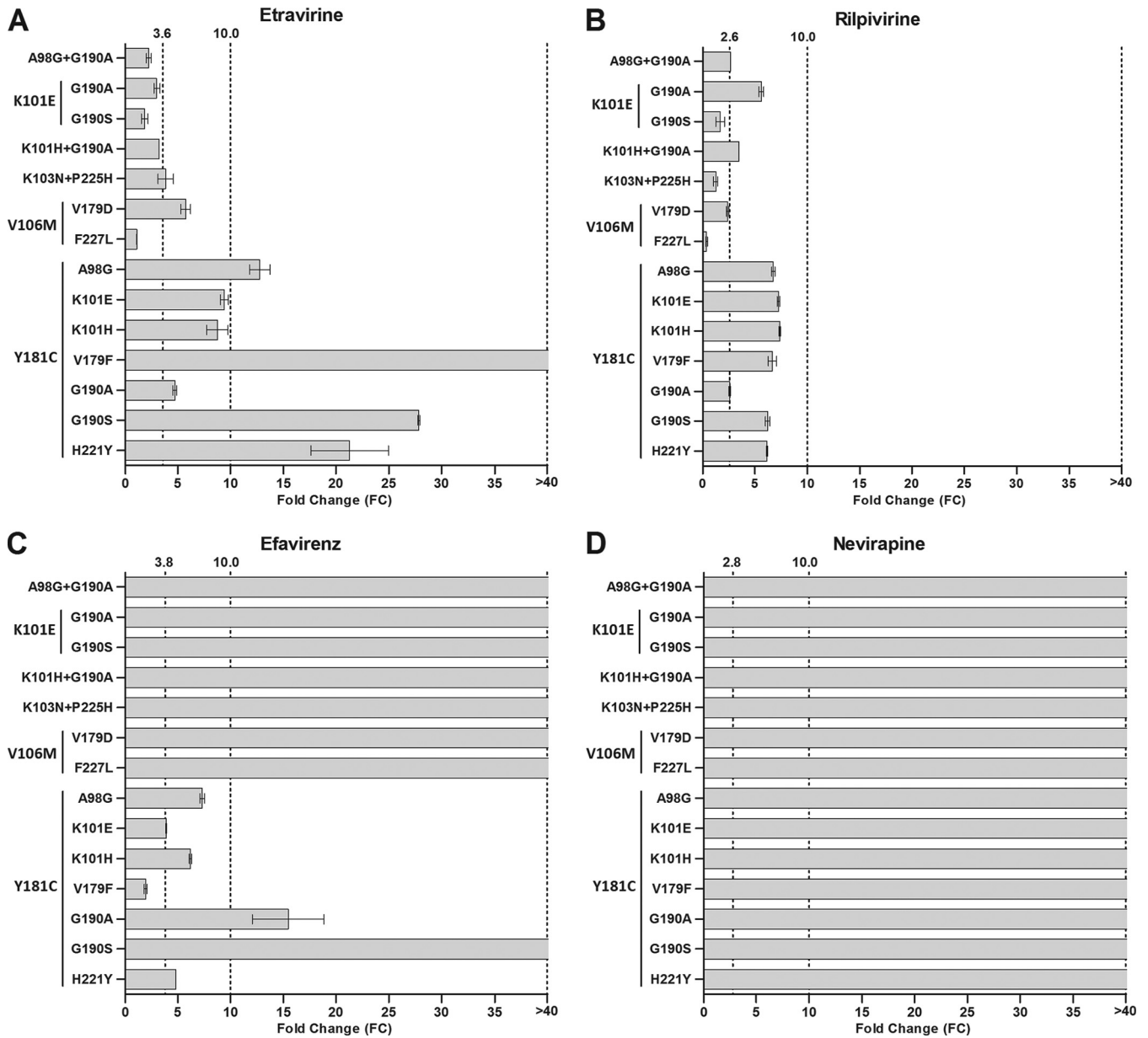


FIG 3 Phenotypic resistance to ETR, RPV, EFV, and NVP of viruses with double resistance-associated amino acid substitutions. Positively associated double mutations ($n = 14$) were introduced into the p8.MJ4 subtype C HIV-1 expression plasmid. Phenotypic susceptibilities to ETR (A), RPV (B), EFV (C), and NVP (D) were determined with a single-cycle nonreplicative phenotypic assay. The lower cutoff values determined for this assay are indicated for each drug. Columns indicate mean values, while error bars indicate the standard errors of the means (SEMs). Each mutant was tested in duplicate in at least two independent experiments.

particularly those containing A98G, V179F, G190S, and H221Y. Mutants containing Y181C had a minor effect on EFV susceptibility, except for combinations with G190A/S (Fig. 3C). Other combinations with G190A/S or V106M, as well as the K103N-P225H combination, caused high-level resistance to EFV. All double NNRTI resistance-associated mutations (with or without Y181C) caused high-level resistance to NVP (Fig. 3D). These data indicate that for the most frequently selected double NNRTI resistance-associated substitutions, there was considerably less resistance to ETR than to EVP and NVP and no high-level resistance to RPV.

Impact of the M184I/V NRTI amino acid substitution on NNRTI susceptibility. The frequent selection of the nucleoside reverse transcriptase inhibitor (NRTI) resistance-associated amino acid substitution M184I/V with E138K in the ECHO and THRIVE trials implicated this combination in reduced susceptibility to RPV (22, 37). To investigate this in a subtype C background, E138K with M184I or M184V was created in p8.MJ4 and the clones were tested for phenotypic susceptibility. The single M184I and M184V substitutions did not reduce susceptibility to any of the NNRTIs, while the single E138K substitution caused reduced susceptibility to RPV, as expected (Fig. 4). A low level of

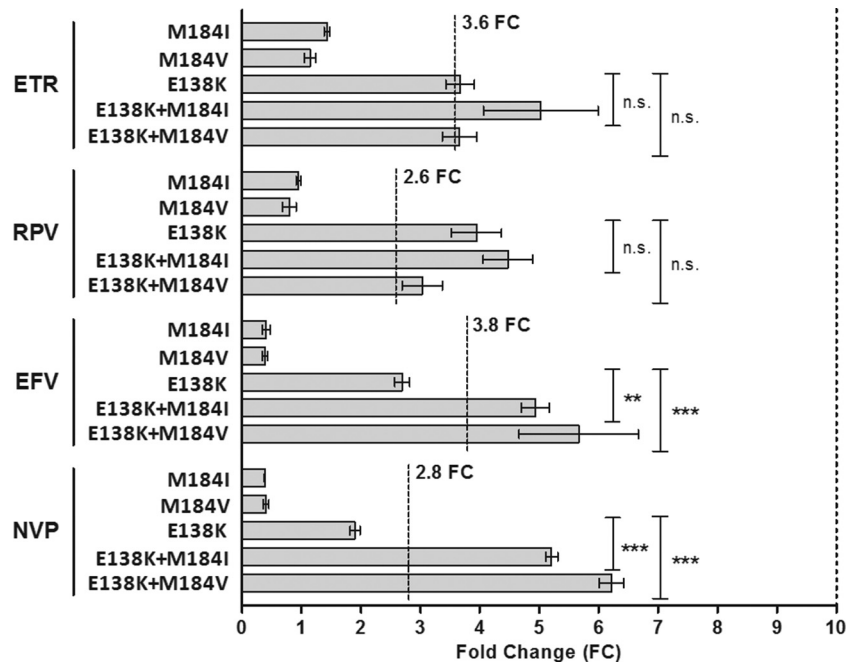


FIG 4 Impact of E138K and M184I/V on phenotypic susceptibility to ETR, RPV, EFV, and NVP. The phenotypic susceptibility to ETR, RPV, EFV, and NVP of site-directed mutants containing amino acid substitutions M184I, M184V, E138K, and their combinations were determined. The lower cutoff values determined for this assay are indicated for each drug. Columns indicate mean values, while error bars indicate the standard errors of the mean (SEMs). Each mutant was tested in duplicate in at least two independent experiments. n.s., not significant; **, $P < 0.01$ (2-way analysis of variance); ***, $P < 0.001$ (2-way analysis of variance).

reduction in ETR susceptibility was also observed with this amino acid substitution. However, the E138K-M184I/V combination reduced susceptibility to all four NNRTIs. Thus, despite E138K-M184V/I being associated with RPV resistance, the largest decreases in susceptibility were observed for EFV and NVP, and we also noted a minor effect on ETR susceptibility.

Sensitivity of mutants with prevalent NNRTI resistance-associated mutations to ETR and RPV. To gauge the likely impact of NNRTI resistance-associated mutations on ETR and RPV efficacy, the phenotypic data for the susceptibility of all the single and double mutants to each drug were ranked according to mutation prevalence in the subtype C data set (Fig. 5). The most prevalent mutations (>10%) caused no major reduction in ETR or RPV susceptibility and included the signature EFV- and NVP-associated substitutions K103N, V106M, Y181C, and G190A. All mutations that caused high-level resistance to ETR or RPV were present at a $\leq 3.5\%$ prevalence. For ETR this included Y181C-H221Y, Y181V/I, and E138Q/R, while for RPV this was restricted to K101P and Y181V/I. While a significant number of mutants showed reduced susceptibility to ETR and RPV, these were not detected in our HIV-1 subtype C data set. Overall, of the 50 mutants tested, far fewer showed high-level resistance to RPV ($n = 3$, 6%) and ETR ($n = 8$, 16%) than to EFV ($n = 18$, 36%) and NVP ($n = 30$, 60%).

Susceptibility of clinical samples to ETR and RPV. In order to assess whether samples with resistance mutations from patients who failed EFV or NVP treatment would be susceptible to ETR and RPV, we tested 38 clinical patient samples using our in-house phenotypic assay. For this, the reverse transcriptase gene was amplified from the plasma RNA of each patient and inserted into the p8.MJ4 vector. Clones with one ($n = 12$), two ($n = 13$), three ($n = 10$), or four ($n = 3$) NNRTI resistance-associated mutations were

selected. This panel included sequences with the major EFV/NVP-related NNRTI resistance-associated substitutions K103N (47.3%), V106M (26.3%), Y181C (18.4%), and G190A (13.2%).

As expected, all samples showed high-level resistance to EFV and NVP, except for one sample that was susceptible to EFV, while another sample showed a reduced susceptibility to NVP (Fig. 6). In contrast, more than half the samples were fully susceptible to ETR ($n = 22/38$, 58%) and RPV ($n = 24/38$, 63%), with less than a quarter showing high-level resistance to these newer NNRTIs. Resistance to ETR and RPV was generally associated with the number of mutations. Thus, of the 12 patients with single NNRTI resistance-associated substitutions, including K103N and V106M, 10 (83%) remained susceptible to ETR and RPV. The patient with M230L as the single NNRTI resistance-associated substitution (patient 6) showed reduced susceptibility, as expected. One patient (patient 12) showed high-level resistance to all NNRTIs but had only a single Y181C amino acid substitution, which confers resistance to NVP exclusively. Overall, the presence of more than one NNRTI resistance-associated mutation was associated with a higher risk of resistance, although some samples with 3 or 4 NNRTI resistance-associated mutations remained susceptible or showed only reduced susceptibility to ETR and RPV.

Closer inspection of the sample from patient 12 revealed the presence of the connection domain (CN) substitution T369I, in addition to Y181C. In order to determine if this mutation was responsible for the high level of resistance to all NNRTIs, two site-directed mutations (T369I and Y181C-T369I) were created in p8.MJ4. Phenotypic testing showed that T369I alone had no impact on susceptibility to ETR, RPV, or EFV but conferred decreased susceptibility to NVP (Fig. 7). However, when T369I was combined with Y181C, a significant decrease in susceptibility to

Mutant	Fold Change (FC)								Mutant Prevalence (%)
	ETR		RPV		EFV		NVP		
	Average	SD	Average	SD	Average	SD	Average	SD	
K103N	0.8	0.3	1.0	0.1	36.4	1.8	>40.0	0.0	52.1
V106M	1.6	0.3	1.0	0.1	20.9	2.0	31.1	3.0	25.3
Y181C	3.9	1.2	1.2	0.1	1.0	0.3	>40.0	0.0	15.3
G190A	0.7	0.0	1.0	0.2	7.8	1.1	>40.0	0.0	15.1
K101E	2.6	0.4	2.7	0.1	5.4	0.6	7.8	1.2	9.6
E138A	2.6	0.5	1.7	0.1	1.3	0.1	1.1	0.1	9.0
P225H	0.6	0.0	0.4	0.1	1.4	0.3	2.0	0.0	8.1
K103N, P225H	3.8	1.0	1.3	0.3	>40.0	0.0	>40.0	0.0	7.7
V108I	0.7	0.0	0.6	0.2	1.4	0.1	2.5	0.7	7.3
H221Y	1.7	0.0	1.6	0.1	2.0	0.5	4.4	1.9	6.2
V179D	1.6	0.8	1.0	0.4	3.0	0.9	1.3	0.8	6.0
K101E, G190A	3.0	0.4	5.6	0.3	>40.0	0.0	>40.0	0.0	5.6
Y188L	2.9	0.7	7.9	0.0	>40.0	0.0	>40.0	0.0	5.5
A98G	1.5	0.2	2.0	0.3	3.6	0.2	7.9	0.0	5.5
V90I	1.3	0.8	1.2	0.5	1.1	0.5	1.3	0.6	5.1
M230L	9.7	1.4	6.9	0.0	15.6	2.0	>40.0	1.3	4.7
V106M, V179D	5.7	0.6	2.4	0.2	>40.0	0.0	>40.0	0.2	4.4
V106M, F227L	1.1	0.1	0.4	0.1	>40.0	0.0	>40.0	0.0	4.0
Y181C, H221Y	21.3	6.4	6.1	0.1	4.8	0.0	>40.0	0.0	3.5
L100I	2.0	1.3	0.7	0.2	14.7	3.6	2.5	1.0	3.2
Y188C	0.1	0.0	0.2	0.1	3.7	0.5	>40.0	0.0	1.8
K101P	4.2	0.0	21.7	1.2	39.8	3.6	>40.0	0.0	1.6
K103S	1.7	0.2	1.7	0.2	14.1	3.4	>40.0	1.1	1.5
K101H	1.1	0.4	2.0	0.1	3.2	0.2	7.2	3.2	1.5
E138K	3.7	0.3	3.9	0.6	2.7	0.2	1.9	0.1	1.2
G190S	0.5	0.1	0.1	0.1	>40.0	0.0	>40.0	0.0	1.2
V106I	0.6	0.2	0.5	0.1	0.6	0.1	0.7	0.1	0.9
Y188H	0.2	0.1	0.6	0.1	4.5	0.5	2.2	0.0	0.9
Y181V	>40.0	0.0	21.5	0.0	2.2	0.8	>40.0	0.0	0.6
Y181I	31.1	3.0	31.1	3.2	1.1	0.2	>40.0	0.0	0.6
E138Q	12.4	3.6	7.1	0.7	6.9	0.8	10.2	3.2	0.6
E138G	4.4	0.9	2.8	0.3	1.7	0.4	2.2	0.2	0.6
V106A	1.2	0.2	0.7	0.1	3.1	0.2	>40.0	0.0	0.6
V179T	1.1	0.1	1.2	0.1	0.7	0.1	0.8	0.3	0.4
E138R	11.7	1.8	6.8	1.0	4.7	0.7	4.8	0.6	0.1
V179F, Y181C	>40.0	0.0	6.7	0.5	1.9	0.2	>40.0	0.0	0.0
Y181C, G190S	27.8	0.1	6.2	0.3	>40.0	0.0	>40.0	0.0	0.0
A98G, Y181C	12.8	1.3	6.7	0.2	7.3	0.3	>40.0	0.0	0.0
K101E, Y181C	9.4	0.6	7.2	0.2	3.9	0.1	>40.0	0.0	0.0
K101H, Y181C	8.7	1.4	7.4	0.1	6.2	0.2	>40.0	0.0	0.0
M230I	8.7	0.2	5.6	0.5	10.6	0.2	15.6	0.5	0.0
*E138K, M184I	5.0	1.4	4.5	0.6	4.9	0.3	5.2	0.1	0.0
Y181C, G190A	4.7	0.3	2.6	0.1	15.5	4.8	>40.0	0.0	0.0
*E138K, M184V	3.7	0.4	3.0	0.5	5.7	1.4	6.2	0.3	0.0
F227C	3.6	0.9	4.1	0.7	5.1	1.5	17.7	2.6	0.0
K101H, G190A	3.2	0.0	3.5	0.0	>40.0	0.0	>40.0	0.0	0.0
A98G, G190A	2.2	0.3	2.7	0.0	>40.0	0.5	>40.0	0.0	0.0
K101E, G190S	1.8	0.4	1.7	0.6	>40.0	0.0	>40.0	0.0	0.0
V179L	0.7	0.2	0.5	0.1	0.4	0.1	0.6	0.1	0.0
V179F	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.0	0.0

FIG 5 Phenotypic susceptibility of mutants with single and double NNRTI resistance-associated mutations. The *in vitro* phenotypic responses of mutants with site-directed single and double NNRTI resistance-associated mutations, ranked according to the prevalence of the mutation among the 1,433 sequences with NNRTI resistance-associated mutations used in this analysis, are summarized. Some mutations (*) were not observed among the sequences but were included, as they are implicated in ETR and RPV resistance. Light gray shading, FCs of >3.6 (ETR), >2.6 (RPV), >3.8 (EFV), and >2.8 (NVP); dark gray shading, FC of >10.

ETR, RPV, and EFV was observed. Since Y181C confers high-level resistance to NVP, it was not possible to see any additional effect of T369I on susceptibility to NVP. These data suggest that for ETR, RPV, and EFV, the T369I connection domain amino acid substitution has a major impact on NNRTI susceptibility when present in combination with Y181C.

A second connection domain amino acid substitution (N348I) was observed in 2 patient samples. The N348I site-directed mutant was susceptible to all NNRTIs except NVP (FC, 5.6) (Fig. 8). However, the mutant with K103N, which singly has no effect on susceptibility to ETR (Fig. 5), showed reduced susceptibility when N348I was combined with K103N. Similarly, combinations with Y181C resulted in significant effects, particularly on susceptibility to ETR. This, once again, confirmed the negative influence of combinations with Y181C on ETR and RPV susceptibility.

DISCUSSION

In this study, we investigated the potential cross-resistance between NNRTIs in the context of HIV-1 subtype C. We showed that the prevalent single NNRTI resistance-associated amino acid substitutions, such as K103N, V106M, Y181C, or G190A that cause high-level resistance to EFV and/or NVP had no effect on susceptibility to ETR and RPV. Combinations of such amino acid substitutions, particularly those containing Y181C, were more likely to cause resistance to ETR and RPV, but such genotypes occurred rarely in subtype C data sets. Among samples from patients failing EFV/NVP-based therapies, more than half retained full susceptibility to ETR and/or RPV. This suggests that ETR and RPV would be suitable alternatives for subtype C patients failing EFV- or NVP-containing regimens.

Our data largely correspond with previously published data on

Pt.	Genotype				Phenotype (F.C.)											
	NRTI amino acid substitutions	NNRTI amino acid substitutions	Other	No. of NNRTI substitutions	ETR			RPV			EFV			NVP		
					MEAN	STD	Stanford	MEAN	STD	Stanford	MEAN	STD	Stanford	MEAN	STD	Stanford
1	-	V106M	P225S	1	0.3	0.1	0	0.1	0.0	0	16.2	0.5	60	13.4	0.6	60
2	-	K103N	-	1	0.8	0.2	0	0.8	0.1	0	19.6	2.1	60	39.0	2.1	60
3	-	K103N	-	1	1.0	0.1	0	0.6	0.0	0	21.0	3.7	60	>40.0	9.1	60
4	-	K103N	-	1	1.3	0.0	0	1.3	0.2	0	>40.0	0.0	60	>40.0	0.0	60
5	-	V106M	-	1	1.3	0.3	0	0.5	0.2	0	21.8	6.5	60	5.2	0.3	60
6	T69N, M184V	M230L	-	1	7.2	0.1	30	4.7	0.8	45	13.4	0.3	45	16.0	0.3	60
7	-	K103N	-	1	1.4	0.1	0	1.6	0.2	0	23.6	2.0	60	>40.0	0.3	60
8	-	V106M	-	1	1.6	0.0	0	0.9	0.1	0	29.2	1.5	60	>40.0	2.3	60
9	M184V	K103N	-	1	1.6	0.1	0	1.2	0.2	0	>40.0	1.8	60	>40.0	0.0	60
10	K70R, M184V	K103N	-	1	1.8	0.2	0	1.3	0.3	0	>40.0	0.0	60	>40.0	0.0	60
11	M184V	K103N	-	1	2.7	0.0	0	1.9	0.3	0	>40.0	0.0	60	>40.0	0.0	60
12	M184V	Y181C	T369I	1	>40.0	1.2	30	17.1	0.2	30	15.1	0.0	30	>40.0	0.0	60
13	M184V	V106M, Y188C	N348I	2	0.5	0.1	0	0.6	0.3	0	>40.0	0.0	130	>40.0	0.0	135
14	M184V	A98G, K103N	-	2	1.3	0.2	10	1.0	0.2	15	>40.0	0.0	70	>40.0	0.0	90
15	M41L, M184V, T215F	K101H, G190A	-	2	1.4	0.0	25	0.8	0.0	25	>40.0	0.0	60	>40.0	0.0	75
16	M184V	K103N, V108I	N348T	2	1.4	0.3	0	1.0	0.1	0	>40.0	0.0	70	>40.0	0.0	75
17	M184V	V106M, G190A	-	2	1.5	0.1	15	0.8	0.1	15	>40.0	0.0	105	>40.0	0.0	120
18	-	V106M, Y188C	-	2	3.1	0.1	0	1.7	0.1	0	>40.0	0.0	120	>40.0	0.0	120
19	T69D, K70R, M184V	V106M, Y181C	-	2	4.5	0.4	30	1.2	0.4	30	>40.0	0.0	90	>40.0	0.0	120
20	-	K103N, Y181C	-	2	9.5	1.2	30	5.7	0.7	30	29.8	2.5	90	>40.0	0.0	120
21	M184V	V108I, Y181C	-	2	9.7	1.0	30	3.3	1.3	30	>40.0	1.4	40	>40.0	0.0	75
22	M184I	K101E, Y188L	-	2	11.2	1.3	30	>40.0	0.0	110	>40.0	0.0	75	>40.0	0.0	90
23	D67N, K70R, V118I, M184V, T215F, K219E	L100I, K103N	N348I	2	18.6	7.6	30	12.4	1.0	60	>40.0	0.0	115	>40.0	0.0	120
24	M184V	Y188H, H221Y	Y181S	2	40.0	3.5	25	8.3	0.1	25	>40.0	0.0	55	>40.0	0.0	130
25	M184V	Y181C, H221Y	-	2	>40.0	0.0	40	23.3	1.5	40	30.7	1.6	40	>40.0	0.0	70
26	M184V	V108I, Y181C, H221Y	-	3	0.4	0.0	40	0.2	0.1	40	0.3	0.1	50	22.7	0.4	85
27	M184V	K101H, K103N, G190A	-	3	0.7	0.1	25	1.1	0.2	25	>40.0	0.0	120	>40.0	0.0	135
28	K65R	K103N, V106A, G190A	-	3	1.3	0.3	15	1.9	0.3	15	>40.0	0.0	150	>40.0	0.0	180
29	M184I, T215I, K219R	V90I, K103N, P225H	-	3	2.3	0.1	0	1.7	0.3	0	>40.0	0.0	90	>40.0	0.0	90
30	K70E, V75A, M184V, L210F, K219R	K103N, H221Y, P225H	-	3	3.2	0.1	10	1.6	0.3	10	>40.0	0.0	100	>40.0	0.0	100
31	M184V	K103N, E138A, P225H	-	3	5.3	0.8	10	7.4	0.3	15	>40.0	0.0	90	>40.0	0.0	90
32	A62V, M184V	V106M, V179D, Y188C	-	3	12.6	1.0	10	2.4	0.0	10	>40.0	0.0	130	>40.0	0.0	130
33	M41L, M184V, T215Y	V106M, Y188L, H221Y	-	3	>40.0	0.0	25	>40.0	0.0	70	>40.0	0.0	130	>40.0	0.0	130
34	M41L, L74V, M184V, L210W, T215Y	A98G, Y181C, M230L	-	3	>40.0	0.0	70	16.9	2.4	90	28.9	0.8	85	>40.0	0.0	150
35	M184V	K101E, V106M, E138A	F227L	3	3.2	0.8	25	1.8	0.0	45	>40.0	0.0	90	>40.0	0.0	120
36	M184V, T215Y	A98G, K103N, V108I, P225H	V189I	4	4.8	0.1	10	5.0	0.7	15	>40.0	0.0	110	>40.0	0.0	135
37	M41L, L74V, M184V, T215Y	K103N, V108I, Y181C, H221Y	E399D	4	5.4	0.5	40	4.9	1.2	40	>40.0	0.0	110	>40.0	0.0	145
38	M184V	K101E, G190A, H221Y, M230L	-	4	14.5	0.4	70	18.3	1.0	100	>40.0	0.0	115	>40.0	0.0	160

FIG 6 Genotypic and phenotypic analysis of samples from EFV/NVP-experienced patients. Patient samples with NNRTI resistance-associated NNRTI resistance-associated amino acid substitutions were screened for NNRTI susceptibility in an *in vitro* phenotypic assay. The Stanford HIV Drug Resistance Database genotypic resistance scores are included for reference purposes. Light gray shading, FCs of >3.6 (ETR), >2.6 (RPV), >3.8 (EFV), and >2.8 (NVP); dark gray shading, FC of >10. Pt. patient number; STD, standard deviation.

site-directed mutations in HIV-1 subtype B (19, 21, 22, 38). Similar to others, we demonstrated the contribution of K101P, E138K/Q/R, Y181I/V, and M230I/L to ETR and/or RPV resistance (19, 22–24, 38, 39). However, some of our subtype C mutants showed a level of resistance to ETR and RPV slightly higher than that reported for subtype B mutants. These included E138Q/R and M230I/L mutations for ETR and E138K/Q/R, Y188L, and M230I/L mutations for RPV. Only E138Q/R changed the classification for ETR from susceptible in subtype B to resistant in subtype C. For RPV, E138K/Q/R, Y188L, and M230I/L also changed the classification from susceptible to reduced susceptibility. Differences in susceptibility between subtypes B and C with substitutions at position 138, including E138A, which did not confer resistance in our study, were also noted in another study (40).

In a comprehensive analysis of genotype-phenotype correlations performed predominantly on clinical subtype B sequences, amino acid substitutions K101P, Y181I/V, Y188L, and M230L have also been shown to contribute toward decreased susceptibility or resistance to ETR/RPV (41). The K101P amino acid substitution conferred a level of reduction in ETR susceptibility higher than that found in the current study. Some additional amino acid substitutions were also shown to contribute to ETR/RPV resistance in the genotype-phenotype subtype B correlation: A98G, L100I, and V179T affected both ETR and RPV susceptibility, while E138A and Y188H reduced susceptibility to RPV only. The F227C amino acid substitution conferred resistance to ETR and RPV in the genotype-phenotype correlation, while only a small

reduction in susceptibility was observed in our study. However, unlike E138K/Q in the current study, no change in the susceptibility to ETR/RPV was observed in isolates with these mutations in the subtype B genotype-phenotype correlation. The effect of E138R on ETR/RPV susceptibility was not reported in that study.

There are a few possible explanations for the discrepancies observed between our data for subtype C and the published data for subtype B. First, the variation between different assay types (replicating versus nonreplicating, method of quantitation, etc.) could make some assays more sensitive at detecting phenotypic resistance than others (42). Second, polymorphic amino acid residues that inherently differ between virus isolates could lead to a range of phenotypic responses to the same drug (43). In the current study, only a single subtype C reference isolate was used, and the phenotypic responses observed could be isolate specific. Third, the variation in responses could be subtype specific, and several studies have shown differences between subtypes regarding both replication capacity and phenotypic susceptibility (44–46). The screening of additional subtype C isolates could validate the phenotypic differences that were observed between our subtype C mutants and the published subtype B mutants.

The NNRTI binding pocket is flexible, which allows similar but structurally unique NNRTIs to bind to the same compartment on HIV-1 RT (47). EFV and NVP are structurally related, and substantial cross-resistance exists between these two drugs. For this reason, the recycling of EFV/NVP after first-line treatment failure is not advised (48). Since the data sets and samples used in this study were derived

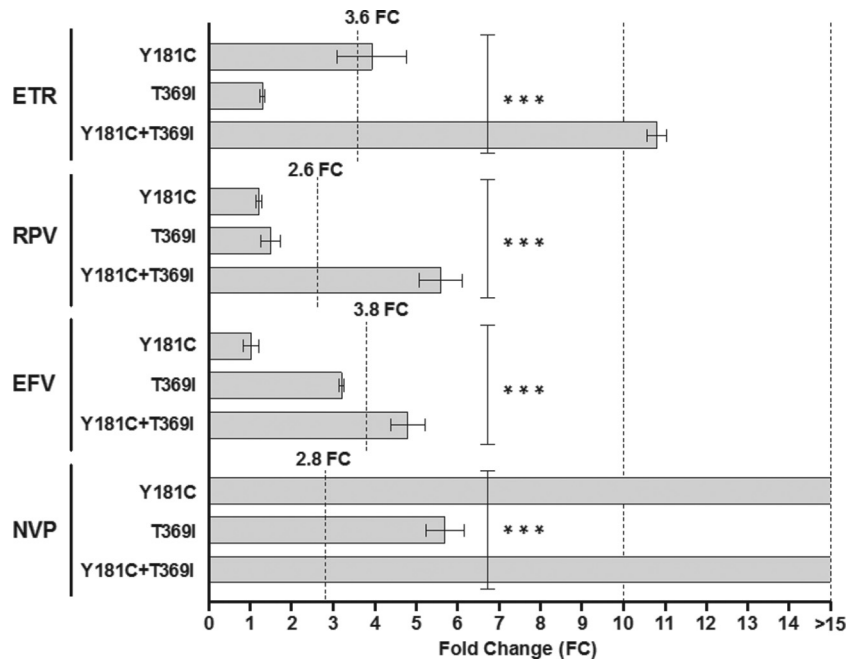


FIG 7 Contribution of T369I to NNRTI resistance. Site-directed mutants containing the T369I amino acid substitution, an amino acid substitution found in the connection domain of patient 12 (Fig. 6), in combination with Y181C were tested for susceptibility to ETR, RPV, EFV, and NVP. The lower cutoff values are indicated for each drug. Columns indicate mean values, while error bars indicate the standard errors of the means (SEMs). Each mutant was tested in duplicate in at least two independent experiments. ***, $P < 0.001$ (2-way analysis of variance).

from patients with NVP and EFV treatment failures, the high levels of resistance seen confirm the limited efficacy of these older NNRTIs against circulating strains. ETR and RPV are structurally distinct from EFV and NVP and are effective against most EFV- and/or NVP-

resistant strains (49). This was confirmed in our study, where we showed, using site-directed mutants, that the most prevalent EFV- and NVP-related NNRTI resistance-associated substitutions caused no decrease in ETR or RPV susceptibility. Overall, the number of

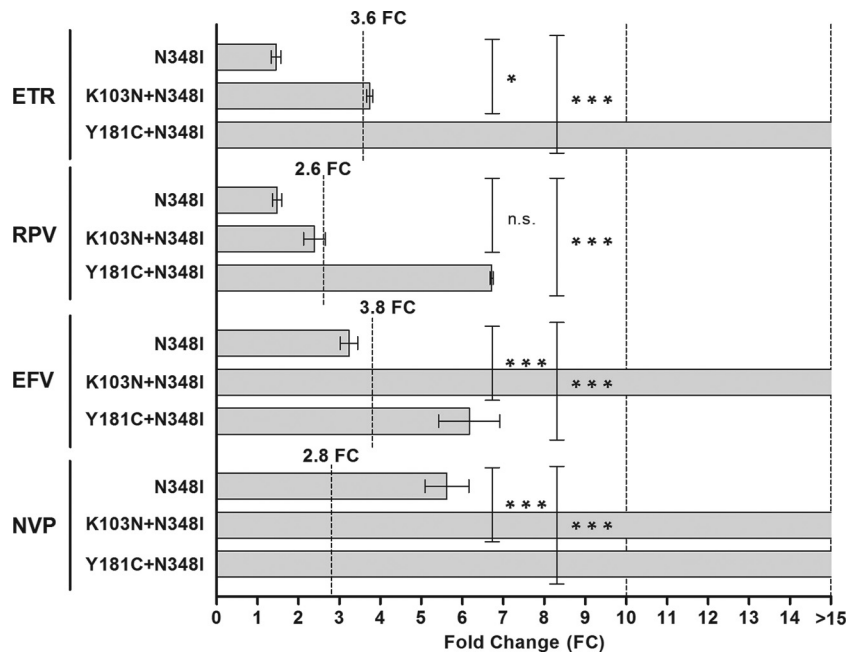


FIG 8 Contribution of N348I to NNRTI resistance. Site-directed mutants containing the N348I amino acid substitution, an amino acid substitution in the connection domain of some patients (Fig. 6), in combination with K103N or Y181C were tested for susceptibility to ETR, RPV, EFV, and NVP. The lower cutoff values are indicated for each drug. Columns indicate mean values, while error bars indicate the standard errors of the mean (SEMs). Each mutant was tested in duplicate in at least two independent experiments. n.s., not significant; *, $P < 0.05$ (2-way analysis of variance); ***, $P < 0.001$ (2-way analysis of variance).

NNRTI resistance-associated substitutions that conferred resistance to ETR/RPV substantially was lower than the number that conferred resistance to EFV/NVP.

The Y181C amino acid substitution, which is one of the most prevalent NNRTI resistance-associated substitutions (50), had no phenotypic impact on ETR or RPV susceptibility. However, combinations of Y181C with other NNRTI resistance-associated substitutions conferred variable degrees of resistance to ETR and, to a lesser extent, RPV. This was observed in both the double mutants and clinical patient samples and has also been reported for HIV-1 subtype B (21, 22, 51, 52). This suggests that RPV may show higher levels of efficacy against viruses with Y181C and that ETR may not be a suitable alternative in patients that harbor Y181C. However, these data are based on an arbitrary *in vitro* cutoff, and so it is unclear whether this would hold true in a clinical setting.

The E138K amino acid change causes low-level resistance to RPV that is moderately increased in the presence of the NRTI resistance-associated substitution M184I but not M184V in subtype B (22, 53). Some studies have suggested a mutual compensatory role of these amino acid changes regarding enzyme processivity (54–56), while others have shown that they confer a decrease in viral fitness (53). While we showed that E138K also conferred low-level resistance to RPV and ETR, we did not observe a significantly enhanced increase when it was combined with M184I/V. However, we did notice a significant increase when this combination was tested against EFV and NVP. Resistance to EFV was also observed in the ECHO and THRIVE trials at week 48 in patients with E138K-M184I/V, although resistance to NVP occurred less often (22). Thus, NRTI resistance-associated substitutions, such as M184I/V, can impact susceptibility to NNRTIs, and as such, the E138K substitution needs to be taken into account before switching to RPV.

Although the resistance-associated mutations have been clearly defined for ETR and RPV, some additional mutations that fall outside the scope of traditional genotypic resistance testing have been identified. Mutations in the connection domain (CN) of HIV-1 reverse transcriptase have been shown to influence mutational pathways and contribute to resistance to both NRTIs and NNRTIs (57). The frequently selected N348I amino acid substitution, also observed in some of our patient samples, has been shown to decrease NVP susceptibility (58). When it occurs in combination with other NNRTI resistance-associated amino acid substitutions, such as K103N and Y181C, N348I has been shown to contribute to EFV, ETR, and RPV resistance (59, 60). In addition, RPV resistance is enhanced by this substitution in the background of E138K (61). We have confirmed in HIV-1 subtype C that N348I alone does not affect ETR/RPV susceptibility but can significantly reduce ETR and/or RPV susceptibility when it occurs in combination with other NNRTI resistance-associated substitutions, particularly Y181C. A less common CN amino acid change, T369I, was observed in conjunction with Y181C in one of our phenotypically resistant patient samples. Previous publications have shown that T369I can influence EFV, NVP, and ETR susceptibility in subtype B (62, 63). Reconstruction of the mutations in p8.MJ4 demonstrated that T369I significantly decreased susceptibility to all NNRTIs in subtype C, including RPV, when it occurred in combination with Y181C. However, by itself, T369I reduced susceptibility to NVP only. The Stanford HIV Drug Resistance Database reports a prevalence of 15.5% and 2.1% for N348I and T369I, respectively, among HIV-1 subtype C NNRTI treatment failures. While T369I is unlikely to be a major factor contributing to ETR or RPV

resistance due to its low prevalence, N348I may play a more significant role. It may therefore be important in future to genotype regions outside those genotyped in standard resistance tests.

Mutations shown to cause resistance to ETR and/or RPV had a low prevalence (<6%) in our data set, and the prevalence was even lower (<2%) for those causing high-level resistance. According to the Stanford HIV Drug Resistance Database, the prevalences of these amino acid substitutions are similar in subtypes B and C, except for M230L, which is more prevalent in subtype C (4.7%) than subtype B (0.9%). The E138A amino acid substitution, which also occurs more frequently in subtype C (9% versus 3% in subtype B), was not shown in our assay to decrease susceptibility to any of the NNRTIs, as has been reported by others (40). All double mutations that caused resistance to ETR and/or RPV in subtype C had a prevalence of <8%, and only 5 of the 14 tested combinations were actually observed in our subtype C data sets.

In conclusion, we have shown that ETR and RPV are active against HIV-1 subtype C isolates with the prevalent EFV/NVP resistance-related mutations and would therefore be expected to be efficacious in the treatment of HIV-1 subtype C in patients that fail an EFV- or NVP-based regimen. However, the presence of Y181C, especially after failure on an NVP-based treatment, could hamper the effectiveness of ETR and, to a lesser extent, RPV. Overall, the development of newer NNRTIs with the ability to retain activity against clinically relevant mutant strains has kept this important class of drugs in the front line of antiviral therapies.

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