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Panel of Prototypical Recombinant Infectious Molecular Clones Resistant to Nevirapine, Efavirenz, Etravirine, and Rilpivirine

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We created a panel of 10 representative multi-nonnucleoside reverse transcriptase inhibitor (NNRTI)-resistant recombinant infectious molecular HIV-1 clones to assist researchers studying NNRTI resistance or developing novel NNRTIs. The cloned viruses contain most of the major NNRTI resistance mutations and most of the significantly associated mutation pairs that we identified in two network analyses. Each virus in the panel has intermediate- or high-level resistance to all or three of the four most commonly used NNRTIs.

New nonnucleoside reverse transcriptase (RT) inhibitors (NNRTIs) are usually tested against a range of NNRTI-resistant clinical HIV-1 isolates. However, because no standard sets of NNRTI-resistant isolates have been identified, it is often not possible to compare the activity of a new NNRTI with those of other licensed or investigational NNRTIs. We have previously shown that NNRTI resistance mutations occur in common mutational clusters (8, 10). Here we modified our previous analyses to identify clusters of viruses with major NNRTI resistance mutations, defined as those that alone cause intermediate- to high-level resistance to ≥ 1 NNRTI. We used these clusters to identify samples with distinct mutational patterns to create a panel of prototypical multi-NNRTI-resistant molecular infectious clones. We chose to create recombinant infectious molecular clones from clinical samples rather than from site-directed mutants because such virus constructs are more likely to have replication characteristics similar to those of clinical isolates.

Selection of HIV-1 plasma samples. As part of an Institutional Review Board-approved protocol, we selected cryopreserved plasma samples from patients in Northern California undergoing standard genotypic resistance testing. Because the mutations that occur most commonly in patients receiving nevirapine and/or efavirenz do not cause high-level etravirine resistance (2, 14), etravirine usually has a higher genetic barrier to resistance than these earlier NNRTIs. Therefore, for this study, we selected available virus samples with ≥ 1 major etravirine resistance mutations and/or a statistically significant pair or cluster of major NNRTI resistance mutations, i.e., L100I, K101E/P, K103N, V106M, E138K, Y181C/I/V, Y188L, G190A/S/E, and M230L (3, 11–13). Major etravirine resistance mutations were defined as those mutations with the highest weights in the etravirine genotypic susceptibility score, i.e., L100I, K101P, Y181C/I/V, and M230L (15).

To identify clusters of NNRTI resistance mutations, we performed a correlation network analysis of viruses with ≥ 1 major NNRTI resistance mutations in 8,035 isolates from 7,751 individuals in the Stanford HIV Drug Resistance Database (9) (see Table S1 in the supplemental material). Two isolates with different patterns of major mutations from 284 patients were included. The isolates' subtypes included B (71.2%), C (12.4%), CRF01_AE (5.7%), and other non-B clades (10.5%). Eighty-two percent received an NNRTI, 10% had an unknown treatment history, and 8% were NNRTI naïve.

Each node in Fig. 1 represents a major NNRTI resistance mutation. Each line represents a statistically significant correlation between two mutations present without electrophoretic evidence of an amino acid mixture, making it probable that co-occurring mutations were on the same genome. Figure 1B contains a correlation network analysis of 80 viruses meeting at least two of the following three criteria: >30 -fold-decreased nevirapine susceptibility, >10 -fold-decreased efavirenz susceptibility, and >5 -fold-decreased etravirine susceptibility (by the PhenoSense assay; Monogram, South San Francisco, CA). In Fig. 1B, mutation pairs present in ≥ 2 viruses are connected by a line. Table S2 in the supplemental material contains the complete set of correlation coefficients for each of the mutation pairs.

Creation of recombinant infectious molecular clones. HIV-1 cDNA was generated from HIV-1 RNA extracted from 10 ultracentrifuged plasma samples. An 871-nucleotide amplicon encompassing RT positions 23 to 313 was amplified using the thermostable *Pfu* DNA polymerase (Promega, Madison, WI). Amplicons were digested with MscI and PflM1 and ligated into pNLPFB (4, 5). Following transformation into competent *Escherichia coli*, selected molecular clones were transfected into C8166 cells. Once overcome by syncytia, C8166 cells were cocultured with SupT1 cells. When syncytia were present in most of the cell clusters, 20 1.0-ml aliquots of cell-free virus were harvested and cryopreserved at -80°C . The list of mutations in each clone is in Table S3 in the supplemental material.

Panel of prototypical recombinant infectious molecular clones. Table 1 shows the NNRTI resistance mutations, virus replication characteristics, and *in vitro* susceptibility results obtained using the PhenoSense assay, a single-cycle reporter gene assay of recombinant viruses containing ligated, patient-amplified PCR

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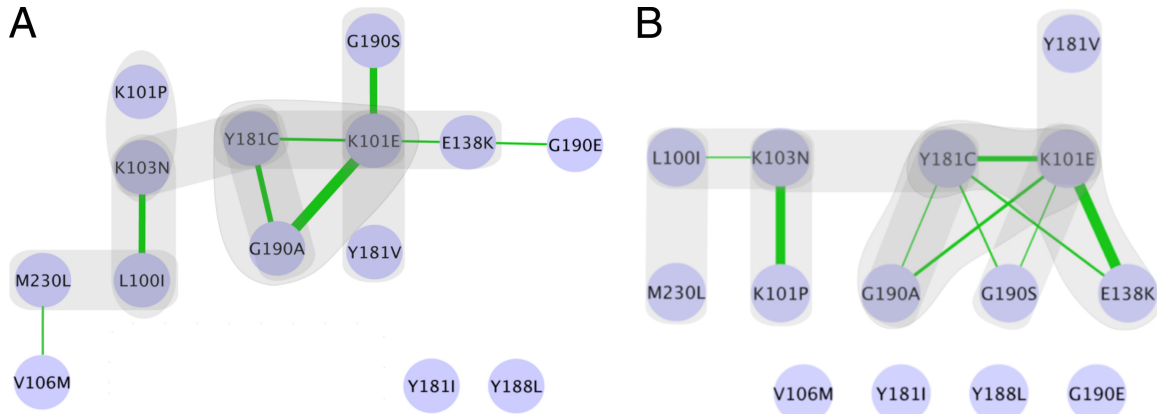


FIG 1 Correlation network analysis of virus sequences with ≥ 1 major NNRTI resistance mutations in 8,035 viruses from 7,751 individuals in the Stanford HIV Drug Resistance Database (A) and in viruses from 80 individuals with intermediate- to high-level resistance to each NNRTI (B). Each node represents a major NNRTI resistance mutation. Each line represents a correlation between a pair of mutations, and the thickness of the line is proportional to the strength of the correlation (Spearman's ρ). The gray ellipses are superimposed on pairs or triplets of major mutations present in the prototypical multi-NNRTI-resistant recombinant infectious molecular clones. None of the clones had the V106M, Y181I, Y188L, or G190E mutation.

fragments encompassing 3' gag, protease, and RT positions 1 to 313 in wild type pNL43 test vectors (7). Susceptibility results were expressed as the ratio of the 50% effective concentration (EC_{50}) of the cloned virus to that of NL43.

Nine of the clones had a major etravirine resistance mutation (Fig. 1). Six of the clones had ≥ 1 of the following correlated major NNRTI resistance mutation pairs or triplets: L100I and K103N; K101P and K103N; K101E and G190S; Y181C and G190A; Y181C, K101E, and E138K; and K101E and G190S. One of the clones had K103N and Y181C in combination with V179F, an accessory NNRTI resistance mutation that is strongly associated with Y181C (8). Although K103N and Y181C are the two most common NNRTI resistance mutations, they are not significantly correlated in viruses from individuals having ≥ 1 NNRTI resistance mutations (Fig. 1A; see Table S3 in the supplemental material). Samples with the major etravirine resistance mutation Y181I and with the mutation pairs Y181C and G190S, E138K and G190E, and V106M and M230L were not available.

Each clone had >200 -fold (the assay upper limit)-decreased nevirapine susceptibility. Five clones had >200 -fold-decreased

efavirenz susceptibility; three had 15- to 90-fold-decreased efavirenz susceptibility. Five clones had >10 -fold-decreased etravirine susceptibility (the upper etravirine PhenoSense clinical cutoff), and five had 3 (the lower clinical cutoff)- to 10-fold-decreased etravirine susceptibility. The panel susceptibility results illustrate (i) the lower genetic barrier to resistance of nevirapine than that of efavirenz and (ii) the lower genetic barrier to resistance of efavirenz than those of etravirine and rilpivirine for viruses with mutations other than E138K and/or Y181C/I/V. With the exception of clones 5244 and 2100, rilpivirine and etravirine susceptibilities were highly correlated ($r = 0.83$, $P = 0.02$; Spearman's ρ). The greater decrease in rilpivirine susceptibility than etravirine susceptibility caused by K101P has been previously reported (1).

Conclusion. The 10 clones in the NNRTI resistance panel contain 10 of the 14 major NNRTI resistance mutations and 8 of the 11 correlated mutational pairs. The panel contained viruses that were intermediately or highly resistance to three or more of the four NNRTIs and showed little evidence of decreased replication *in vitro*, as indicated by replication capacities that were generally

TABLE 1 Panel of prototypical multi-NNRTI resistance recombinant infectious molecular clones^a

Sample	NNRTI resistance mutation(s)		Treatment history		Resistance (<i>n</i> -fold)				Replication characteristics		
	Major	Accessory	NNRTI(s)	Duration (mo)	NVP	EFV	ETR	RPV	RC (%)	p24 level (ng/ml)	TCID ₅₀ (IU/ml)
5244	101P, 103N		EFV	13	>200	>200	5.8	92	56	4.5e2	$\geq 3,080$
5485	100I, 103N	221Y	EFV	11	>200	>200	6.8	6.3	NA	2.2e5	1,950
7066	103N, 181C	179F	EFV	14	>200	90	8.8	2.3	83	2.2e5	$\geq 3,080$
5735	101E, 181V		NVP	31	>200	2.1	27	24	78	2.8e5	$\geq 3,080$
1833	101E, 181C, 190A	98G	NVP, EFV	19	>200	>200	15	22	51	3.7e5	$\geq 3,080$
16182	181C, 190A	106I, 221Y	NVP	61	>200	26	6	3.5	117	4.1e4	$\geq 3,080$
5375	101E, 138K, 181C	98G	NVP, DLV	25	>200	3.6	10	9.2	41	2.0e2	1,950
25641	101E, 190S	138G	EFV, NVP	26	>200	>200	3.2	2.6	61	5.3e4	493
2100	100I, 230L	179D	NVP, DLV	15	>200	>200	95	13	69	2.1e2	1,230
1579	230L	138G, 221Y, 227L	DLV, NVP	28	>200	15	21	18	41	1.6e4	1,950

^a Abbreviations: EFV, efavirenz; NVP, nevirapine; DLV, delavirdine; ETR, etravirine; RPV, rilpivirine; RC, replication capacity (percentage of that of a wild-type control virus) as determined by Monogram; NA, not applicable. TCID₅₀, 50% tissue culture infective dose after 10 days in MT2 cells. Resistance levels are *n*-fold differences from those of wild-type control virus NL43. For NVP, EFV, ETR, and RPV, the NL43 EC_{50} s were 0.1 μ M, 0.002 μ M, 0.005 μ M, and 0.0009 μ M, respectively. Resistance levels of >10 -fold are in bold.

above 50% (6) and virus titers (Table 1). We hypothesize that NNRTIs that retain activity against the viruses in this panel are likely to retain activity against most of the clinically relevant NNRTI-resistant viruses observed *in vivo*. However, because the numbers of sequences from patients with etravirine and/or rilpivirine failure is low, we plan to extend this panel as new commonly observed mutational clusters are reported.

Nucleotide sequence accession numbers. The GenBank accession numbers for the samples in this paper are JQ814886, JQ814884, JQ814890, JQ814888, JQ814891, JQ814889, JQ814892, JQ814887, JQ814885, and JQ814893. We have submitted this panel to the NIH AIDS Research and Reference and Reagent Program (<http://www.aidsreagent.org>), where it is available without restriction to researchers developing and testing novel NNRTIs.

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