In Vitro Cross-Resistance Profiles of Rilpivirine, Dapivirine and MIV-150: NNRTI Microbicides in Clinical Development for the Prevention of HIV-1 Infection

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Running title: **Cross-Resistance Profiles of RPV, DPV and MIV-150**

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Rilpivirine, dapivirine (DPV) and MIV-150 are in development as microbicides. It is not known whether they will block infection of circulating NNRTI-resistant HIV-1 variants. Here, we demonstrate that the activity of DPV and MIV-150 are compromised by many resistant viruses containing single or double substitutions. High DPV genital tract concentrations from DPV-ring use may block replication of resistant viruses. However, MIV-150 genital tract concentrations may be insufficient to inhibit many resistant viruses, including those harboring K103N or Y181C.

Nonnucleoside reverse transcriptase (RT) inhibitors (NNRTIs) comprise a group of small amphiphilic compounds with diverse chemical structures that inhibit HIV-1 (but not HIV-2) replication. They interact with HIV-1 RT by binding to a single site, termed the NNRTI-binding pocket, on the p66 subunit of the p66/p51 heterodimeric enzyme (1). In many low and middle income countries (LMIC), particularly in sub-Saharan Africa, NNRTIs are used in both HIV-1 treatment and prevention strategies (2). Specifically, the NNRTIs nevirapine, efavirenz or rilpivirine (RPV) are used in first-line antiretroviral therapies; whereas etravirine is reserved for salvage therapy. For prevention of HIV-1 infection, nevirapine is used to block mother-to-child transmission; a dapivirine (DPV)-containing ring provided moderate efficacy in HIV-1 negative female participants, particularly in compliant women over 25 years of age (3, 4); a microbicide gel formulation (PC-1005) containing the phenylethylthiazolylthiourea derivative MIV-150 is in phase I clinical studies (5); and an injectable long-acting RPV formulation was evaluated in the clinical study HPTN 076 for pre-exposure prophylaxis (6, 7).

Due to their extensive use in LMIC, there have been significant increases in acquired NNRTI drug resistance, and consequently the proportion of newly infected patients with transmitted drug resistance has also increased (8, 9). In this regard, four NNRTI-resistance mutations — K101E, K103N, Y181C, and G190A — account for > 80% of NNRTI-associated transmitted drug resistance in all regions and subtypes (10). Currently, it is unknown whether the NNRTIs used in prevention strategies (e.g. DPV, RPV or MIV-150) will prevent infection of circulating NNRTI-resistant HIV-1 variants. Importantly, and relevant to this study, there is also a paucity of information in regard to the resistance and cross-resistance profiles of DPV and MIV-150. To address these important knowledge gaps, we constructed by site-directed

mutagenesis twenty-eight subtype B HIV-1^{LAI} infectious viruses containing single NNRTI resistance mutations spanning 17 different codons (V90I; L100I/V; K101E/P; K103N/S; V106I; V108I; E138A/K; V179D/F, G190A/S; I181C/I/V; Y188C/H/L; H221Y; P225H; F227C/L; M230L; P236L; N348I). We also constructed six subtype B HIV-1^{LAI} infectious viruses containing two NNRTI resistance mutations (K101E/G190A; K101E/K103N; K101E/Y181C; K103N/G190A; K103N/Y181C; Y181C/G190A). Drug susceptibility in a single cycle assay using TZM-bl cells was determined for RPV (Selleckchem, TX, USA), DPV (Selleckchem, TX, USA) and MIV-150 (Cayman Chemical Company, MI, USA) as described previously (11, 12). Low-, intermediate- and high-level resistance was defined as 2→8, 8→20, and > 20-fold changes in drug susceptibility compared to the wild type (WT) virus. Of the three NNRTIs studied, RPV exhibited the best antiviral activity across the panel of mutant viruses tested, and retained full sensitivity against 19 of 28 variants containing a single substitution; and 2 of 6 variants containing double substitutions (Table 1; Fig. 1). The E138A/K, F227C, K101E, Y188L, M230L, K101E/G190A and K103N/Y181C substitutions conferred low-level RPV resistance, while the Y181I/V and K101P substitutions conferred high-level resistance. The RPV resistance profile reported in this study is consistent with those previously published (13, 14). In contrast to RPV, DPV only retained activity against 15 of the 28 viruses containing a single substitution; and 1 of 6 viruses containing double substitutions (Table 1; Fig. 1). The K101E, E138K, K103N/S, F227C, Y181C and K101E/G190A substitutions conferred low-level resistance to DPV; whereas the L100I/V, M230L, K101E/K103N and Y181C/G190A substitutions, and the Y188L, K101P, Y181I/V and K101E/Y181C and K103N/Y181C substitutions were found to confer intermediate and high-level resistance, respectively. The DPV cross-resistance profile reported in our study is consistent with prior *in vitro* studies of DPV resistance selection and

93 cross-resistance profiling (15-17). Additionally, Penrose *et al* recently reported that there was
94 frequent cross-resistance to DPV in subtype C-infected individuals after first-line therapy failure,
95 and reported that L100I and K103N were significantly more frequent in samples with >500-fold
96 resistance to DPV compared to samples with a ≤500-fold resistance (18). However, the
97 limitation of this latter study is that each clinical isolate contained on average 3 NNRTI
98 resistance mutations, making it difficult to identify the genetic determinants for resistance.
99 Similar to DPV, MIV-150 was also found to be active against only 15 of the HIV-1 variants
100 containing single NNRTI substitutions tested; and 2 of 6 variants containing two substitutions
101 tested. However, high-level resistance was more frequently observed for MIV-150 than for either
102 DPV or RPV (Table 1; Fig. 1). Notably, the M230L, K103S, K103N, Y181V, K101P, Y181I,
103 Y188L, K101E/K103N, K101E/Y181C and K103N/Y181C substitutions all conferred high-level
104 resistance. The F227C and Y181C substitutions, and the L100L/V, K101E and K101E/G190A
105 substitutions, were found to confer intermediate- and low-level MIV-150 resistance, respectively
106 (Table 1, Fig. 1). To our knowledge, this is the first study to define in detail the cross-resistance
107 profile for MIV-150, although one prior study identified different combinations of E138K,
108 Y181I, Y181C, K103N, L100I or K101E in SHIV-RT viruses exposed to MIV-150 in rhesus
109 macaques, although no phenotypic data were provided (19). Additionally, prior studies have
110 reported on the resistance profiles of the MIV-150 analogs, namely MIV-160 and MIV-170
111 (16,17).
112
113 Recently we reported that an E138A substitution occurs more frequently in subtype C
114 (range: 5.9-7.5%) than B (range: 0-2.3%) sequences from treatment-naïve individuals ($p<0.01$)
115 (11). Because E138A in subtype C HIV-1 decreases RPV susceptibility, we previously proposed

that this polymorphism may impact prevention (and treatment) strategies that include RPV in geographic areas where subtype C infection is prevalent (11). Accordingly, in this study we synthesized (GenScript, NJ, USA) and cloned into our HIV-1^{LAI} viral vector (as described previously (12)) full length subtype C RT sequences from two antiretroviral-naïve individuals that did not harbor E138A, and from 6 antiretroviral-naïve individuals that contained E138A. Phenotypic analyses revealed that 2 of the recombinant viruses that contained E138A conferred low-level resistance (2.4- and 2.0-fold, respectively) to RPV (Table 2). In contrast, 4 of the 6 recombinant viruses that contained E138A conferred decreased susceptibility to DPV (range; 2.1- to 4.7-fold) and MIV-150 (range: 1.9- to 3.4-fold) (Table 2). These data highlight that the RT genetic backbone influences, at least to some extent, the ability of E138A to decrease NNRTI susceptibility; and suggests that the low level resistance conferred by E138A is unlikely to impact RPV, DPV or MIV-150 activity.

In summary, this study provides the first detailed insights into the antiviral activity of RPV, DPV and MIV-150 against a broad panel of recombinant viruses containing substitutions that are known to decrease NNRTI susceptibility. We also evaluated their activity against WT subtype C RTs that contained E138A. The pharmacokinetics of the long-acting RPV formulation has been investigated in healthy individuals in two different studies (20,21). In cervicovaginal fluid (CVL), RPV concentrations at day 28 post-administration were 12, 15 and 98 ng/mL (68, 107 and 232 nM) following injected doses of 300, 600 and 1200 mg, respectively. In the rectal fluid (RF), RPV concentrations at day 28 post-administration were 11.9 ng/mL (32 nM), following a 600 mg injection. The RPV concentrations in the CVL and RF exceed the EC_{50} values for all of the NNRTI-resistant variants listed in Table 1, suggesting that RPV may prevent

infection from transmitted NNRTI-resistant viruses. With regard to DPV, pharmacokinetic studies have shown that the vaginal fluid concentration on day 28 of DPV ring use ranged from 14.9 – 65 µg/mL (45-198 µM) (22, 23). These concentrations far exceed the reported EC_{50} values for the WT and mutant HIV-1 in Tables 1 and 2, suggesting that the ring would effectively inhibit replication of all the resistant viruses tested. (Note, exact EC_{50} values for DPV for the K101P and Y181I/V HIV-1 viruses could not be determined as they exceeded the highest concentration of drug used in the assay.) In contrast, pharmacokinetic studies of PC-1005 (MIV-150 and zinc acetate in a carrageenan gel) yielded concentrations of MIV-150 in cervicovaginal lavages ranging from ~ 100–170 nM (5). In this regard, it is questionable whether these concentrations will effectively block the mutant viruses which exhibited high-level MIV-150 resistance (K101P, K103N/S, Y181C/I/V, F227C, M230L, K101E/K103N, K101E/Y181C and K103N/Y181C) and for which EC_{50} values range from 10->100 nM. Importantly, both K103N and Y181C, which are frequently associated with transmitted NNRTI resistance, fall into this category.

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None

Conflict of Interest

None to declare

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295 **TABLE 1: Susceptibility of HIV-1 containing single or double NNRTI resistance mutations**

296 **to RPV, DPV and MIV-150.**

Virus	RPV		DPV		MIV-150	
	EC_{50} (nM)[1]	Fold-R^2 (p-value)	EC_{50} (nM)[1]	Fold-R^2 (p-value)	EC_{50} (nM)[1]	Fold-R^2 (p-value)
WT	0.33 ± 0.13	-	0.64 ± 0.13	-	0.68 ± 0.09	-
V90I	0.59 ± 0.13	1.8	1.09 ± 0.47	1.7	0.98 ± 0.14	1.4
L100I	0.36 ± 0.13	1.1	9.36 ± 1.37	14.7 (<0.01)	5.03 ± 1.35	7.4 (<0.01)
L100V	0.27 ± 0.13	0.8	6.15 ± 1.79	9.6 (<0.01)	5.06 ± 2.42	7.5 (0.02)
K101E	1.12 ± 0.24	3.5 (<0.01)	2.29 ± 0.68	3.6 (<0.01)	3.25 ± 0.75	4.8 (<0.01)
K101P	13.10 ± 1.70	40.1 (<0.01)	≥ 62.5	≥ 100 (<0.01)	≥ 62.5	≥ 100 (<0.01)
K103N	0.53 ± 0.11	1.6	3.03 ± 0.47	4.8 (<0.01)	23.40 ± 2.81	34.6 (<0.01)
K103S	0.49 ± 0.11	1.5	4.61 ± 0.43	7.2 (<0.01)	15.30 ± 2.03	22.7 (<0.01)
V106I	0.48 ± 0.12	1.5	0.94 ± 0.14	1.5	0.78 ± 0.11	1.20
V108I	0.38 ± 0.01	1.2	0.96 ± 0.20	1.5	0.89 ± 0.15	1.3
E138A	0.80 ± 0.41	2.5 (0.06)	1.29 ± 0.33	2.0 (0.02)	1.32 ± 0.23	2.0 (0.01)
E138K	0.96 ± 0.18	2.9 (<0.01)	2.79 ± 0.70	4.4 (<0.01)	1.89 ± 0.17	2.8 (<0.01)
V179D	0.39 ± 0.15	1.2	0.81 ± 0.33	1.3	0.73 ± 0.35	1.1
V179F	0.003 ± 0.002	0.01 (>0.05)	0.20 ± 0.01	0.3 (<0.01)	0.03 ± 0.01	0.1
G190A	0.42 ± 0.03	1.3	0.77 ± 0.15	1.2	0.40 ± 0.09	0.6
G190S	0.25 ± 0.17	0.8	0.84 ± 0.01	1.3	0.09 ± 0.01	0.1
Y181C	0.60 ± 0.25	1.8	5.06 ± 1.50	7.9 (<0.01)	10.20 ± 1.60	15.1 (<0.01)
Y181I	7.68 ± 0.78	23.5 (<0.01)	≥ 62.5	≥ 100 (<0.01)	≥ 62.5	≥ 100 (<0.01)
Y181V	7.36 ± 1.20	22.6 (<0.01)	≥ 62.5	≥ 100 (<0.01)	39.60 ± 4.10	58.6 (<0.01)
Y188C	0.08 ± 0.04	0.3 (>0.05)	0.39 ± 0.20	0.6	0.21 ± 0.05	0.3 (>0.05)
Y188H	0.11 ± 0.04	0.3 (>0.05)	0.69 ± 0.33	1.1	1.16 ± 0.48	1.7
Y188L	1.69 ± 0.23	5.2 (<0.01)	55.30 ± 6.53	86.7 (<0.01)	≥ 62.5	≥ 100 (<0.01)
H221Y	0.45 ± 0.20	1.37	0.85 ± 0.20	1.3	0.91 ± 0.19	1.3
P225H	0.29 ± 0.17	0.9	0.69 ± 0.25	1.1	0.76 ± 0.32	1.1
F227C	1.11 ± 0.27	3.40 (0.01)	4.37 ± 0.78	6.9 (<0.01)	10.01 ± 3.16	14.8 (<0.01)
F227L	0.28 ± 0.26	0.9	0.61 ± 0.19	1.0	0.88 ± 0.31	1.3
M230L	2.58 ± 1.13	7.9 (0.01)	10.10 ± 0.38	15.8 (<0.01)	15.30 ± 2.87	22.6 (<0.01)
P236L	0.55 ± 0.10	1.7	1.00 ± 0.18	1.6	0.63 ± 0.07	0.9
N348I	0.50 ± 0.13	1.5	1.07 ± 0.47	1.7	0.99 ± 0.34	1.5
K101E/G190A	1.42±0.22	4.4 (<0.01)	4.22±0.31	6.6 (<0.01)	1.45±0.21	2.2 (<0.01)

K101E/K103N	0.50±0.07	1.5	12.60±1.84	19.7 (<0.01)	38.10±7.38	56.3 (<0.01)
K101E/Y181C	3.12±0.48	9.5 (<0.01)	40.70±9.86	63.8 (<0.01)	35.50±13.80	52.5 (<0.01)
K103N/G190A	0.02±0.01	0.06 (<0.01)	0.98±0.17	1.5	1.07±0.25	1.6
K103N/Y181C	1.87±0.34	5.7 (<0.01)	56.20±3.06	88.0 (<0.01)	182.00±51.20	≥ 250 (<0.01)
Y181C/G190A	0.70±0.10	2.1 (<0.01)	7.22±2.27	11.3 (<0.01)	1.26±0.39	1.9

[1] The concentrations of drug required to inhibit viral replication by 50% (EC_{50}) from 3 independent experiments. Data reported as a mean ± standard deviation from at least 3 independent experiments.

[2] Mean fold change in EC_{50} of mutant versus WT virus. EC_{50} values were compared for statistically significant differences (p-value < 0.05) using a non-paired, 2 sample equal variance (homoscedastic) test.

TABLE 2: Susceptibility of recombinant viruses containing full-length patient-derived WT subtype C RT sequences with and without E138A to RPV, DPV and MIV-150.

Virus[1]	E138A Present	RPV		DPV		MIV-150	
		EC_{50} (μM)[2]	Fold-R[3] (p-value)	EC_{50} (μM)[1]	Fold-R[2] (p-value)	EC_{50} (μM)[1]	Fold-R[2] (p-value)
AF361879	No	0.30 ± 0.03	-	0.68 ± 0.10	-	0.56 ± 0.10	
AY043176	No	0.24 ± 0.04	-	0.49 ± 0.02	-	0.36 ± 0.05	
Avg[4]	No	0.27 ± 0.04	-	0.58 ± 0.13	-	0.46 ± 0.13	
DQ351238	Yes	0.40 ± 0.12	1.5	1.20 ± 0.33	2.1 (<0.01)	1.40 ± 0.59	3.1 (<0.01)
AY901981	Yes	0.64 ± 0.14	2.4 (<0.01)	1.80 ± 0.51	3.0 (<0.01)	1.50 ± 0.23	3.2 (<0.01)
AF443097	Yes	0.41 ± 0.12	1.5	1.30 ± 0.50	2.3 (<0.01)	0.86 ± 0.16	1.9 (<0.01)
AY253303	Yes	0.23 ± 0.04	0.9	0.83± 0.13	1.4	0.60 ± 0.05	1.3
AY734559	Yes	0.36 ± 0.14	1.3	0.77 ± 0.26	1.3	0.63 ± 0.15	1.4
FJ199637	Yes	0.54 ± 0.07	2.0 (<0.01)	2.70 ± 0.19	4.7 (<0.01)	1.60 ± 0.31	3.4 (<0.01)

[1] The GenBank sequence identifier for the full-length subtype C RT gene

[2] The concentrations of drug required to inhibit viral replication by 50% (EC_{50}) are reported as a mean ± standard deviation from at least 3 independent experiments.

[3] Mean fold change in EC_{50} of WT-E138A vs WT virus. EC_{50} values from 3 independent experiments were compared for statistically significant differences (p-value < 0.05) using a non-paired, 2 sample equal variance (homoscedastic) test.

[4] The median EC_{50} value for the 2 viruses (AF361897 and AY043176) that did not harbor E138A was used as the WT reference for determination of Fold-R.

334 **Figures Legends**

335

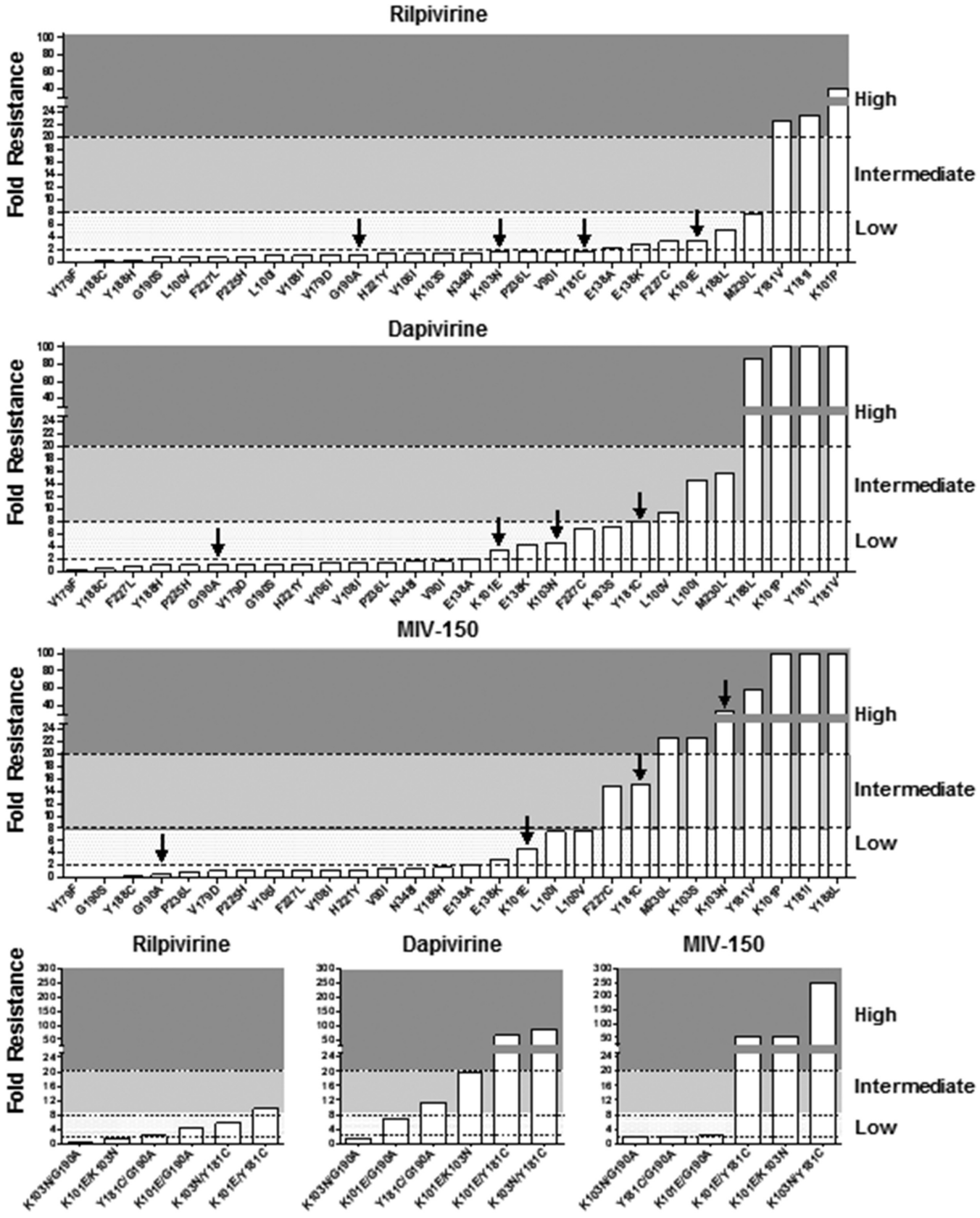
336 **Figure 1: NNRTI cross-resistance profiles for RPV, DPV and MIV-150.** Low-,

337 intermediate- and high-level resistance was defined as 2→8, 8→20, and >20-fold changes in

338 drug susceptibility compared to the WT virus. Arrows indicate the four most commonly

339 transmitted drug resistance mutations, G190A, K101E, Y181C, and K103N.

340



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