Altered Viral Fitness and Drug Susceptibility in HIV-1 Carrying Mutations That Confer Resistance to Nonnucleoside Reverse Transcriptase and Integrase Strand Transfer Inhibitors

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ABSTRACT

Nonnucleoside reverse transcriptase (RT) inhibitors (NNRTI) and integrase (IN) strand transfer inhibitors (INSTI) are key components of antiretroviral regimens. To explore potential interactions between NNRTI and INSTI resistance mutations, we investigated the combined effects of these mutations on drug susceptibility and fitness of human immunodeficiency virus type 1 (HIV-1). In the absence of drug, single-mutant viruses were less fit than the wild type; viruses carrying multiple mutations were less fit than single-mutant viruses. These findings were explained in part by the observation that mutant viruses carrying NNRTI plus INSTI resistance mutations had reduced amounts of virion-associated RT and/or IN protein. In the presence of efavirenz (EFV), a virus carrying RT-K103N together with IN-G140S and IN-Q148H (here termed IN-G140S/Q148H) mutations was fitter than a virus with a RT-K103N mutation alone. Similarly, in the presence of EFV, the RT-E138K plus IN-G140S/Q148H mutant virus was fitter than one with the RT-E138K mutation alone. No effect of INSTI resistance mutations on the fitness of RT-Y181C mutant viruses was observed. Conversely, RT-E138K and -Y181C mutations improved the fitness of the IN-G140S/Q148H mutant virus in the presence of raltegravir (RAL); the RT-K103N mutation had no effect. The NNRTI resistance mutations had no effect on RAL susceptibility. Likewise, the IN-G140S/Q148H mutations had no effect on EFV or RPV susceptibility. However, both the RT-K103N plus IN-G140S/Q148H and the RT-E138K plus IN-G140S/Q148H mutant viruses had significantly greater fold increases in 50% inhibitory concentration (IC_{50}) of EFV than viruses carrying a single NNRTI mutation. Likewise, the RT-E138K plus IN-G140S/Q148H mutant virus had significantly greater fold increases in RAL IC_{50} than that of the IN-G140S/Q148H mutant virus. These results suggest that interactions between RT and IN mutations are important for NNRTI and INSTI resistance and viral fitness.

IMPORTANCE

Nonnucleoside reverse transcriptase inhibitors and integrase inhibitors are used to treat infection with HIV-1. Mutations that confer resistance to these drugs reduce the ability of HIV-1 to reproduce (that is, they decrease viral fitness). It is known that reverse transcriptase and integrase interact and that some mutations can disrupt their interaction, which is necessary for proper functioning of these two enzymes. To determine whether resistance mutations in these enzymes interact, we investigated their effects on drug sensitivity and viral fitness. Although individual drug resistance mutations usually reduced viral fitness, certain combinations of mutations increased fitness. When present in certain combinations, some integrase inhibitor resistance mutations increased resistance to nonnucleoside reverse transcriptase inhibitors and vice versa. Because these drugs are sometimes used together in the treatment of HIV-1 infection, these interactions could make viruses more resistant to both drugs, further limiting their clinical benefit.

Antiretroviral therapy (ART) prevents morbidity and mortality associated with human immunodeficiency virus type 1 (HIV-1) infection and can protect against transmission of HIV-1 (1). However, the transmission or emergence of drug-resistant variants of HIV-1 can blunt the efficacy of ART. Combination ART that effectively suppresses HIV-1 replication can prevent the emergence of drug resistance, but partial viral suppression (e.g., in the setting of inconsistent adherence) can select for multiclass drug resistance (2).

The HIV-1 *pol* gene encodes three enzymes that are essential for the viral life cycle: protease (PR), reverse transcriptase (RT), and integrase (IN). The mature enzymes are derived from the same polyprotein precursor, suggesting the potential for interactions among them (3, 4). Integrase promotes reverse transcription through specific interactions with the HIV-1 reverse transcription complex (5, 6). Integrase binds the HIV-1 RT heterodimer (p66/p51); conversely, the individual RT subunits, p51 and p66, are

each able to bind IN (7). These interactions appear to promote viral replication, although some studies show that RT inhibits the enzymatic activities of IN (8, 9).

Nonnucleoside reverse transcriptase inhibitors (NNRTI) are key components of ART. Mutations conferring resistance have been described for each of the currently approved NNRTI (10). The RT-K103N and -Y181C substitutions are the most frequently observed resistance mutations in HIV-1 from patients treated

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with efavirenz (EFV) and nevirapine (NVP), respectively (11–13), whereas the RT-E138K is the principal mutation associated with resistance to rilpivirine (RPV) (14–16).

Integrase strand transfer inhibitors (INSTI) approved for the treatment of HIV-1 infection include raltegravir (RAL), elvitegravir (EVG), and dolutegravir (DTG) (17–19). Raltegravir resistance is conferred by mutations at IN residue 143, 148, or 155 together with associated secondary mutations (20). The IN-N155H mutant viruses emerge first and are eventually replaced by IN-Q148H mutant viruses, usually in combination with an IN-G140S mutation (21–24). Mutations that confer resistance to RAL generally confer cross-resistance to EVG and vice versa (25–27). These mutations have different effects on susceptibility and viral fitness (23, 28). Presence of a mutation at IN codon 148 together with additional INSTI resistance mutations reduces susceptibility to DTG (29–31).

Although it is known that functional interactions occur between the HIV-1 RT and IN, data on the relative contributions of RT and IN to viral fitness are limited. Preliminary data suggest that the combination of NNRTI and INSTI resistance mutations impairs HIV replication capacity (32, 33). In order to explore the interactions of these mutations, we investigated the combined effects of NNRTI (RT-K103N, -E138K, and -Y181C) and INSTI (N-G140S and IN-Q148H [hereinafter referred to as IN-G140S/Q148H]) resistance mutations on drug susceptibility and viral fitness.

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MATERIALS AND METHODS

Cells and reagents. EFV, RPV, RAL, MT2 cells, and TZM-bl cells were provided by the AIDS Research and Reference Reagent Program. Human embryonic kidney cells (293T) were purchased from the American Type Culture Collection (Manassas, VA). The 293T and TZM-bl cells were cultivated in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 U/ml); MT-2 cells were propagated in R-10 medium (RPMI 1640 [Cellgro, Herndon, VA] supplemented with 10% FBS, 2 mM L-glutamine, penicillin [100 U/ml], and streptomycin [100 µg/ml]).

Construction of HIV-1 vectors with a *pol* gene deletion. We constructed a variety of vectors carrying a deletion of the entire HIV-1 *pol* gene in an otherwise intact molecular clone of HIV-1 (NL4-3). These vectors either have a wild-type (WT) *nef* or have *nef* replaced with a sequence tag (for performing growth competition assays) or a reporter gene (firefly or renilla luciferase, for performing replication capacity assays). These vectors allowed us to insert *pol* gene cassettes from various HIV-1 isolates carrying mutations of interest in PR, RT, and/or IN.

Site-directed mutagenesis. The full-length *pol* gene of HIV-1_{NL4-3} was amplified by PCR and cloned into the pGEM-T Easy vector (Promega, Madison, WI). The K103N, E138K, and Y181C mutations in RT and the E92Q, Y143C, N155H, E92Q/N155H, and G140S/Q148H mutations in IN were introduced into the cloned *pol* gene using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The presence of mutant sequences was confirmed by automated sequencing of the final plasmid clone with an ABI 377 automated sequencer.

Generation of virus stocks. Infectious recombinant marker viruses expressing WT or mutant RTs or INs or combinations of RT and IN mutants were generated by cotransfecting 293T cells with the *pol*-deleted proviral clone of NL4-3 together with the PCR-amplified *pol* gene of interest. Virus-containing supernatants were harvested 72 h after transfection, and the recombinant virus stocks were expanded and titers were determined as described previously (34). The RT- and IN-coding regions

of *pol* were amplified from proviral DNA of infected cells at the end of virus culture and analyzed by automated DNA sequencing to verify the presence of the correct alleles at RT codons 103, 138, and 181 and at IN codons 92, 140, 143, 148, and 155.

Drug susceptibility assay. The susceptibility of HIV-1 recombinants to EFV, RPV, and RAL was determined by infectivity assay as described previously (23). Briefly, 2-fold serial dilutions of drug (range of 0.39 to 100 nM for EFV, 0.039 to 100 nM for RAL, and 0.0039 to 1 nM for RPV) were added to the wells of a 96-well microtiter plate. An amount of virus sufficient to produce β-galactosidase activity was added to each well except for uninfected control wells, after which the 1×10^4 TZM-bl cells suspended in 50 µl of medium were added to each well; the final volume in each well was 200 µl. After incubation at 37°C for 48 h, β-galactosidase activity was quantified using a Galacto-Light Plus β-galactosidase activity assay kit (Applied Biosystems, Foster City, CA). All infections were performed in triplicate and experiments repeated at least twice. Percent inhibition was expressed as counts per second generated at various inhibitor concentrations relative to results with the no-drug control. Drug susceptibility was calculated by plotting the percent inhibition of virus replication versus the log₁₀ drug concentration to derive the 50% inhibitory concentration (IC₅₀). Drug susceptibility curves were fitted by nonlinear regression using GraphPad Prism 5 (GraphPad Software, Ann Arbor, MI).

Replication capacity assay. Viral replication capacity was determined by luciferase assay as described previously (35). Briefly, the TZM-bl cells were plated in 24-well plates at 4×10^4 cells/well the day before infection. After removal of the medium, virus stocks at a multiplicity of infection (MOI) of 0.05 were adsorbed onto cells in triplicate wells for 2 h. Subsequently, DMEM was added to a final volume of 1 ml/well, and cultures were incubated at 37°C in the absence and presence of 8 nM EFV, 0.08 nM RPV, and/or 0.8 nM RAL. After 3 days, the medium was removed; cells were washed with phosphate-buffered saline (PBS) and lysed in 500 µl of lysis buffer (5 mM MgCl₂ and 0.1% NP-40 in PBS). Luciferase activity was quantified in cell lysates by using a luciferase activity assay system (Promega, Madison, WI). Experiments were performed twice and the results averaged.

Infectivity and fitness profile assays. Viral infectivity was determined as described previously (23). Briefly, virus stock titers were determined according to β-galactosidase activity on TZM-bl cells. Two-fold serial dilutions of HIV inhibitors (0.39 to 100 nM for EFV, 0.0039 to 1 nM for RPV, and 0.039 to 10 nM for RAL) were added to triplicate wells of 96-well microtiter plates in a total volume of 200 µl of DMEM. After incubation at 37°C for 48 h, β-galactosidase activity was quantified using Galacto-Light Plus (Applied Biosystems, Foster City, CA) and expressed as chemiluminescence units. Infectivity was determined from the mean chemiluminescence units in the triplicate wells.

To determine the replicative advantage of mutant viruses as a function of HIV inhibitor concentration, the infectivity ratio of the mutants to the wild type (measured as relative β-galactosidase activity) was determined at each concentration of EFV, RPV, or RAL. The ratios were then interpolated as a continuous profile across the range of different drug concentrations tested using GraphPad Prism 5 (GraphPad Software, Ann Arbor, MI). Assays were performed in triplicate, and independent experiments were repeated twice.

Western blots. To determine the relative amounts of correctly processed RT and IN present in the mutant virion particles, viral stocks were harvested from infected MT-2 cell cultures and the virions were pelleted by centrifugation at $36,756 \times g$ in a Sorvall Biofuge for 1 h at 4°C. Virus pellets were resuspended and lysed by adding lysis buffer. Viral input was standardized by p24 antigen. Virus lysates were heated to 95°C for 5 min and loaded onto a 4 to 15% sodium dodecyl sulfate-polyacrylamide gel. Virion proteins were separated by electrophoresis and transferred by blotting onto a nitrocellulose membrane. Membranes were blocked using 5% milk in 50 mM Tris (pH 7.4), 0.15 M NaCl, and 0.1% Tween 20 (TBST) and incubated with a 1:200 dilution of mouse monoclonal antibodies

TABLE 1 Susceptibility to efavirenz, rilpivirine, and raltegravir for HIV-1 mutants carrying NNRTI and/or INSTI resistance mutations^a

Wild-type or mutant strain	EFV		RPV		RAL	
	IC ₅₀ ^b (nM)	Fold change ^c	IC ₅₀ (nM)	Fold change	IC ₅₀ (nM)	Fold change
Wild type	0.96 ± 0.20	—	0.10 ± 0.01	—	10.4 ± 1.7	—
RT-K103N	19.5 ± 6.81	20.2 ± 4.9	0.11 ± 0.03	1.1 ± 0.2	10.9 ± 2.4	1.0 ± 0.1
RT-E138K	1.12 ± 0.33	1.2 ± 0.2	0.22 ± 0.05	2.3 ± 0.3	11.8 ± 3.1	1.1 ± 0.5
RT-Y181C	3.91 ± 0.28	4.1 ± 0.6	0.23 ± 0.05	2.4 ± 0.4	10.8 ± 1.9	1.0 ± 0.3
IN-Q140S/Q148H	1.19 ± 0.26	1.2 ± 0.2	0.11 ± 0.02	1.2 ± 0.2	1,870 ± 247	185 ± 46
K103N+Q140S/Q148H	39.8 ± 5.6	42.4 ± 9.1*	0.11 ± 0.03	1.1 ± 0.2	1,992 ± 146	201 ± 50
E138K+Q140S/Q148H	4.23 ± 0.53	4.5 ± 1.1**	0.20 ± 0.06	2.1 ± 0.5	2,911 ± 191	284 ± 32***
Y181C+Q140S/Q148H	3.67 ± 0.62	3.8 ± 0.4	0.18 ± 0.05	1.8 ± 0.3	2,593 ± 99	258 ± 39****

^a EFV, efavirenz; RPV, rilpivirine; RAL, raltegravir. *, RT-K103N+IN-Q140S/Q148H versus RT-K103N ($P = 0.021$); **, RT-E138K+IN-Q140S/Q148H versus RT-E138K ($P = 0.006$); ***, RT-E138K+IN-Q140S/Q148H versus IN-Q140S/Q148H ($P = 0.012$); ****, RT-Y181C + IN-Q140S/Q148H versus IN-Q140S/Q148H ($P = 0.08$).

^b IC₅₀, 50% inhibitory concentration.

^c Fold change, fold change in IC₅₀ compared to results for the wild type. —, not applicable. Data shown are the means (± standard deviations) of three or four independent determinations. Mean fold change was determined by taking the mean of the fold change (mutant versus wild type) determined within each replicate assay.

against HIV-1-p24 and IN (purchased from Santa Cruz Biotechnology, Inc.). After being washed three times with TBST, the membranes were incubated with a 1:5,000 dilution of horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (Bio-Rad) and developed by using an ECL chemiluminescence kit (Thermo Scientific). The membranes were developed, washed three times with TBS, immersed in stripping buffer (Thermo Scientific), and incubated for 10 to 15 min at room temperature. After being washed three times in TBS, the membranes were blocked again for 1 h or overnight and incubated with a 1:2,000 dilution of rabbit polyclonal antibody against HIV-1 RT (obtained from Thermo Scientific). The membranes were washed three times with TBST, incubated with a 1:5,000 dilution of HRP-conjugated-goat anti-rabbit antibody, and developed using the ECL chemiluminescence kit as above. The relative amount of IN or RT normalized to the amount of p24 in the sample was determined by densitometry using ImageJ version 1.47v software (Wayne Rasband, National Institutes of Health, USA; <http://imagej.nih.gov/ij>).

Reverse transcriptase assay. Virion-associated RT activity of the wild-type and mutant virus stocks was assayed using a colorimetric RT assay (Roche Applied Science, Indianapolis, IN) as described previously (35). Briefly, the viruses harvested from infected MT-2 cells were pelleted by centrifugation at $36,756 \times g$ for 1 h at 4°C. Virus pellets containing 100 ng of p24 capsid protein were resuspended and lysed by adding 40 μl lysis buffer. Reactions were performed in triplicate by following the manufacturer's protocol; experiments were performed twice and the results averaged.

Statistical analysis. Results are presented as means ± standard deviations (SD). Mean IC₅₀s and fold changes in drug susceptibility were compared by two-tailed *t* test. Mean replication capacities, relative band densities on the Western blot, and virion-associated RT activity were compared by analysis of variance. Posttest comparisons (performed only if the *P* value was <0.05) were made with the two-sample comparison test. *P* values of <0.05 were considered significant. Because *P* values were not corrected for multiple comparisons, findings of statistical significance should be considered provisional.

RESULTS

Drug susceptibility. Table 1 shows the IC₅₀ for EFV, RPV, and RAL of viruses carrying NNRTI and/or INSTI resistance mutations. The RT-K103N and RT-Y181C mutations conferred 20-fold and 4-fold resistance to EFV, respectively; RT-E138K and IN-G140S/Q148H mutations had no effect on EFV susceptibility. The combination of IN-G140S/Q148H and RT-K103N or RT-E138K mutations significantly increased the level of EFV resistance compared to that of the single-mutant viruses, conferring 42-fold ($P = 0.021$) or 4.5-fold ($P = 0.006$) greater resistance to

EFV, respectively. In contrast, the addition of IN-G140S/Q148H to the RT-Y181C mutation had no significant effect on the IC₅₀ for EFV. The RT-E138K and RT-Y181C mutations conferred 2.3- and 2.4-fold greater resistance to RPV, respectively; RT-K103N and IN-G140S/Q148H mutations had no effect on RPV susceptibility. Adding the IN-G140S/Q148H mutations to the NNRTI resistance mutations had no significant effect on RPV susceptibility. The IN-G140S/Q148H mutations conferred 185-fold greater resistance to RAL; as expected, the NNRTI resistance mutations had no effect on RAL susceptibility. However, the combination of RT-E138K together with IN-G140S/Q148H mutations significantly increased the level of RAL resistance, resulting in 284-fold greater resistance ($P = 0.012$).

Replication capacity of NNRTI- and INSTI-resistant viruses.

The replication capacity assay showed that in the absence of drug, the RT-K103N, -E138K, and -Y181C mutant viruses had replication capacity (RC) values that were 65%, 62%, and 65% of that of the wild-type virus, respectively; the IN-G140S/Q148H mutant had an RC value that was 61% of that of the wild type. Viruses carrying NNRTI plus INSTI resistance mutations showed further decreases in RC (Fig. 1). In the presence of 8 nM EFV (Fig. 2A), the viruses carrying the combination of RT-K103N and IN-G140S/Q148H mutations had a significant replication advantage compared to those with the RT-K103N mutation alone ($P = 0.008$). Similarly, the RT-E138K plus IN-G140S/Q148H mutant virus was fitter than that with the RT-E138K mutation alone ($P = 0.003$). In contrast, the addition of IN-G140S/Q148H mutations to the RT-Y181C mutation did not increase RC compared to results with the RT-Y181C mutation ($P = 0.42$). In the presence of 0.08 nM RPV, the addition of the IN-G140S/Q148H mutations reduced the RC of viruses carrying the RT-K103N, RT-E138K, or RT-Y181C mutation (Fig. 2B). In the presence of 0.8 nM RAL, viruses carrying IN-G140S/Q148H plus RT-K103N mutations had RC values similar to those of the IN-G140S/Q148H mutant virus ($P = 0.59$). In contrast, viruses carrying the RT-E138K or Y181C mutations in addition to the IN-G140S/Q148H mutations had significantly greater RC values than those of the IN-G140S/Q148H mutant virus ($P = 0.004$ and $P = 0.002$, respectively) (Fig. 2C).

Fitness profiles of NNRTI- and INSTI-resistant viruses. The infectivity of NNRTI- and INSTI-resistant viruses was compared to that of the wild-type virus over a range of drug concentrations. Results of these experiments were consistent with those of the RC

FIG 1 Replication capacity (RC) in the absence of drug of HIV-1 recombinants carrying NNRTI resistance mutations RT-K103N, -E138K, or -Y181C and INSTI resistance mutations IN-G140S/Q148H alone or in combination. The y axis shows the relative RC for each mutant as a percentage of the wild-type value, which was set at 100%. The data shown are the means $\pm$ SD of results of two experiments.

assays. In the presence of EFV, addition of the IN-G140S/Q148H mutations improved the fitness profile of the RT-K103N and -E138K mutant viruses but not of the RT-Y181C mutant virus (Fig. 3A to C). In contrast, addition of the IN-G140S/Q148H mutations reduced the replicative fitness of the NNRTI-resistant mutant viruses in the presence of RPV (Fig. 3D to F). Addition of the RT-E138K or -Y181C mutation enhanced the replication fitness of the IN-G140S/Q148H mutant virus in the presence of RAL, but the RT-K103N mutation had no effect (Fig. 3G to I).

Western blot analysis. To determine whether the NNRTI and/or INSTI resistance mutations altered the amounts of correctly processed RT and IN proteins present in virion particles, viral lysates were analyzed by Western blotting (Fig. 4). The relative amounts of each protein were normalized to the amount of p24 in each sample compared to that in the wild type. The amount of RT protein was reduced in the RT-E138K and RT-Y181C mu-

tant viruses, but increased in the RT-K103N mutant virus, compared to that in the wild type (Fig. 4A to C). The amount of IN was reduced in each of the RT mutant viruses, most notably in the RT-Y181C mutant virus (Fig. 4A and D). In contrast, the amount of IN and RT protein present in the IN-G140S/Q148H mutant virus was increased compared to that in the wild type. The combined presence of the RT-K103N and IN-G140S/Q148H mutations resulted in a reduction in the amount of RT and IN compared to that for the RT-K103N single-mutant virus; the amount of IN also was reduced compared to that for the IN-G140S/Q148H mutant virus. The virus with RT-E138K plus IN-G140S/Q148H mutations had reduced amounts of IN and RT compared to those for the IN-G140S/Q148H mutant virus but similar amounts of these proteins compared to those for the RT-E138K single-mutant virus. Similarly, the RT-Y181C plus IN-G140S/Q148H mutant virus had smaller amounts of IN and RT than those in the IN-G140S/Q148H mutant virus; the amount of IN was also lower than that in the RT-Y181C mutant, but the amount of RT was not reduced further compared to that in the RT-Y181C mutant.

Virion-associated RT polymerase activity of NNRTI- and INSTI-resistant viruses. Fig. 5 shows the relative virion-associated RT activity of the wild-type and mutant viruses. Viruses carrying the single NNRTI resistance mutations or the IN-G140S/Q148H INSTI resistance mutations each reduced RT activity relative to that of the wild type, with the exception of the RT-K103N mutant virus, which had increased virion-associated RT activity. Whereas introduction of the G140S/Q148H mutation into the E138K or Y181C mutant virus resulted in comparable RT activity ($P = 0.65$ and $P = 0.49$, respectively), introduction of these IN mutations into the RT-K103N mutant virus resulted in significantly reduced RT activity compared to that of the RT-K103N single-mutant virus ($P < 0.001$).

DISCUSSION

We determined the drug susceptibility, replication capacity, fitness profiles, virion-associated RT and IN protein levels, and virion-associated RT activity of recombinant HIV-1 carrying mutations that confer resistance to NNRTI and INSTI. When present alone, NNRTI resistance mutations (RT-K103N, -E138K, and -Y181C) and INSTI resistance mutations (IN-Y143C, -N155H,

FIG 2 Replication capacity of HIV recombinants carrying NNRTI (RT-K103N, -E138K, and -Y181C) and INSTI (IN-G140S/Q148H) resistance mutations in the presence of 8 nM EFV (A), 0.08 nM RPV (B), or 0.8 nM RAL (C). The y axis shows the relative RC for each mutant as a percentage of the wild-type value, which was set at 100%. The data shown are the means $\pm$ SD of results of two experiments.

FIG 3 Fitness profiles of HIV-recombinants carrying NNRTI (RT-K103N, -E138K, and -Y181C) and INSTI (IN-G140S/Q148H) resistance mutations. The infectivity ratio of the mutants to the wild type (measured as relative β -galactosidase activity) was determined in the presence of 0.39 to 100 nM etravirine (ETV; panels A to C), 0.0039 to 1 nM rilpivirine (RPV; panels D to F), or 0.039 to 10 nM raltegravir (RAL; panels G to I).

and -G140S/Q148H) led to viral RCs that were reduced compared to that of the wild type. The combined presence of both NNRTI and INSTI resistance mutations resulted in further decreases in RC in the absence of drug. This observation confirms and extends the findings of a preliminary report that noted interactions between NNRTI and INSTI resistance mutations with respect to viral RC (33).

Some of the effect of NNRTI and INSTI resistance mutations on RC may have been mediated by reductions in the amount of RT and IN incorporated into virions. For example, the RT-Y181C/IN-G140S/Q148H triple-mutant virus had the lowest RC compared to that of the WT and also had reduced quantities of both RT and IN as determined by Western blotting. This observation suggests that the combined presence of these mutations results in reduced packaging of these enzymes in the virion or reduced stability of the mutant polypeptide precursor. Although RT and IN are produced from the same polypeptide precursor, differential levels of stability of the mature proteins could account for differ-

ences in the relative virion contents of these two proteins. However, the RT-K103N, RT-E138K, and RT-Y181C single mutations each resulted in the same reduced RC (~60% of the wild-type value), even though the RT-K103N mutant virus had an increased amount of virion-associated RT protein and a slightly reduced amount of IN protein compared to that of the wild type, whereas the RT-E138K and RT-Y181C mutant viruses had reduced quantities of virion-associated RT and IN. Thus, differences in enzyme stability and/or packaging cannot fully account for the observed differences in RC.

An alternative explanation for the fitness differences we observed is the effect on virion-associated RT activity of the different mutant viruses. We found that viruses carrying the RT-E138K or -Y181C mutation or the IN-G140S/Q148H mutations each had reduced RT activity relative to that of the WT. Introduction of the IN-G140S/Q148H mutations into the RT-K103N mutant virus significantly reduced RT activity and viral fitness. In contrast, introduction of the G140S/Q148H mutation into the E138K or

FIG 4 (A) Western blot analysis of HIV-recombinants carrying NNRTI (RT-K103N, -E138K, and -Y181C) and INSTI (IN-G140S/Q148H) resistance mutations with anti-RT, anti-IN, and anti-p24 antibodies. Virus-containing supernatants were harvested from infected MT-2 cells. The virions were pelleted by ultracentrifugation, and viral input was standardized by p24 antigen concentration. Virion proteins were separated by electrophoresis on 4 to 15% sodium dodecyl sulfate-polyacrylamide gradient gels and transferred to nitrocellulose membranes. Blots were developed with antibodies against Gag (p24), integrase (p32), and reverse transcriptase (p51 and p66). Arrows indicate the positions of each protein. The relative amounts of reverse transcriptase p66 (B) or p51 (C) or integrase (D) were determined by densitometry and normalized to the amount of p24 in each sample compared to that in the wild type. Data shown represent the means $\pm$ SD of three or more densitometry tracings. *, $P < 0.05$ for the RT mutant virus versus the WT; **, $P < 0.001$ for the RT mutant virus versus the WT; ⊗, $P < 0.05$ for the IN-G140S/Q148H mutant virus versus the WT; ⊗⊗, $P = 0.071$ for the IN-G140S/Q148H mutant virus versus the WT; ψ, $P < 0.05$ for the RT mutant virus with the IN-G140S/Q148H mutations versus the IN-G140S/Q148H mutant virus; ψψ, $P < 0.001$ for the RT mutant virus plus the IN-G140S/Q148H mutations versus the IN-G140S/Q148H mutant virus; ♣, $P < 0.05$ for the RT-K103N plus IN-G140S/Q148H mutant virus versus the RT-K103N single-mutant virus; ♣♣, $P < 0.001$ for the RT-K103N plus IN-G140S/Q148H mutant virus versus the RT-K103N single-mutant virus.

Y181C mutant virus had no significant effect on RT activity. These results demonstrate that INSTI resistance mutations have a differential effect on RT function. A previous study showed that an IN-H12N mutation and deletion of the IN C-terminal domain reduce levels of virion-associated RT and IN, but that study did not examine the effect of INSTI resistance mutations (36). Previous studies have shown that NNRTI mutations can affect virion RT content and RNase H activity (37) and that mutations in the connection and RNase H domains can affect susceptibility to nucleoside and nonnucleoside RT inhibitors (38–41). It will be interesting to know how the combinations of NNRTI and INSTI resistance mutations affect the RNase H activity.

In several cases, the combination of NNRTI and INSTI resistance mutations led to an unexpected increase in RC and relative

fitness in the presence of drug. This interaction was observed in the presence of EFV when the IN-G140S/Q148H mutations were added to a virus with the RT-K103N or -E138K mutations and in the presence of RAL when the RT-E138K or -Y181C mutations were added to a virus with the IN-G140S/Q148H mutations (Fig. 2A and B). The greater RC and replicative fitness of the combination mutant viruses in the presence of EFV could be explained by the significantly greater fold change in IC_{50} for EFV when the INSTI resistance mutations were present together with the RT-K103N or -E138K mutation, even though the level of virion-associated RT activity was lower (Table 1 and Fig. 5). Similarly, the greater RC and relative fitness of the combination mutants in the presence of RAL could be explained by the greater fold change in IC_{50} for RAL when the RT-E138K mutation was present together

FIG 5 Virion-associated RT activity. The y axis shows the relative RT activity for each mutant virus as a percentage of wild-type activity, which was set at 100%. The data shown are the means $\pm$ SD of results of two independent experiments.

with the IN-G140S/Q148H mutations (Table 1). In the case of the RT-K103N plus IN-G140S/Q148H mutant virus, the amount of virion-associated RT and IN protein was lower than in the RT-K103N single-mutant virus. In contrast, in the case of the RT-138K plus IN-G140S/Q148H mutant virus, the amount of RT p66 and IN protein was the same, although RT p51 was slightly decreased compared to that in the RT-E138K mutant virus. Differences in the amounts of these two proteins, therefore, do not seem sufficient to explain the observed phenotypes. The structural and biochemical bases for the increased level of EFV and RAL resistance in the combined presence of these mutations remain to be determined.

The combination of NNRTI and INSTI resistance mutations had the opposite effect on viral RC and relative fitness in the presence of RPV—susceptible viruses carrying the RT-K103N, -E138K, or -Y181C mutations plus IN-G140S/Q148H each had lower RC and relative fitness than viruses carrying NNRTI resistance mutations alone. In contrast to the effect on EFV susceptibility, the IN-G140S/Q148H had no effect on RPV susceptibility when present together with NNRTI resistance mutations (Table 1). Thus, the differences in RC and relative fitness observed for the combination mutants cannot be explained on the basis of altered RPV susceptibility.

One limitation of our study is that we examined the interactions among the NNRTI and INSTI mutations only in an HIV-1_{NL4-3} backbone. In this regard, it is noteworthy that the reduction in RC associated with the K103N mutation was unexpected, given the long persistence of mutation in the absence of ongoing NNRTI therapy (42). Different results might be achieved using other viral backbones. In addition, we were not able to assess the effects of the resistance mutations on virion-associated integrase activity. It is possible that variation in regions of the viral genome encoding other viral proteins could also have important effects on NNRTI and INSTI susceptibility and viral fitness, as observed for resistance to HIV-1 protease inhibitors (43, 44). Finally, the clinical significance of the differences we observed *in vitro* remains to be confirmed in clinical studies.

In summary, our results suggest that mutational interactions between RT and IN are important for NNRTI and INSTI resistance and viral fitness. Extensive crystallographic, enzymological,

and molecular modeling studies have contributed much toward understanding resistance to INSTI (45–48) and NNRTI (49–53). It will be interesting to know the biochemical and structural mechanisms that underlie the interactions between the NNRTI and INSTI resistance mutations we have observed.

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