

The Level of Reverse Transcriptase (RT) in Human Immunodeficiency Virus Type 1 Particles Affects Susceptibility to Nonnucleoside RT Inhibitors but Not to Lamivudine

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We investigated the relationship between the level of reverse transcriptase (RT) in human immunodeficiency virus type 1 (HIV-1) particles and susceptibility to nonnucleoside reverse transcriptase inhibitors (NNRTIs). HIV-1 virions containing different active levels of RT were generated. Susceptibility to the NNRTIs efavirenz and nevirapine was inversely proportional to the level of enzymatically active RT. However, the sensitivity of HIV-1 to the nucleoside analog 3TC was not affected by the level of RT per particle. These data indicate that the susceptibility of HIV-1 to NNRTIs is influenced by RT activity.

The development of resistance by human immunodeficiency virus type 1 (HIV-1) to antiretroviral drugs poses a major problem in the treatment of HIV-infected individuals. Mutations conferring antiviral resistance arise in response to all known classes of anti-HIV-1 drugs. There are two classes of reverse transcriptase (RT) inhibitors: nucleoside analogs (nucleoside RT inhibitors; NRTIs) and nonnucleoside RT inhibitors (NNRTIs). NRTIs are incorporated into viral DNA during reverse transcription and terminate the synthesis of the viral DNA chain, whereas NNRTIs bind directly to RT near the polymerase active site, blocking the chemical step of DNA synthesis and preventing RT from copying the viral RNA genome into DNA.

Previously, we characterized a chimeric HIV-1/simian immunodeficiency virus called RT-SHIV_{mne} (1). HIV-1 RT was incorporated into particles with reduced efficiency, decreasing the replicative capacity of the virus. The NNRTI concentration required to inhibit viral replication by 50% was approximately twofold lower for RT-SHIV_{mne} than HIV-1. By contrast, the 50% inhibitory concentration values for NRTIs did not differ significantly between HIV-1, RT-SHIV_{mne}, and the mne strain of simian immunodeficiency virus. These observations prompted us to ask whether the amount of RT activity in a virion would affect the susceptibility of the virus to NNRTIs but not to NRTIs.

We created phenotypically mixed HIV-1 that had differing ratios of (i) wild-type (WT) RT and (ii) RT containing two mutations, D110E and Y181I. The D110E mutation renders the polymerase inactive, and the Y181I mutation makes it resistant to most NNRTIs by hindering their physical interaction. Phenotypically mixed virions pseudotyped with vesicular stomatitis virus G envelope were generated by transfecting 293T cells with differing amounts of two replication-defective HIV vectors: one encoding WT RT (pNLN_{go}MIVR⁻E⁻.HSA) and the other encoding RT with the D110E/Y181I mutations as previously described (9). Consistent with the published

study, the specific infectivity of HIV-1, which was determined from infecting GHOST-Hi5 indicator cells (4) and correcting for the amount of capsid (p24 enzyme-linked immunosorbent assay; Beckman Coulter, Miami, FL), decreased with increasing amounts of defective RT (Fig. 1). The D110E/Y181I RT mutant allowed a stoichiometric incorporation of viral protease and integrase in the phenotypically mixed particles but introduced a nonfunctional RT that should not compete for NNRTI binding. Thus, the effects of the level of active RT on inhibitor sensitivity can be evaluated.

Susceptibility of these viruses to the NNRTIs efavirenz (EFV) and nevirapine (NVP; AIDS Reference and Reagent Program, Rockville, MD) and the NRTIs zidovudine (AZT; Sigma, St. Louis, MO) and lamivudine (3TC; Moravex, Brea, CA) was analyzed in an *in vitro* replication assay using JC53 BL13+ cells as previously described (1). Infection of the phenotypically mixed HIV-1 particles was inhibited by EFV and NVP (Fig. 2). Viruses with 50% or 25% WT RT were inhibited to a greater extent by NNRTIs than was the virus containing 100% WT RT. This result was not affected by changing the multiplicity of infection (data not shown).

NNRTIs reduce the number of enzymatically active RT molecules in a virion core. Once the number of active RTs is reduced below a critical threshold, there is not enough polymerase activity to produce a complete copy of the viral DNA. If the viral core is already deficient in RT molecules (or RT activity), it is easier to reduce the polymerase activity below this threshold. In contrast, the ultimate target of NRTIs is viral DNA. Therefore, reducing the amount of RT activity should not affect NRTI sensitivity.

As predicted, infection of the phenotypically mixed viruses was inhibited to the same extent by 3TC as was the virus with 100% WT RT (Fig. 3). However, AZT inhibited the virus containing only 25% WT RT to a slightly greater extent than did the viruses with 100% or 50% WT RT. This sensitivity could be due to the fact that WT HIV-1 RT can excise AZT from viral DNA to a limited extent (2, 3, 12). The smaller amount of active RT could reduce excision in virions containing only 25% WT RT relative to virions that contained 50% or 100% WT RT. Consistent with our findings, the reduction of

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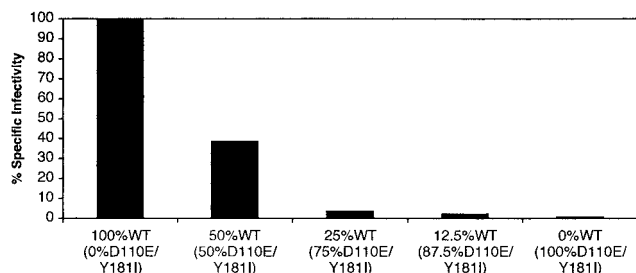


FIG. 1. The specific infectivity of HIV-1 correlates with the amount of polymerase-active RT per virion. Phenotypically mixed viruses were made by cotransfecting different ratios of plasmids encoding a WT HIV-1 vector and an HIV-1 vector containing the D110E and Y181I mutations in RT. Specific infectivity was measured as IU/p24, and HIV-1 containing 100% WT RT was normalized to 100%.

active RT levels in protease inhibitor-resistant HIV-1 has also been reported to increase AZT sensitivity (5). In contrast, inhibition by 3TC, which is not efficiently excised by WT HIV-1 RT (7, 15), is not affected by the overall level of polymerase activity in virions.

Based on our hypothesis, we would expect that increasing the amount of RT in virions would decrease susceptibility to NNRTIs. We employed a Vpr-RT fusion construct that can incorporate RT into virions in *trans* (19). 293T cells were transfected with pNLN_goMIVR^{-E}.HSA and the Vpr-RT ex-

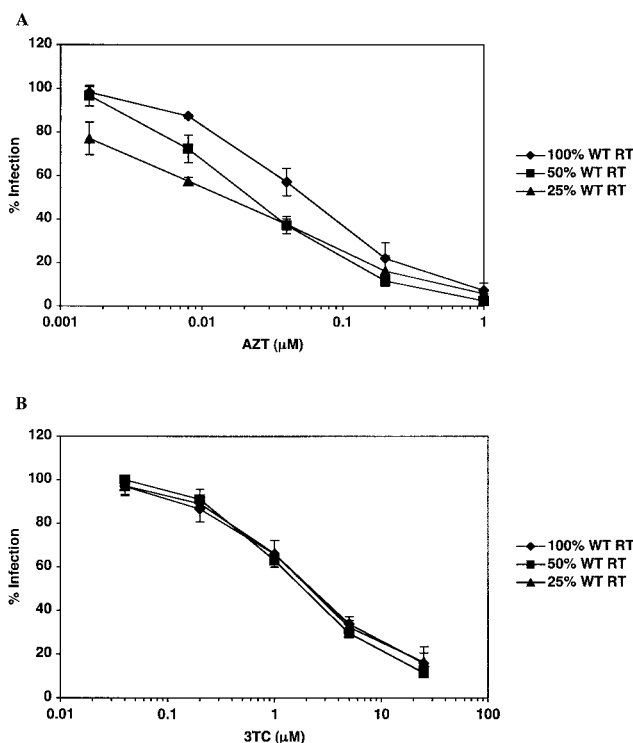


FIG. 3. Inhibition of HIV-1 by 3TC is not affected by the RT activity in particles. Phenotypically mixed viruses (100%, 50%, or 25% WT RT) were inhibited in a single round of infection assay at similar multiplicities of infection by AZT (A) or 3TC (B). Error bars represent the standard deviations of three or two independent experiments, respectively. Infection level was normalized to 100% for virus in the absence of drug.

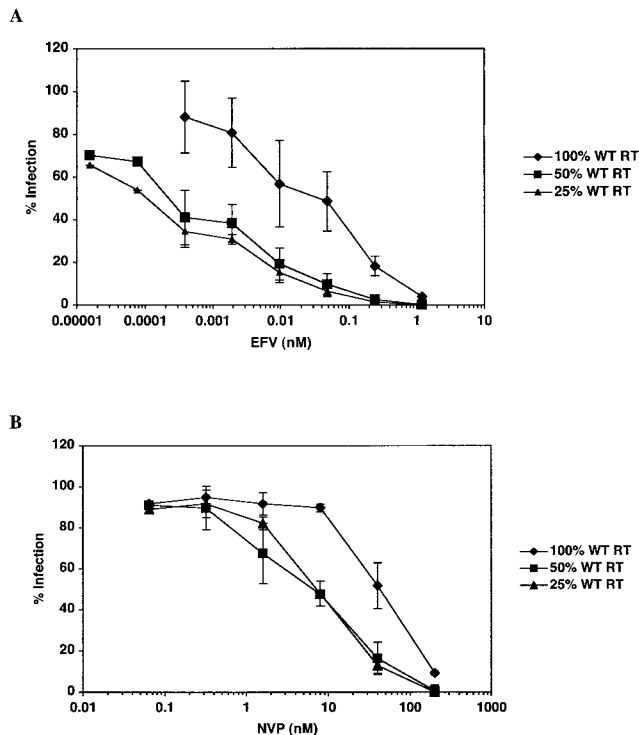


FIG. 2. Sensitivity to NNRTIs depends on the RT activity in particles. Phenotypically mixed viruses (100%, 50%, or 25% WT RT) were inhibited in a single round of infection at similar multiplicities of infection by EFV (A) or NVP (B). Error bars represent the standard deviations of three or two independent experiments, respectively. Infection level was normalized to 100% for virus in the absence of drug.

pression construct to create virions that contain higher amounts of RT. To control for the effects of the Vpr-RT protein, virus was also made by transfection of the HIV vector with the Vpr-RT plasmid encoding the D110E/Y181I double mutation (Vpr-RT DM), which should have WT levels of normal RT and excess RT that is polymerase inactive and resistant to NNRTIs.

The three virus preparations (WT HIV, HIV with Vpr-RT, and HIV with Vpr-RT DM) were purified as previously described (1). To assay Vpr-RT incorporation, viruses were pelleted through a sucrose cushion prior to analysis by immunoblotting. The blots were probed with an anti-p24 polyclonal goat antibody (courtesy of J. Mirro), followed by anti-goat immunoglobulin G-horseradish peroxidase (Calbiochem, San Diego, CA), or a set of murine anti-RT monoclonal antibodies (courtesy of M. Parniak), followed by anti-mouse immunoglobulin G-horseradish peroxidase (Amersham Pharmacia, Piscataway, NJ) and development with ECL (Amersham Pharmacia). The anti-Gag immunoblot indicated that the virus preparations contained similar amounts of p55 and p24 Gag proteins and that there was slightly more virus in the Vpr-RT virion preparation (Fig. 4A). The immunoblot probed with anti-RT antibodies showed that the HIV lysates contained p66 and p51 subunits. The virions made in cells expressing Vpr-RT or Vpr-RT DM also contained uncleaved Vpr-p66 (Fig. 4B).

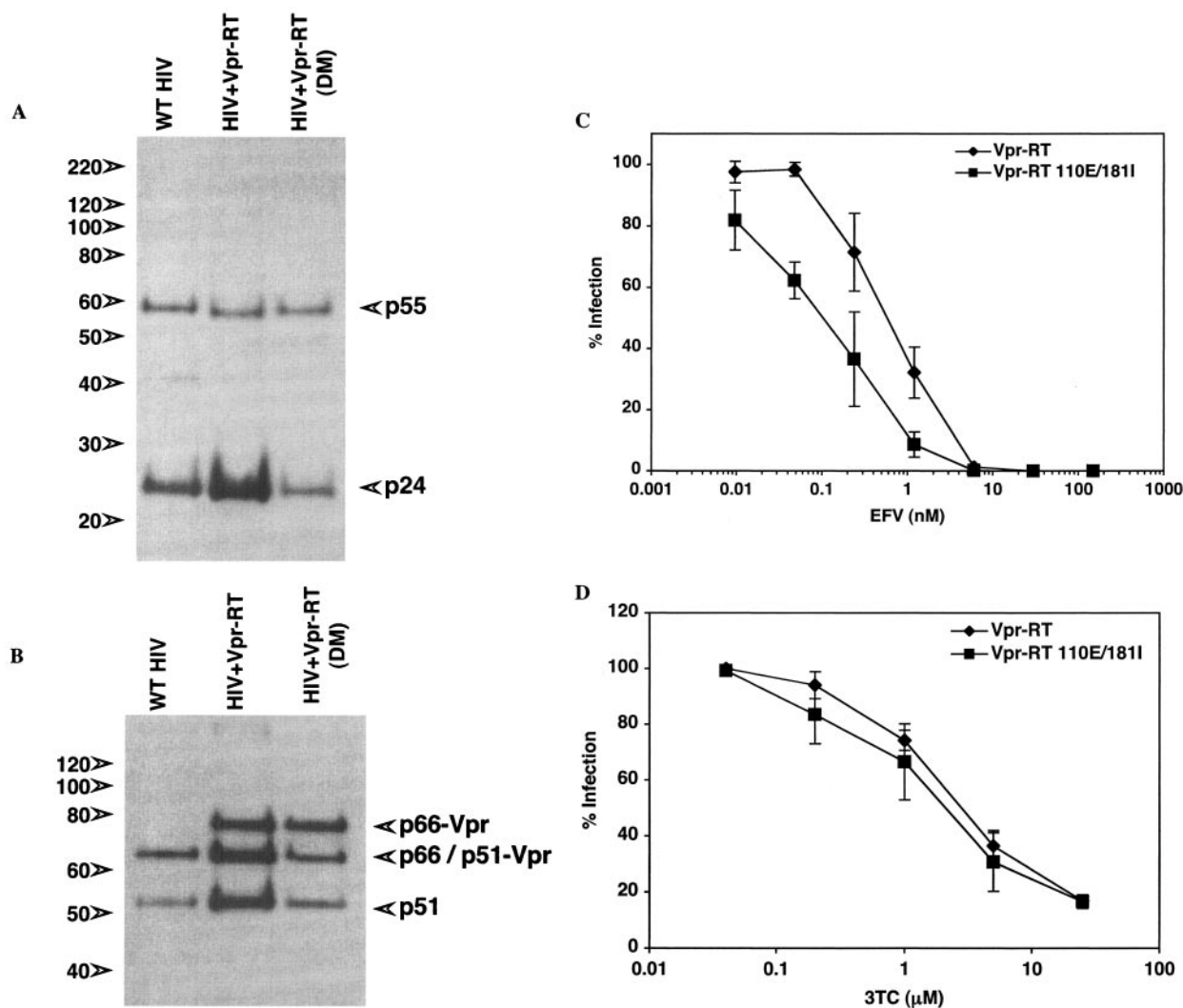


FIG. 4. Extra WT RT delivered to particles via Vpr-RT changes the susceptibility of HIV-1 to EFV but not 3TC. Immunoblotting was performed on purified WT HIV-1, HIV with Vpr-RT, or HIV with Vpr-RT DM lysates, and blots were probed with an anti-Gag antibody (A) or anti-RT antibodies (B). HIV containing Vpr-RT or Vpr-RT DM was inhibited in a single round of infection at similar multiplicities of infection by EFV (C) or 3TC (D). Error bars represent the standard deviations of three independent experiments. Infection level was normalized to 100% for virus in the absence of drug.

HIV containing Vpr-RT was less susceptible to EFV than was HIV with Vpr-RT DM (Fig. 4C). The difference between the inhibition curves of the two viruses was modest, possibly due to inefficient cleavage of p66 from Vpr and/or to constraints on the interaction of Vpr-RT with the NNRTI. As expected, both viruses had similar sensitivities to 3TC (Fig. 4D).

Based on these results, one might expect that NNRTI-containing therapies would select HIV-1 with increased levels of RT per virion. However, physical constraints make such a mechanism of resistance unlikely because virions have a limited capacity to incorporate Gag-Pol. Studies in which *pol* products were significantly overexpressed in *trans* or by mutation of the *gag-pol* frameshift site showed that the incorporation of large amounts of Gag-Pol led to the formation of aberrant and noninfectious particles (11, 13, 14).

An alternative mechanism for NNRTI resistance would be an increase in RT activity, allowing the virus to replicate efficiently with fewer active RT molecules. Increased enzymatic

activity is biochemically equivalent to increased levels of a less active enzyme. Strikingly, HIV-1 isolates have been obtained from NNRTI-naïve patients with variations in RT activity per RNA or infectious unit, suggesting that natural variation in RT activity per virion is common (6, 10). It will be interesting to determine if NNRTIs can select for an increase in RT activity in HIV-1 isolated from NNRTI-experienced individuals.

Conversely, NNRTIs may be more efficacious against HIV-1 with protease inhibitor- or NRTI-associated resistance mutations that reduce the level of RT activity in virions; specific NRTI resistance mutations are associated with hypersensitivity to NNRTIs (5, 8, 16–18). Our findings suggest that NNRTI hypersensitivity is related to a reduction in RT activity.

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