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Analysis of Nonnucleoside Drug-Resistant Variants of Human Immunodeficiency Virus Type 1 Reverse Transcriptase

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A number of chemically distinct nonnucleoside inhibitors of human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) have been reported. Several lines of evidence, including the isolation of RT mutants that show cross resistance, suggest that, despite their structural diversity, many of these inhibitors bind to a common site on HIV-1 RT. We have recently reported that, on the basis of analyses of HIV-1/HIV-2 chimeras, the natural product calanolide A may interact with a different site or sites in HIV-1 RT. We have used *Bsp*MI cassette mutagenesis to prepare a collection of HIV-1 RT mutants that show resistance to the known members of the general class of nonnucleoside inhibitors. This collection of mutants can be used to determine whether a new drug will show cross resistance with known inhibitors and to define amino acid positions critical for the action of the drugs. The mutants were used to analyze calanolide A, 1*H*,3*H*-thiazolo[3,4-a]benzimidazole(4*i*), and the acyclic nucleoside analog 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine. These analyses suggest that all three drugs interact with HIV-1 RT within the previously defined common binding site for nonnucleoside inhibitors. However, the drugs respond differently to the panel of drug-resistant HIV-1 RTs, indicating that while the binding sites of the drugs overlap they are not identical.

The reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) has been the target of both nucleoside analogs, such as zidovudine and dideoxyinosine, and nonnucleoside drugs, including nevirapine and the various tetrahydroimidazo[4,5,1-*jk*][1,4]benzodiazepin-2(1*H*)-one and -thione (TIBO) derivatives. However, drug-resistant HIV-1 RT variants have been isolated from clinical materials and from samples of virus in vitro (8, 10–12, 14, 21–23, 26, 28, 32–34, 37). Nonnucleoside drugs are highly specific inhibitors of HIV-1 RT and are not active against the RT of the closely related lentivirus HIV-2 or other retroviral RTs (6, 7, 9, 11, 13, 18, 20, 24, 27, 29, 35). This suggests that HIV-1 RT contains specific structural elements which the other RTs do not possess that interact with nonnucleoside inhibitors. The recently published structure of HIV-1 RT complexed with nevirapine shows that this drug binds to a hydrophobic pocket just underneath the polymerase active site and appears to be interacting directly with the tyrosine residues at positions 181 and 188 (19). Since several other nonnucleoside inhibitors have been reported to show cross resistance with nevirapine, these other nonnucleoside inhibitors may bind to this site as well. Unfortunately, HIV-1 RT is able to accommodate substantial changes in the amino acids that surround this critical binding pocket so that drug-resistant mutants are easily obtained. This makes it especially important to identify drugs that bind to parts of the enzyme that the virus is not able to change so easily. We have used *Bsp*MI cassette mutagenesis (3, 4) to construct various HIV-1 RT mutants that have been reported to play a role in resistance to several nonnucleoside drugs. Since the mutant enzymes differ only at the designated sites of mutation, differences in resistance to the drugs can be attributed directly to the specific mutations. This collection of mutants can be used to test new nonnucleoside inhibitors to determine whether they bind to the same hydrophobic pocket at which the known drugs appear to bind and to investigate which amino acids are involved in binding a particular drug.

In the present study, these variant HIV-1 RTs have been assayed for sensitivity to two nonnucleoside drugs, calanolide A (18) and 1*H*,3*H*-thiazolo[3,4-a]benzimidazole(4*i*) (TBA) (6, 7), and to the acyclic nucleoside analog 1-[(2-hydroxyethoxy)methyl]-6-(phenylthio)thymine (HEPT) (1, 2, 10, 25). Calanolide A (Fig. 1A) is a coumarin derivative isolated from extracts of the fruit and twigs of the tree *Calophyllum lanigerum* (18). This compound inhibits HIV-1 replication and cytopathicity by noncompetitive inhibition of HIV-1 RT and is inactive against HIV-2 RT (18). Calanolide A is fully active against HIV-1 strain A17 (26), which reportedly is resistant to many known nonnucleoside drugs, such as TIBO and BI-RG-587 (nevirapine). Analysis of the resistance and sensitivity of a series of enzymatically active HIV-1/HIV-2 RT chimeras suggested that calanolide A does not interact with the same segments of HIV-1 RT as the TIBO compounds (16). This suggests that calanolide A belongs to a pharmacologic class different from that of the other known nonnucleoside RT-inhibitory drugs (18). TBA (Fig. 1B) is a synthetic compound which also has been reported to inhibit HIV-1 RT noncompetitively but not to inhibit HIV-2 RT (6, 7). The A17 strain (26), however, is resistant to this compound, which suggests that TBA may fall into the known class of nonnucleoside inhibitors (5). HEPT (1, 2, 10, 25) (Fig. 1C) is an acyclic nucleoside analog, but, unlike other nucleoside analogs such as dideoxyinosine, HEPT does not appear to inhibit HIV-1 RT by acting as a competitive inhibitor or a chain terminator when poly (rC) · oligo(dG) is used as the substrate (1, 2, 10). HEPT appears to be mechanistically similar to the class of noncompetitive inhibitors which includes nevirapine and TIBO (10). Like the nonnucleoside inhibitors, HEPT is inactive against

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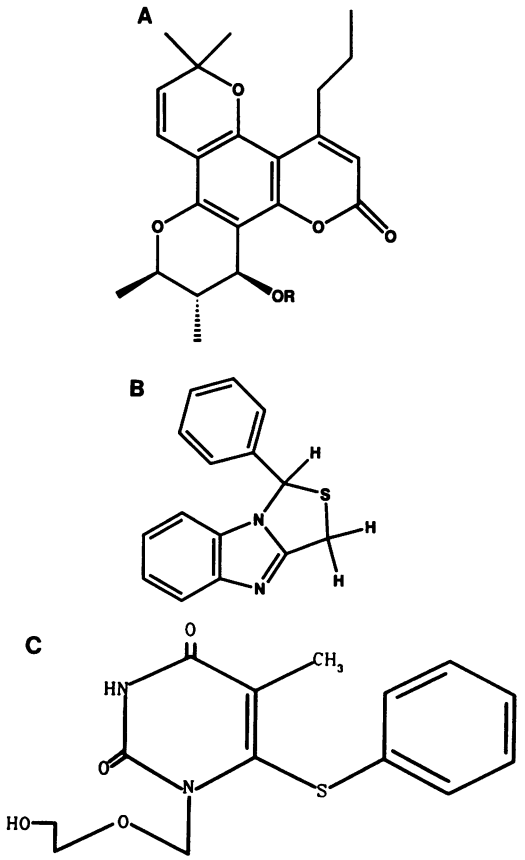


FIG. 1. Structures of the nonnucleoside drugs. (A) Calanolide A (15). (B) TBA (4, 5). (C) HEPT (2, 8, 23).

HIV-2 and other retroviral RTs. Most of the other nucleoside analogs, such as zidovudine and 2′,3′-dideoxycytidine, are equally effective against HIV-1 or HIV-2 RT (2). The results obtained in this study indicate that although the binding sites of the three drugs overlap with each other and with those of other known nonnucleoside inhibitors, the drugs differ in precisely how they interact with the enzyme.

Results and discussion. We have constructed eight single amino acid substitutions and four multiple amino acid substitutions within the polymerase domain of HIV-1 RT (Table 1). The amino acid substitutions correspond to mutations that have been reported to play a role in resistance to nonnucleoside inhibitors (8, 10, 11, 14, 23, 26, 28, 30, 32, 35, 37). The substitutions were generated with *Bsp*MI cassettes (3, 4), which are derivatives of the plasmid HIV-1 RT. The parental plasmid HIV-1 RT induces expression of the 66-kDa form of HIV-1 RT in *Escherichia coli* (15, 17). Once a clone was constructed, the presence of the mutation was verified by dideoxynucleotide sequencing. Appropriate expression of each of the variant HIV-1 RTs was determined by fractionation of the *E. coli* extracts on sodium dodecyl sulfate (SDS)-polyacrylamide gels. All of the variant HIV-1 RTs and the normal HIV-1 RT expressed equivalent amounts of RT. The variant HIV-1 RTs were assayed for RNA-dependent DNA polymerase activity with bacterial extracts and the template-primer poly(rC) · oligo(dG) as previously described (3). All of the variant HIV-1 RTs, except Leu-100→Ile and Ile-135→Thr, have approximately the same amount of polymerase activity as wild-type HIV-1

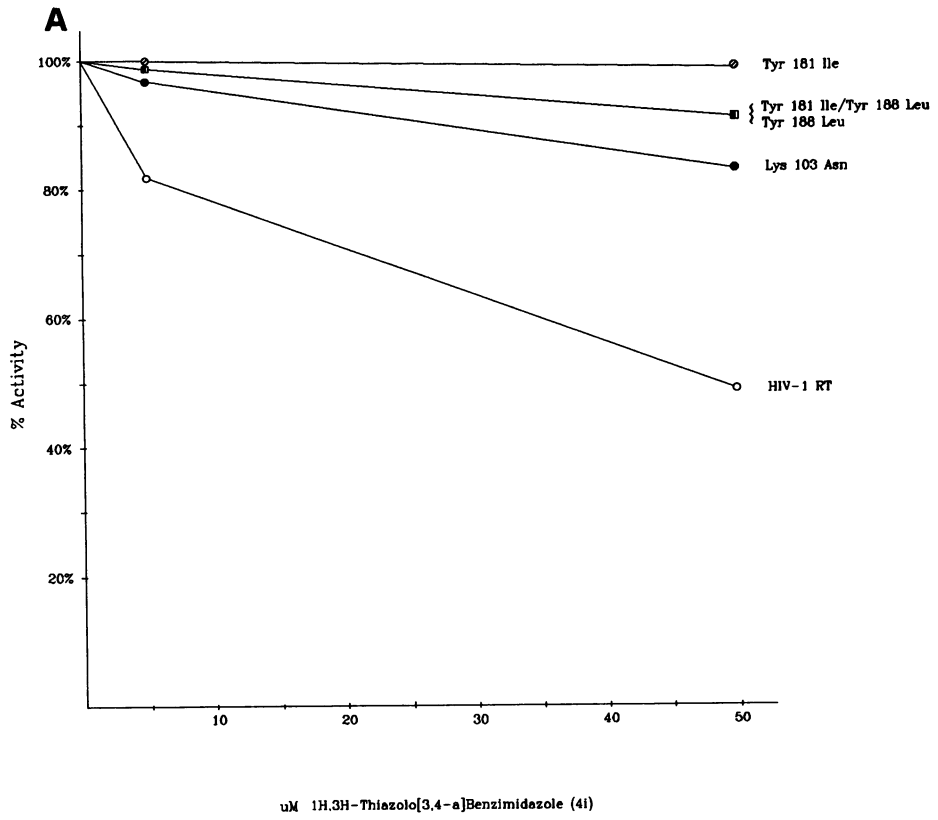
TABLE 1. RNA-dependent DNA polymerase activities of the variant HIV-1 RTs[a]

Mutation	% Polymerase activity
None	100
Leu-100→Ile	40
Lys-103→Asn	110
Lys-103→Asn/Tyr-181→Ile	100
Lys-103→Asn/Tyr-188→Leu	100
Lys-103→Asn/Tyr-181→Ile/Tyr-188→Leu	100
Val-108→Ile	100
Ile-135→Thr	20
Val-179→Asp	100
Tyr-181→Ile	110
Tyr-181→Ile/Tyr-188→Leu	120
Tyr-188→Leu	110
His-221→Tyr	130

[a] The assays were performed as described previously (3) by with poly(rC) · oligo(dG) as the template-primer. Activity of the wild-type HIV-1 RT was set at 100%, and the activities of the mutant RTs were normalized to the wild-type activity.

RT. These two mutants have approximately 40 and 20% the wild-type level of polymerase activity, respectively. All of the assays were done on three separate occasions, with independently prepared extracts. All three assays gave similar results.

This panel of drug-resistant mutants was constructed to simplify the testing of new nonnucleoside inhibitors of HIV-1 RT. We have tested two nonnucleoside inhibitors, calanolide A (18) and TBA (6, 7), and an acyclic nucleoside analog, HEPT (1, 2, 10, 25), against the various mutant forms of HIV-1 RT. A number of research groups have shown that the tyrosine residues at positions 181 and 188 are critically involved in sensitivity to many nonnucleoside drugs, including the pyridinones (26, 30, 37), nevirapine (23, 28, 35), R82150 (11, 30), and 5-ethyl-1-ethoxymethyl-6-(phenylthio)uracil, which is related to HEPT (11). Substitution of the lysine residue at position 103 with an asparagine has been shown to confer partial resistance to pyridinone-class drugs and has a large effect on drug resistance when combined with substitutions at position 181 (26, 30). The drugs were initially tested against variant HIV-1 RTs with single amino acid substitutions at Lys-103, Tyr-181, and Tyr-188 and with a variant that has amino acid substitutions at both Tyr-181 and Tyr-188. The tyrosine at 181 was changed to an isoleucine residue and leucine was substituted for the tyrosine at position 188, since these amino acids are found at the equivalent positions in HIV-2 RT. The lysine at position 103 was changed to an asparagine residue, which is the same substitution that was reported by Nunberg et al. (26). As shown in Fig. 2A, mutant forms of HIV-1 RT with each of these amino acid substitutions were all resistant or partially resistant to TBA. A mutant HIV-1 RT with the amino acid substitution Tyr-181→Ile was completely resistant to the drug up to a concentration of 50 μM, indicating that Tyr-181 is important in the sensitivity of HIV-1 RT to TBA. Interestingly, the HIV-1 RT with the double amino acid substitution Tyr-181→Ile/Tyr-188→Leu was slightly less resistant to TBA than was the mutant with the single substitution Tyr-181→Ile. We have no simple explanation for this observation. The panel of HIV-1 RT variants showed a different pattern of resistance when tested with calanolide A (Fig. 2B). Although HIV-1 RT mutants with the amino acid substitutions Tyr-181→Ile and Lys-103→Asn were partially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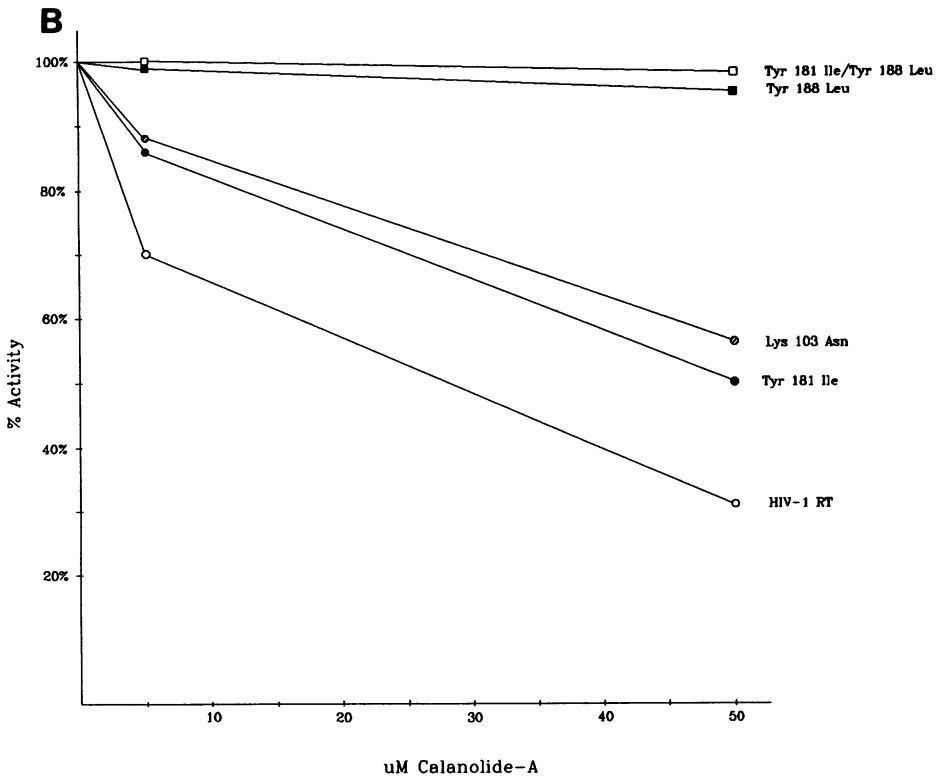
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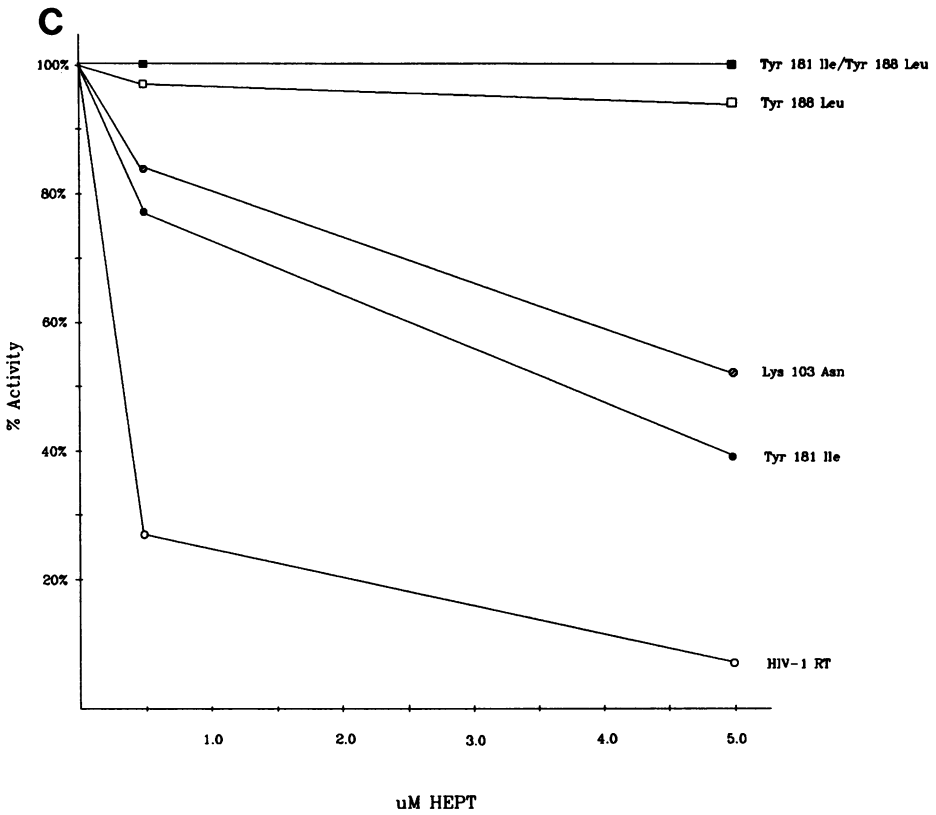
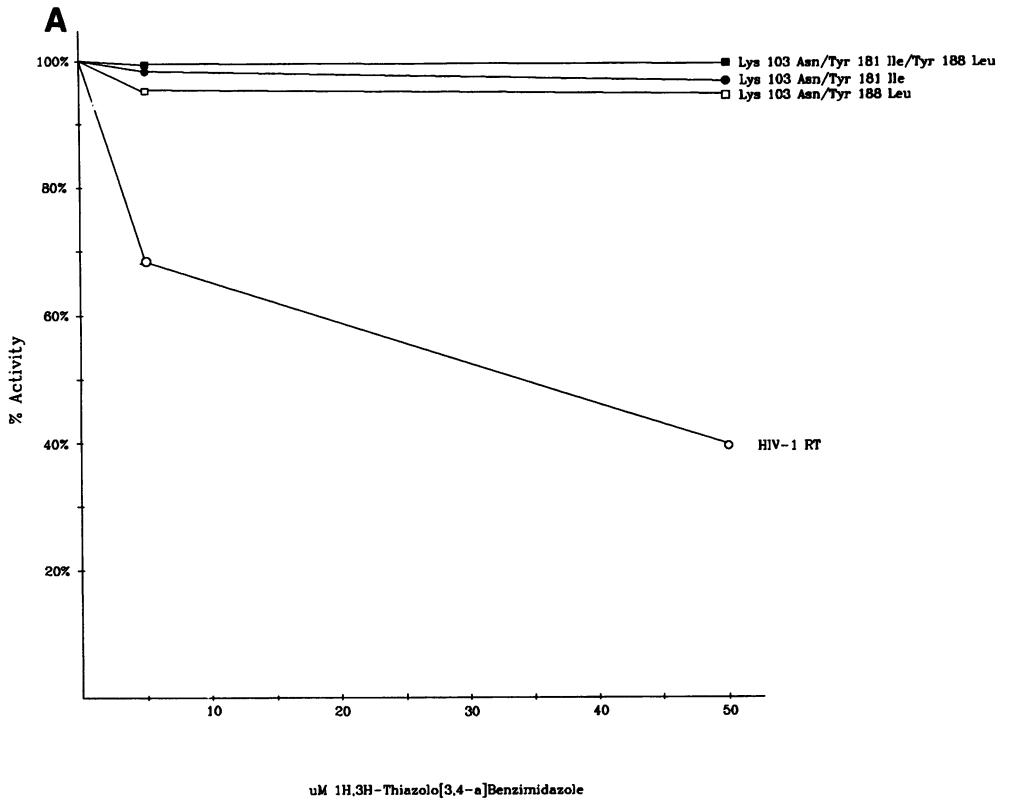


FIG. 2. Drug resistance of variant HIV-1 RTs with the single amino acid substitutions Lys-103→Asn, Tyr-181→Ile, and Tyr-188→Leu and the double amino acid substitution Tyr-181→Ile/Tyr-188→Leu. Assays were performed as previously described (3) by using poly(rC) · oligo(dG) as the template-primer. The variant HIV-1 RTs were assayed with the indicated amounts of TBA (A), calanolide A (B), and HEPT (C). Activity of each RT in the absence of an inhibitor was considered to represent 100% activity. Activity of each RT in the presence of an inhibitor was normalized to this value.

resistant to calanolide A, the level of resistance was much lower than that seen with TBA (Fig. 2A). Mutants that contained the amino acid substitutions Tyr-188→Leu and Tyr-181→Ile/Tyr-188→Leu showed substantial resistance to calanolide A, suggesting that Tyr-188 is very important for calanolide A sensitivity in HIV-1 RT. The observation that Tyr-181 and Lys-103 are less important for calanolide A sensitivity than for TBA sensitivity suggests that while the binding sites for the two drugs partially overlap they are not identical. The HIV-1 RT mutants showed a pattern of resistance to HEPT (Fig. 2C) which was similar to that shown for calanolide A (Fig. 2B). This suggests that the two drugs, while structurally very dissimilar, may bind at similar locations within the hydrophobic pocket.

A mutant HIV-1 RT with two amino acid substitutions, Tyr-181→Ile/Tyr-188→Leu, had a high level of resistance to TBA, calanolide A, and HEPT. Therefore, we constructed the other possible combinations of the amino acid substitutions and tested them for resistance to the three drugs. As shown in Fig. 3A, all of the mutant HIV-1 RTs with multiple mutations were highly resistant to TBA compared with the normal HIV-1 RT. For calanolide A, however, (Fig. 3B), only the RTs with the amino acid substitutions Lys-103→Asn/Tyr-188→Leu and Lys-103→Asn/Tyr-181→Ile/Tyr-188→Leu showed high levels of resistance. The double mutant Lys-103→Asn/Tyr-181→Ile gave only partial resistance, suggesting that these amino acids are not as important for sensitivity to calanolide A as for sensitivity to TBA. The results of the in vitro enzyme assays mirror the previously reported in vitro antiviral data; the pyridinone-resistant virus A17 (26), which contains an alteration at Tyr-181 (to a cysteine) and an alteration at Lys-103 (to an asparagine), is resistant to TBA (5) but is sensitive to calanolide A (18). HEPT did not show the same pattern of resistance as calanolide A in the analysis with the multiple-mutation RT variants (Fig. 3C). For example, while the double mutant Lys-103→Asn/Tyr-181→Ile was only partially resistant to calanolide A, it was almost completely resistant to HEPT.

Other amino acid substitutions have been reported to be present in HIV-1 RT variants which are resistant to nonnucleoside drugs. HIV-1 RT variants containing these alterations, Leu-100→Ile (31, 37), Val-108→Ile (31), Ile-135→Thr (31), Val-179→Asp (36), and His-221→Tyr (31), were constructed with the *Bsp*MI cassette mutagenesis system. These HIV-1 RT variants were likewise tested for resistance to TBA, calanolide A, and HEPT. As shown in Fig. 4A and B, only the Leu-100→Ile mutation gave any significant resistance to either TBA or calanolide A, with the resistance to calanolide A being the most marked. The Leu-100→Ile mutant, however, did not appear to be substantially more resistant to HEPT than the wild-type HIV-1 RT (Fig. 4C). The amino acid substitution Val-179→Asp may confer partial resistance to HEPT (Fig. 4C) but does not seem to affect sensitivity to TBA or calanolide A. The other amino acid substitutions did not confer significant resistance to TBA, HEPT, or calanolide A. While these amino acids changes do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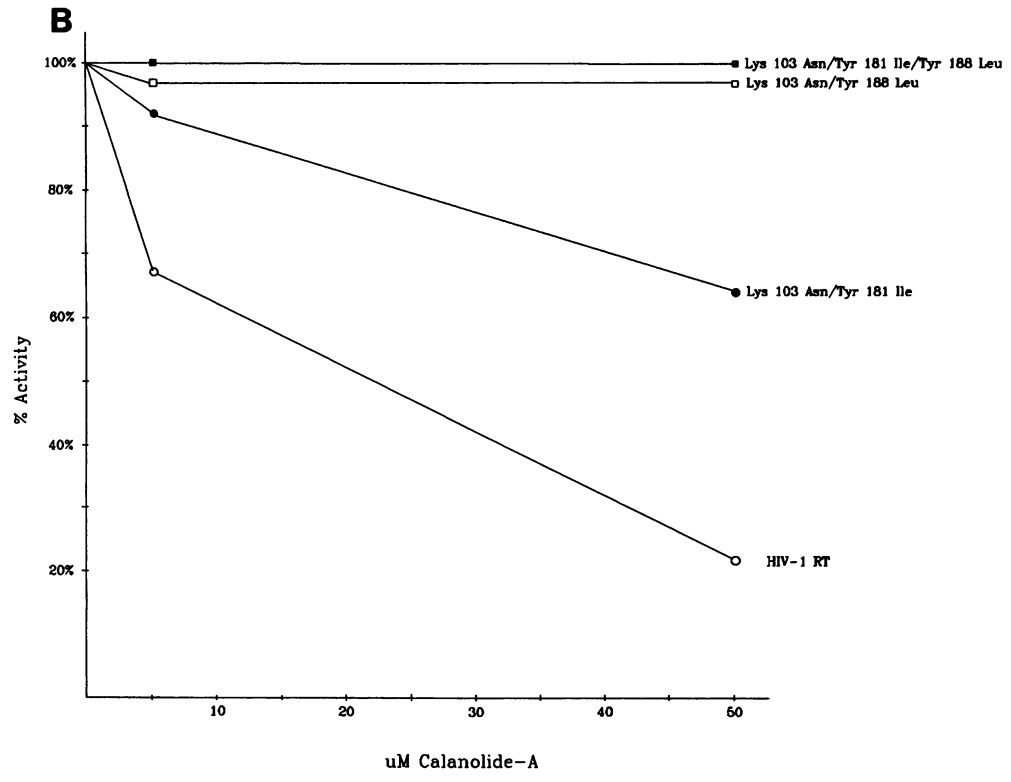
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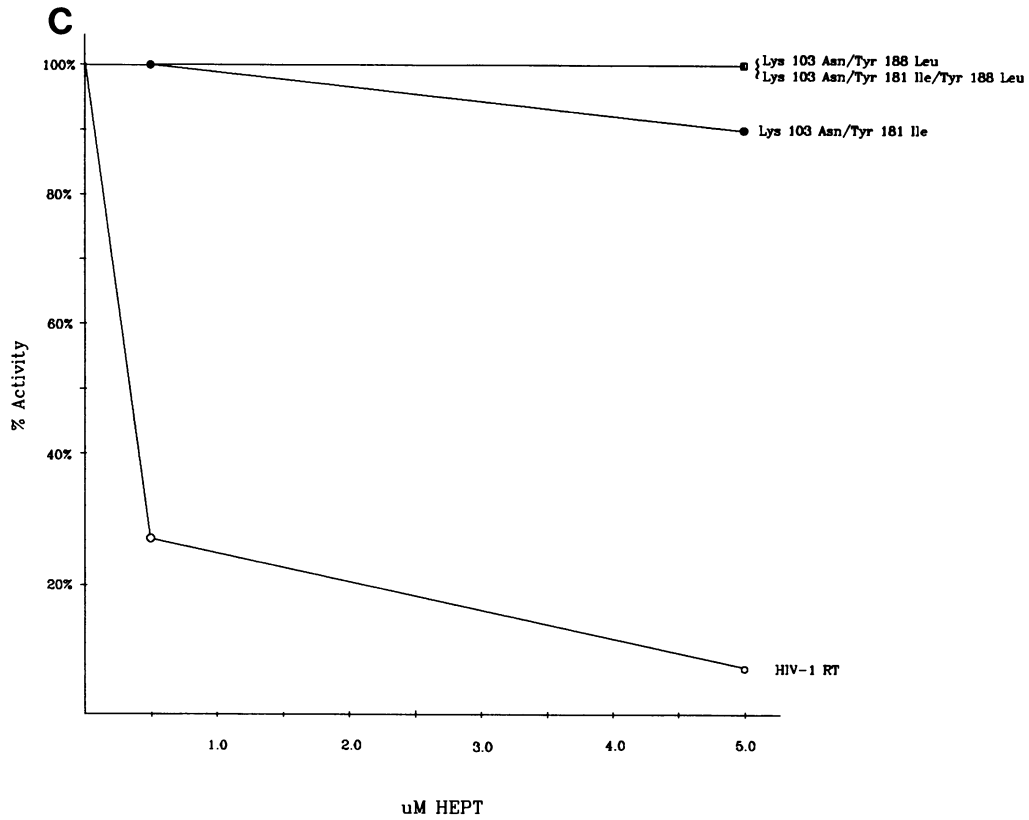
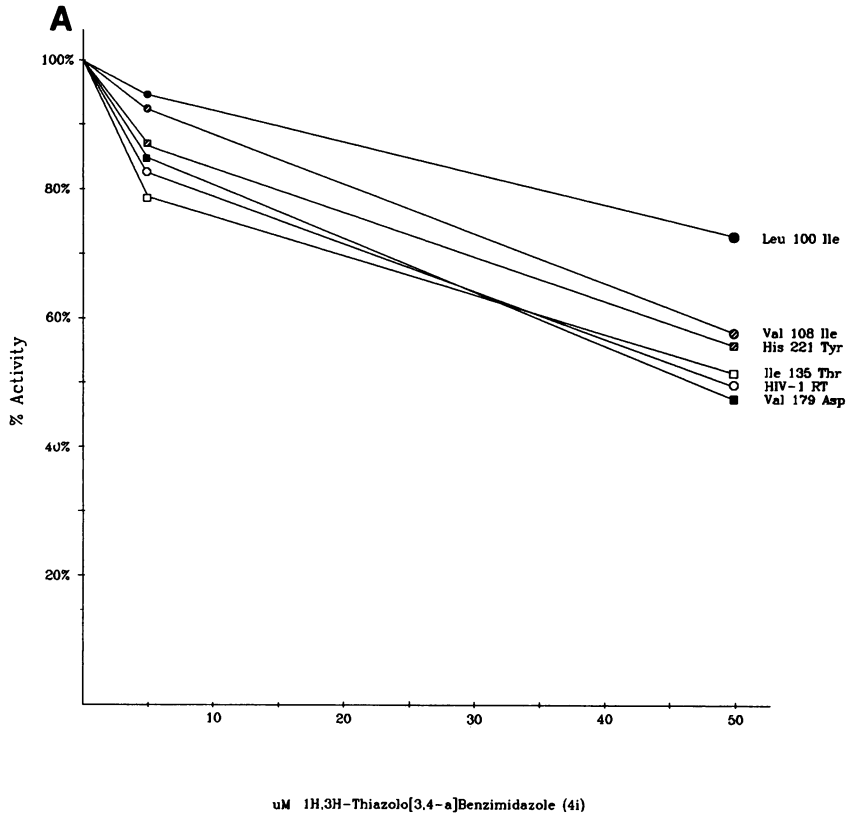


FIG. 3. Drug resistance of variant HIV-1 RTs with the multiple amino acid substitutions Lys-103→Asn/Tyr-181→Ile, Lys-103→Asn/Tyr-188→Leu, and Lys-103→Asn/Tyr-181→Ile/Tyr-188→Leu. The variant RTs were assayed with the indicated amounts of TBA (A), calanolide A (B), and HEPT (C).

not, by themselves, appear to be important in determining the sensitivity of HIV-1 RT to TBA, HEPT, or calanolide A, it is still possible that these substitutions could have an effect if they were tested against other nonnucleoside drugs or if they were to occur as part of a cluster of amino acid substitutions.

In the analysis with the point mutants, TBA appears to behave like many previously described nonnucleoside drugs. Calanolide A, however, is an unusual compound and appears to belong to a pharmacologic class different from that of the other known nonnucleoside RT-inhibitory drugs. Calanolide A is fully active against HIV-1 strain A17 (26), and analysis of the resistance and sensitivity of a series of enzymatically active HIV-1/HIV-2 RT chimeras suggested that calanolide A does not interact with the same segments of HIV-1 RT as the TIBO compounds (15). While the pattern of calanolide A resistance and sensitivity of the point mutants agrees with the A17 data, the pattern of calanolide A sensitivity of the HIV-1/HIV-2 chimeras is more difficult to explain (16). With the chimeras, sensitivity to calanolide A appeared to be primarily dependent on two regions from HIV-1 RT, amino acids 93 to 157 and amino acids 224 to 427. The region from 93 to 157 contains amino acids 100 and 103, which the present data derived with the point mutants suggest are important for sensitivity to calanolide A. Moreover, the structure of HIV-1 RT complexed with nevirapine revealed that the region that contains these amino acids lies close to the bound drug (19). The region between 224 and 427 contains portions of the connection domain that also appear to be quite close to the drug binding pocket (19). It is not difficult to imagine how alteration of this region could affect the drug binding pocket either directly or indirectly. What is more difficult to reconcile with the present results are the data with chimera C1, in which the body of the chimera is from HIV-1 RT and the region between amino acids 158 and 190 derives from HIV-2 RT. On the basis of the results with the drug-resistant point mutants, we would have expected that this chimera, which is resistant to TIBO, should also be resistant to calanolide A. It is, in fact, only slightly less sensitive (50% inhibitory concentration about threefold higher) to calanolide A than the wild-type enzyme. The C1 chimera is much more sensitive to calanolide A than is the HIV-1 RT with the two mutations Tyr-181→Ile and Tyr-188→Leu. This difference in sensitivity is not a result of the particular amino acids that are present at these positions since the C1 chimera and the HIV-1 RT with the two mutations both contain an Ile at position 181 and a Leu at position 188. Therefore, we are left with the hypothesis that sensitivity and resistance to these drugs are the result of the precise geometry of the drug binding pocket and that this geometry may be quite sensitive to changes that do not necessarily manifest themselves as particular sites at which individual amino acid substitutions lead directly to drug resistance. Amino acid positions within RT which have not yet been specifically implicated in drug resistance may still have some direct or indirect role in determining susceptibility to individual drugs. Substitution of relatively large blocks of amino acids could conceivably affect the shape of the drug binding pocket, even if these substitutions do not involve amino acids that directly form the drug binding pocket. This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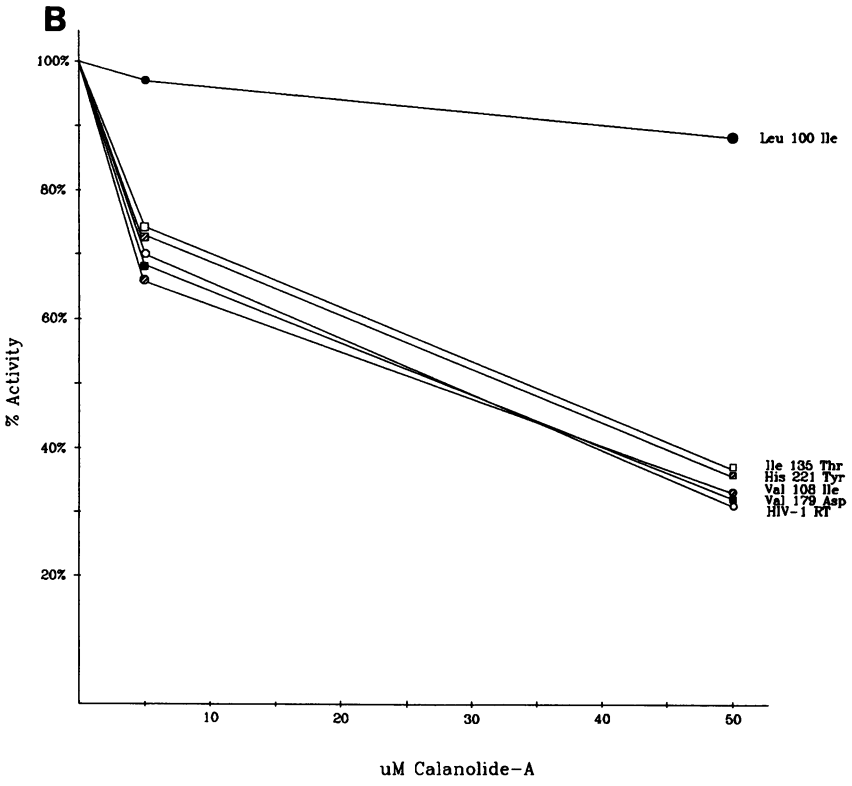
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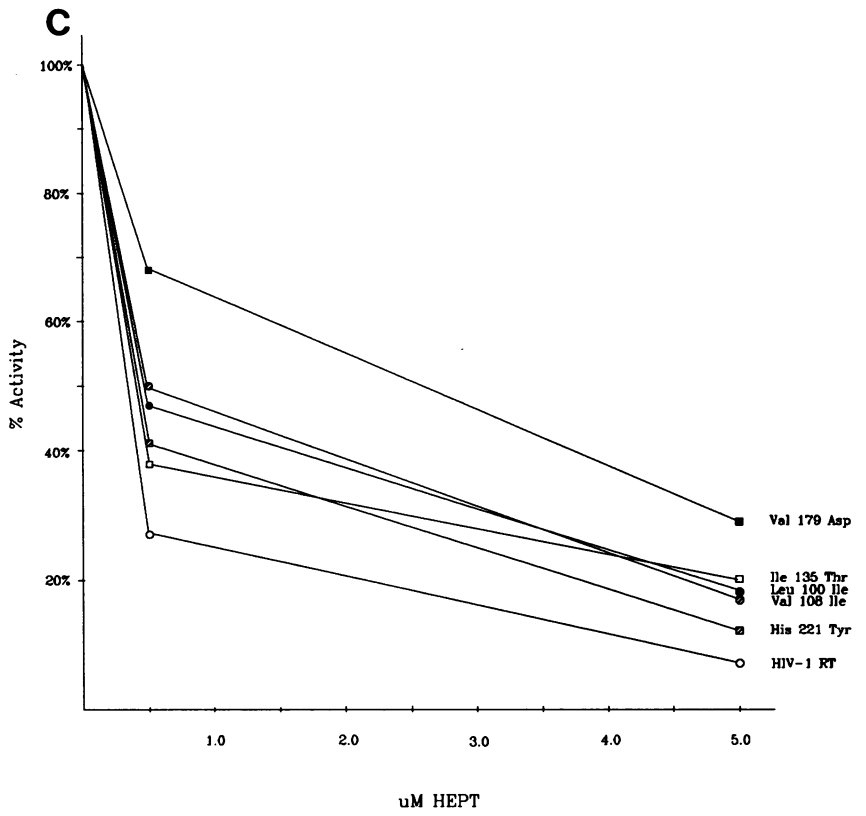

FIG. 4. Drug resistance of variant RTs with the single amino acid substitutions Leu-100→Ile, Val-108→Ile, Ile-135→Thr, Val-179→Asp, and His-221→Tyr. The variant RTs were assayed with the indicated amounts of TBA (A), calanolide A (B), and HEPT (C).

idea is further underscored by the recent observation that an HIV-2 RT with the amino acid substitutions Ile-181→Tyr and Leu-188→Tyr is not sensitive to the nonnucleoside inhibitors nevirapine and R82913 (TIBO) (14, 35). There is hope, however, that additional work on the three-dimensional structure of the RT-drug complexes and of drug-resistant mutants will help to elucidate the exact relationships between the sequence of HIV-1 RT and its structure.

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