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Specific Inhibition of the Reverse Transcriptase of Human Immunodeficiency Virus Type 1 and the Chimeric Enzymes of Human Immunodeficiency Virus Type 1 and Type 2 by Nonnucleoside Inhibitors

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We have studied the effects of four nonnucleoside inhibitors, including the novel natural product inhibitor calanolide A, on molecular chimeras containing complementary segments of human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) reverse transcriptases (RTs). All four compounds specifically inhibited the DNA polymerase activity of HIV-1 RT but had no apparent effect on the RNase H activity of this enzyme or on the DNA polymerase or RNase H activity of HIV-2 RT. Three of these compounds showed the generally expected patterns of resistance and susceptibility with the various chimeric RTs. However, the inhibition patterns of the chimeric RTs by calanolide A provided evidence that there is a segment between residues 94 and 157 in HIV-1 RT that is critical for inhibition. However, the data also suggest that there may be a second segment located between amino acids 225 and 427 in HIV-1 RT that is also important for specifying susceptibility to the drug.

Human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) are etiological agents responsible for AIDS (1a, 33). As is true for all retroviruses, the virus-coded enzyme reverse transcriptase (RT) is a key enzyme in the early steps of the viral life cycle, since RT is required for the synthesis of unintegrated viral DNA from the viral RNA template (40, 42, 43). Reverse transcription, which does not appear to be required for normal cellular processes, is an attractive target for the development of potent anti-HIV drugs (12, 30).

Considering the complex nature of RT and the enzymatic reactions that RT catalyzes, it is not surprising that numerous compounds that inhibit the reverse transcription process have been found. However, most of these inhibitors can be grouped into two distinct classes (12, 30). The first class consists of nucleoside analogs, such as 3′-azido-2′,3′-dideoxythymidine (AZT) and 2′,3′-dideoxyinosine (ddI). These compounds, when incorporated into viral DNA by RT, cause premature chain termination of the nascent DNA strands. However, long-term therapy of HIV-infected patients has been limited by the toxicity of these agents and by the emergence of drug-resistant viral strains (12, 15, 24, 25, 30, 39).

A second class of inhibitors consists of complex aromatic compounds (11, 12, 14, 16, 17, 23, 29, 32) that are potent inhibitors of HIV-1 RT but have no apparent effect on the closely related RT of HIV-2. The members of this class are structurally diverse, and none show structural resemblance to the deoxynucleosides. Inhibition of the RT-catalyzed DNA- and RNA-dependent DNA synthesis by these drugs is noncompetitive with respect to both template primers and deoxynucleoside triphosphate (dNTP) substrates. These compounds include tetrahydroimidazo[4,5,1-*jk*][1,4]-benzodiazepin-2(1H)one and -thione (TIBO) and its derivatives (11–14, 32), pyridobenzodizepinones and dipyridodiazepinones (e.g., B1-RG-587 and nevirapine) (12, 17, 23), pyridinone derivatives (i.e., L-696,040 and L-697,639) (12, 16, 29), and certain bis(heteroaryl)piperazines (35). More recently, several new classes of compounds with similar functional properties have been identified by the National Cancer Institute AIDS antiviral drug screening program (2, 41). These compounds include derivatives of diarylsulfone (27), thiazolobenzimidazole (5–7), and sulfoxamine (unpublished data). Despite their chemical diversity, all of these compounds appear to belong to a common pharmacological class with functional similarity. HIV-1 RT mutants that arose following exposure to individual compounds in this class or that were generated by in vitro site-directed mutagenesis are cross-resistant to the diverse members of this class. For example, mutants that are resistant to TIBO show cross-resistance to nevirapine and to pyridinone compounds (10, 13, 17, 28, 31, 34, 38). It was recently shown that nevirapine binds to a hydrophobic pocket in HIV-1 RT near, but distinct from, the active site of DNA polymerase (22). It seems likely that the other nonnucleoside inhibitors in this group interact with the enzyme in a similar manner.

The calanolides have been recently identified as a new type of nonnucleoside inhibitor of HIV-1 RT (21). Calanolide A, a natural product compound isolated from the tropical rainforest tree *Calophyllum lanigerum* inhibits HIV-1 RT but does not affect HIV-2 RT. However, calanolide A was active not only against the AZT-resistant G910-6 strain of HIV-1 but also against the pyridinone-resistant A17 strain of HIV-1 (21). Since the A17 strain of HIV-1 was highly resistant to the known nonnucleoside inhibitors of HIV-1 (e.g., TIBO

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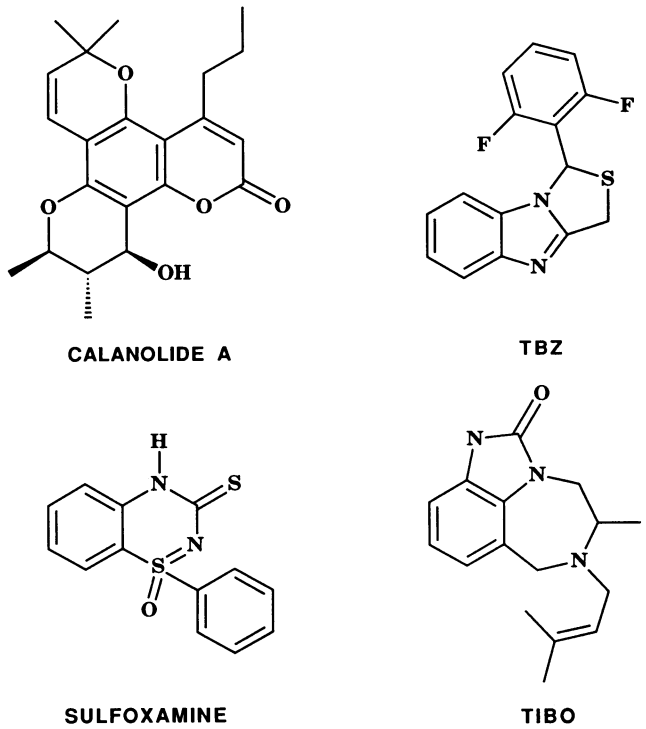


FIG. 1. Chemical structures of nonnucleoside HIV-1 RT inhibitors (complete chemical names for these compounds are given in the text).

and nevirapine), it was suggested that the calanolides might have a different mode of RT inhibition. Therefore, we have used molecular chimeras composed of complementary segments of HIV-1 and HIV-2 RTs to learn more about the molecular basis of RT inhibition by calanolide A.

MATERIALS AND METHODS

Reagents. Calanolide A (Fig. 1) was obtained from extracts of the *Calophyllum lanigerum* tree as previously described (21). All other experimental antiviral agents were obtained through the Drug Synthesis and Chemistry Branch, National Cancer Institute. The other compounds used is this study were TIBO (R79882), 1H,3H-thiazolo[3,4-*a*]benzimidazole (TBZ) (NSC 625487), and 1H-1,2,4-benzothiadiazine-3(4H)-thione, 1-phenyl-, 1-oxide (sulfoxamine) (NSC 287474) (Fig. 1).

Construction of HIV-1-HIV-2 RT chimeras. The construction of the A1, A4, A6, A6/B6, B1, B2, and B6 chimeras has been described previously (37). We made use of the *Bsp*MI cassette mutagenesis system (4) to construct the C1 and C2 chimeras. Briefly, digestion of one of the HIV-1 RT *Bsp*MI cassette plasmids with *Bsp*MI removes a portion of DNA from the region encoding HIV-1 RT. The portion that is excised can be replaced with double-stranded synthetic DNA. In this case, we replaced the segment of HIV-1 DNA encoding either amino acids 158 to 190 or 192 to 224 with the corresponding sequences of HIV-2 DNA. A similar method for the preparation of HIV-1-HIV-2 RT chimeras has been described by others (10). In all cases, the HIV-1-HIV-2 RT plasmids encoding the chimeras were introduced in *Escherichia coli* DH5α.

Enzymes. All RTs used in this study were recombinant enzymes expressed in *E. coli*. The HIV-1 and HIV-2 RT expression plasmids have been described elsewhere (18, 19). HIV-1 and HIV-2 RTs were purified and yielded polypeptides with apparent molecular masses of approximately 66 and 68 kDa for the enzymes from HIV-1 and HIV-2, respectively. HIV-1 RT was purified to homogeneity by the method of Clark et al. (8). HIV-2 RT was purified to about 90% homogeneity by similar methods. Purified enzymes were stored in 50% glycerol (vol/vol), 2 mM dithiothreitol, and 25 mM Tris-HCl (pH 8.0), at −80°C. Samples of these stocks used for routine assays were kept at −20°C. Extracts of bacterial strains expressing HIV-1-HIV-2 RT chimeras were prepared as described previously (37).

Enzymatic assays. In all enzymatic inhibition studies, increasing concentrations of each inhibitor were present in the reaction mixtures. The extent of inhibition was expressed as IC_{50}s, inhibitor concentrations leading to 50% inhibition of the initial enzymatic activities.

RT-associated DNA polymerase assays. The RNA-dependent DNA polymerase activity was assayed by monitoring the rates of the poly(rA)$_n$ · oligo(dT)$_{12-18}$-directed incorporation of [^{3}H]dTTP or by measuring the rates of the poly(rC) · oligo(dG)$_{12-18}$-directed incorporation of [^{3}H]dGTP. The reactions for HIV-1 and HIV-2 RTs were carried out in a final volume of 0.1 ml containing 5 μg of poly(rA)$_n$ · oligo(dT)$_{12-18}$ or poly(rC)$_n$ · oligo(dG)$_{12-18}$ (Pharmacia LKB Biotechnology Inc.) per ml, 25 mM Tris-HCl, 40 mM KCl, 8 mM $MgCl_2$, and the appropriate ^{3}H-labeled dNTP (50 μM; specific radioactivity of about 300 cpm/pmol) at the final pH of 7.5. After 30 min of incubation at 37°C, the amount of polymerized dNTP was measured by adding herring sperm carrier DNA (to a final concentration of 100 μg/ml) and 0.02 M sodium pyrophosphate and then precipitating with ice-cold 10% (wt/vol) trichloroacetic acid. The precipitates were collected on Whatman GF/C fiberglass filters, and the filters were washed with a solution of 5% trichloroacetic acid and ethanol. The dried filters were counted in a toluene-based scintillation fluid in a beta scintillation counter. The DNA-dependent DNA polymerase activities were assayed with activated herring sperm DNA substituting for the synthetic template primer and with all four dNTPs present in the reaction mixtures. The activated DNA was present in the reaction mixture at a final concentration of 20 μg/ml. The three unlabeled dNTPs (dGTP, dCTP, and dATP) and the labeled dNTP (dTTP) were all present in the reaction mixtures at final concentrations of 50 μM (the specific activity of dTTP was approximately 300 cpm/pmol).

RESULTS AND DISCUSSION

Effects of calanolide A on the enzymatic activities of HIV-1 and HIV-2 RTs. The inhibition of RNA-dependent DNA polymerase activities of highly purified recombinant HIV-1 and HIV-2 RTs by calanolide A were previously reported (21). We have extended this analysis to include the DNA-dependent DNA polymerase activity and RNase H activity of these enzymes. The enzymes were preincubated with various concentrations of pure calanolide A for 5 min at 30°C, and then the residual enzymatic activity was compared with the activity of untreated enzyme. Both the RNA-dependent DNA polymerase and the DNA-dependent DNA polymerase activities of HIV-1 RT were quite sensitive to calanolide A (Fig. 2). As expected, the compound did not have any effect on the DNA polymerase activities of HIV-2 RT, even at drug concentrations as high as 300 μM (Fig. 2 and 3). There were, however, some differences in the extent of

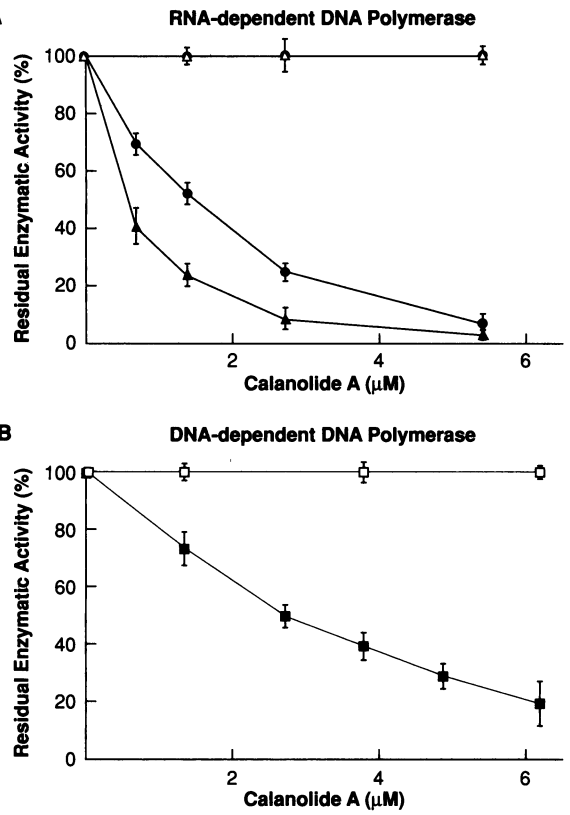


FIG. 2. Effects of calanolide A on the DNA polymerase activities of purified RTs of HIV-1 and HIV-2. Increasing amounts of calanolide A were added to the purified HIV RTs in a solution containing dimethyl sulfoxide at a final concentration of 0.1%. The HIV RTs were expressed in bacteria and purified as described previously (8, 18, 19). After preincubation for 5 min at 30°C, the different catalytic activities were determined as described in detail previously (20, 26). Residual activity was calculated as a percentage of enzymatic activity without inhibitor. Closed symbols refer to HIV-1 RT, and open symbols refer to HIV-2 RT. (A) RNA-dependent DNA polymerase activity. Poly $(rC)_n \cdot$ oligo$(dG)_{12-18}$-directed DNA polymerase activity (△, ▲) and poly$(rA)_n \cdot$ oligo$(dT)_{12-18}$-dependent activity (○, ●). (B) DNA-dependent DNA polymerase activity assayed with gapped-activated herring sperm DNA (20). Each value represents the mean ± 1 standard deviation taken from triplicate determinations.

inhibition of the RNA-directed DNA synthesis exhibited by HIV-1 RT, depending on the choice of template primer. RNA-dependent DNA synthesis with poly$(rC)_n \cdot$ oligo $(dG)_{12-18}$ as the template primer was more sensitive to inhibition by calanolide A than was poly$(rA)_n \cdot$ oligo$(dT)_{12-18}$-directed DNA synthesis (Fig. 2). Similar results have been reported for the inhibition of HIV-1 RT by other nonnucleoside analogs (12, 32). The DNA-dependent DNA polymerase activity, which was assayed with activated herring sperm DNA (20, 26), seemed less sensitive to calanolide A than was RNA-dependent DNA polymerase activity (Fig. 2). It is apparent, from the values calculated for the concentration of the inhibitor that blocked 50% of the initial enzymatic activity (IC_{50}s), that the poly$(rC)_n \cdot$ oligo$(dG)_{12-18}$-dependent synthesis was about twice as sensitive to calanolide A than was poly$(rA)_n \cdot$ oligo $(dT)_{12-18}$-dependent synthesis. DNA-dependent DNA synthesis was two- to fourfold less sensitive to calanolide A than was RNA-dependent DNA synthesis.

Neither the RNase H activity of HIV-1 RT nor that of HIV-2 RT was inhibited by calanolide A (not shown). Likewise, neither the nucleoside analogs nor the previously described nonnucleoside inhibitors had significant effects on the RNase H activities of these enzymes (12).

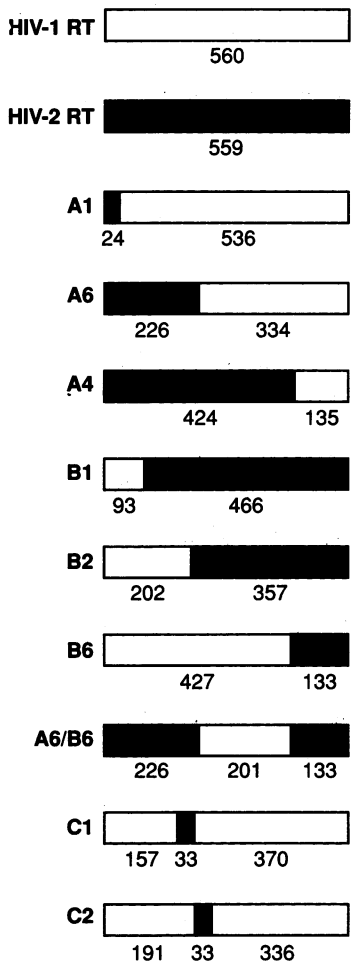
Inhibition of chimeric HIV-1-HIV-2 RT molecules by calanolide A. We have taken advantage of the insensitivity of HIV-2 RT to nonnucleoside inhibitors to study the structural basis for sensitivity to these inhibitors. A series of recombinant HIV-1-HIV-2 RT chimeras in which complementary segments of the chimeric enzymes derive from the two parental molecules has been constructed (Fig. 3). In the initial study, we prepared 12 chimeras, of which 7 showed full or partial enzymatic activity (A1, A6, A4, B1, B2, B6, and A6/B6). We and others have used chimeras of this type to show that TIBO interacts with HIV-1 RT in the vicinity of amino acid 190 (10, 37, 38).

We have recently generated two additional chimeras, C1 and C2 (Fig. 3) that should permit a more precise mapping of the regions that are involved in the sensitivity to nonnucleoside inhibitors. We have found that the C1 is almost completely resistant to TIBO, while C2 is fully sensitive, suggesting that the TIBO binding site involves amino acids that are between positions 158 and 190 (Fig. 3). These results are consistent with other studies using RT mutants resistant to TIBO, nevirapine, and the pyridinone inhibitors that were generated either as a result of drug exposure or were created in vitro by site-directed mutagenesis. These findings suggested that sensitivity to inhibition by these drugs is dependent on regions of RT between residues 176 to 190 with a specific contribution by tyrosines 181 and 188 (28, 31, 34, 35). These studies also suggested that the region between amino acids 101 and 106 is important in determining sensitivity to nonnucleoside RT inhibitors (10, 31, 36). The importance of tyrosines 181 and 188 to the binding of these nonnucleoside analogs was further supported by mapping those portions of the RT molecule that are bound covalently to a labeled nevirapine analog (9) and by the three-dimensional structure of HIV-1 RT bound to nevirapine (22).

In contrast to the results with TIBO, the results with the chimeric RTs and calanolide A do not give a simple pattern that allows us to define a single binding site (Fig. 3). Because the results with calanolide A were more complex, we also tested two additional nonnucleoside inhibitors, TBZ and sulfoxamine (5–7). The C1 chimera was resistant to TBZ and sulfoxamine. However, the B2 and C2 chimeras also showed significant resistance to both compounds. This result implicated residues 158 to 224 in binding TBZ and sulfoxamine. This is not entirely unexpected, since the nevirapine-binding pocket lies near or within this segment of the protein (1, 22). The data for calanolide A are more difficult to explain.

The inhibition patterns of the hybrid HIV-1-HIV-2 RTs by calanolide A (Fig. 3) indicate that these enzymes can be put in four groups as follows: (i) group 1 consists of chimeric RTs that were as resistant as wild-type HIV-2 RT, including those designated A6, A4, B1, and A6/B6; (ii) group 2 consists of hybrid RTs with an intermediate sensitivity to calanolide A (only the B2 chimera); (iii) group 3 consists of chimeras that were quite sensitive to the inhibitor but which had IC_{50}s 2.5- to 3-fold higher than wild-type HIV-1 RT (chimeras C1 and C2); and (iv) group 4 consists of chimeric molecules that were as sensitive to calanolide A as wild-type HIV-1 RT was (this group included A1 and B6).

The scheme of inhibition seen with most of the chimeras (excluding C1 and C2) suggested that the region which is primarily responsible for calanolide A sensitivity is located



	IC_{50} (μM)			
	Calanolide A	TIBO	TBZ	Sulfoxamine
HIV-1 RT	3.4	2	7.3	5.1
HIV-2 RT	>>300	>>300	>>300	>>300
A1	3.5	2	8.7	4
A6	>>300	>>300	>>300	>>300
A4	>>300	>>300	>>300	>>300
B1	>>300	>>300	>>300	>>300
B2	108	30	>>300	>>300
B6	3.8	4.5	13	4.4
A6/B6	>>300	>>300	>>300	>>300
C1	8.2	174	>>300	>>300
C2	9.4	3.4	156	77

FIG. 3. Structures and susceptibilities of the chimeric HIV-1-HIV-2 RTs. The construction of the plasmids used for the expression of the chimeric RTs was described in detail previously (37). Wild-type and chimeric HIV-1-HIV-2 RTs were expressed in the DH5α strain of *E. coli*. The two new chimeras C1 and C2 were constructed by a modified version of the *Bsp*MI cassette mutagenesis method that was used previously for the introduction of point mutations in HIV-1 RT (4). We have replaced segments of the HIV-1 RT gene by the DNA sequences coding for the appropriate peptide sequences of HIV-2 RT. A similar method for the construction of chimeric HIV-1-HIV-2 RTs has been described by others (10). Open boxes represent sequences derived from HIV-1 RT, and solid boxes represent sequences from HIV-2 RT. The number of amino acids in each segment is indicated under the boxes. Extracts of the bacterial strains expressing each recombinant protein were prepared as described previously (18, 19, 37). The extracts were diluted 4.5-fold into a solution of 0.1% dimethyl sulfoxide containing increasing concentrations of the indicated compounds and were preincubated for 5 min at 30°C. The residual poly(rC)$_n$ · oligo(dG)$_{12-18}$-dependent DNA polymerase activities were determined as described previously (18, 19, 37). The IC_{50}s were calculated from the inhibition curves for each recombinant enzyme from triplicate determinations. Most of the data for TIBO (except the analysis with C1 and C2) has already been reported (37).

between residues 94 and 226. Only molecules that contained this entire segment (group 4) from HIV-1 RT were fully sensitive to the drug. However, the hybrid B2 showed a partial sensitivity to the compound (group 2). The junction between the HIV-1 and HIV-2 RT segments in this chimera is close to the nevirapine-binding site, and this chimera showed some resistance to all four nonnucleoside inhibitors. The fact that the two new hybrid RTs (C1 and C2) were both inhibited by calanolide A to almost the same extent as wild-type HIV-1 RT was unexpected. As mentioned above, we have shown previously that the other seven chimeras behave appropriately in response to TIBO on the basis of the sites within HIV-1 RT which are believed to interact with this drug (37).

We believe that the pattern of calanolide A inhibition of the different HIV-1-HIV-2 RT chimeras suggests that there is a crucial segment between residues 94 and 157 in HIV-1 RT. Other recent experiments with point mutants (3) suggest that sensitivity to calanolide A depends on amino acids in this segment, in particular on Leu-100 and to a lesser degree on Lys-103. The analysis with the point mutants also suggested that sensitivity to calanolide A depends on Tyr-188 (3). We cannot easily reconcile that data with the results we have obtained with the C1 chimera. On the basis of the

analysis of the point mutants, we would have expected C1 to be partially resistant to calanolide A. The data generated with the chimeric RTs (especially B2, B6, and C2) suggest that there may be an additional segment that lies between amino acids 225 and 427 that interacts, either directly or indirectly, with calanolide A. This interpretation could explain why the C2 chimera is still quite sensitive to calanolide A and why the B6 chimera was as sensitive to calanolide A as wild-type HIV-1 RT. If this interpretation is correct, there are at least two possible simple explanations. First, there could be two calanolide A-binding sites, each of which binds a separate drug molecule. Alternatively, it is possible that the two segments of HIV RT that confer sensitivity to calanolide A interact with a single inhibitor molecule. We have examined the three-dimensional structure of HIV-1 RT (1, 22) to determine whether these segments could be in sufficiently close contact to form a single binding site for calanolide A. The second region, which is less well-defined (lying between residues 224 and 427) is either in the "thumb" or "connection." The three-dimensional model of HIV-1 RT shows that the hydrophobic pocket that binds nevirapine is in close proximity to the base of the thumb and to parts of the connection (22). The segment from amino acids 100 to 103 and a segment lying just beyond amino acid 224 could both interact with the same calanolide A molecule.

In summary, the results presented here support the initial conclusion (21) that calanolide A is an HIV-1 RT-specific inhibitor that is distinct in its interaction with RT from the known nonnucleoside inhibitors. There are several lines of future investigation that could help elucidate the mechanism by which calanolide A inhibits HIV-1 RT in greater detail. Sequence analysis of calanolide A-resistant HIV strains, should they emerge, might indicate which amino acid modifications in wild-type HIV-1 RT confer resistance. It may also be possible to prepare derivatives of calanolide A that would bind covalently to RT. Such compounds might permit the identification of specific amino acid residues that can interact with the inhibitor.

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