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Inhibition of human immunodeficiency virus type 1 integrase by 3′-azido-3′-deoxythymidylate

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ABSTRACT **The effects of 3′-azido-3′-deoxythymidine (AZT) and three of its intracellular metabolites, azido- thymidine mono-, di-, and triphosphates, on the human immunodeficiency virus type 1 integrase have been determined. AZT mono-, di-, and triphosphate have an IC_{50} for integration between 110 and 150 μM, whereas AZT does not inhibit the integrase. The inhibition by AZT monophosphate can be partially reversed by coincubation with either thymidine monophosphate or 2′,3′-dideoxythymidine monophosphate, suggesting that either of these monophosphates can bind to the integrase but that the azido group at the 3′ position could be responsible for the inhibition. Integrase inhibition is associated with reduced enzyme–DNA binding but does not appear to be competitive with respect to the DNA substrate. Inhibition of an integrase deletion mutant containing only amino acids 50–212 suggests that these nucleotides bind in the catalytic core. Concentrations up to 1 mM AZT monophosphate can accumulate *in vivo*, indicating that integrase inhibition may contribute to the antiviral effects of AZT. The increasing incidence of AZT-resistant virus strains may, therefore, be associated with mutations not only in the reverse transcriptase but also in the human immunodeficiency virus integrase. Finally, these observations suggest that additional strategies for antiviral drug development could be based upon nucleotide analogs as inhibitors of human immunodeficiency virus integrase.**

The first clinically approved drug in the treatment of AIDS was 3′-azido-3′-deoxythymidine (AZT). This thymidine analog is converted to AZT monophosphate (AZTMP), diphosphate (AZTDP), and triphosphate (AZTTP) by thymidine, thymidylate, and nucleoside diphosphate kinases, respectively (1). The antiretroviral effect of AZT is attributed to AZTTP, which interferes with viral DNA replication by two mechanisms (2). (*i*) It can competitively inhibit the human immunodeficiency virus (HIV) reverse transcriptase against normal dTTP for DNA polymerization; and (*ii*) it can act as a chain terminator of the nascent viral DNA chain. The major limitation to AZT therapy is bone marrow suppression, leading to anemia or neutropenia (3). The most likely mechanism for AZT-induced toxicity could be incorporation of AZT into newly synthesized host (i.e., nonviral) DNA and inhibition of strand elongation due to chain termination (4).

Studies on the cellular metabolism of AZT have shown that AZTMP accumulates *in vivo* because it is a competitive substrate inhibitor of thymidylate kinase that converts AZTMP to AZTDP (with a K_i of 8.6 μM) (5). In fact, millimolar concentrations of AZTMP have been observed in cells chronically exposed to AZT (6). Similarly, accumulation of 3′-azido-2′,3′-dideoxyguanosine diphosphate (AZGDP) upon incubation of 3′-azido-2′,3′-dideoxyguanosine with CEM cells has also been demonstrated (7). The present study was initiated to determine the effects of AZTMP and other AZT metabolites on another potentially important viral target, the integrase (8–10).

Retroviruses such as HIV type 1 (HIV-1) encode the integrase protein at the 3′ end of the *pol* gene (11–13). This enzyme, a proteolytic cleavage product of a gag–pol fusion protein precursor, is contained in the virus particle and is required for integrating a double-stranded DNA copy of the RNA genome, synthesized by reverse transcriptase, into a host chromosome (14, 15). During viral infection, integrase catalyzes the excision of the last 2 nt from each 3′ end of the linear viral DNA, leaving the terminal dinucleotide CA-3′-OH at these recessed 3′ ends (16–21). This activity is referred to as the 3′ processing or dinucleotide cleavage. After transport to the nucleus as a nucleoprotein complex, integrase catalyzes the ligation of these ends to the 5′-phosphate ends of a staggered double-stranded break made by integrase in a host chromosome (14, 15).

The present study demonstrates that the normal metabolites of AZT can inhibit the HIV-1 integrase protein at concentrations achieved *in vivo*, indicating that integrase inhibition may contribute to the anti-HIV activity of AZT. This result demonstrates that nucleotide analogs can bind to and inhibit HIV-1 integrase. An HIV-1 integrase mutant (IN^{50-212}) lacking the N-terminal zinc finger (first 49 amino acids) and the C-terminal DNA-binding domain (last 66 amino acids) cannot catalyze the 3′-processing and integration reactions but can perform the disintegration reaction (22). For this reason, IN^{50-212} was used to determine whether the AZT metabolites bind to the catalytic core.

MATERIALS AND METHODS

Nucleotides. AZT was obtained from the Division of Cancer Treatment, National Cancer Institute. AZTMP, AZTDP, and AZTTP were purchased from Moravek Biochemicals (La Brea, CA). The purity of AZT, AZTMP, and AZTTP was determined to be 99%, and these drugs were used without further purification. However, the purity of AZTDP was 85.2%. This preparation was lyophilized, reconstituted in 50 μl of water, and subjected to ascending paper chromatography. Two principal spots were seen: a slower moving spot (lower), which consisted of AZTDP (as determined by HPLC), and a faster moving, well-separated spot consisting of AZTMP along with unidentified contaminants. These spots were eluted and lyophilized before analysis of their integrase-inhibitory properties.

Preparation of Radiolabeled DNA Substrates. The following oligonucleotides were purchased from Midland Certified Reagent (Midland, TX): AE118, 5′-GTGTGGAAAATCTCT-AGCAGT-3′; AE146, GGACGCCATAGCCCCGGCGCGG-TCGCTTTC-3′; AE156, 5′-GTGTGGAAAATCTCTAG-

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Abbreviations: AZT, 3′-azido-3′-deoxythymidine; AZTMP, AZT monophosphate; AZTDP, AZT diphosphate; AZTTP, AZT triphosphate; HIV-1, human immunodeficiency virus type 1.

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CAGGGGCTATGGCGTCC-3'; AE117, 5'-ACTGCT-AGAGATTTTCCACAC-3'; AE157, 5'-GAAAGCGAC-CGCGCC-3'. These oligonucleotides were purified by HPLC. The AE117 and AE118 oligonucleotides correspond to the U5 end of the HIV long terminal repeat (8, 16). For the 3'-processing and integration assay, AE118 was 5'-end-labeled using polynucleotide T4 kinase and [γ-^{32}P]ATP. The kinase was heat-inactivated, and an equimolar amount of oligonucleotide AE117 was added. The mixture was heated at 95°C and allowed to cool slowly to room temperature. The reaction was then run on a G-25 Sephadex quick spin column (Boehringer Mannheim) to separate annealed double-stranded oligonucleotide from unincorporated label and single-stranded oligonucleotides. For the disintegration assay (22), oligonucleotide AE157 was 5'-end-labeled as above. Equimolar amounts of oligonucleotides AE117, AE156, and AE146 were added. The mixture was annealed and run on a G-25 Sephadex quick spin column as above.

3'-Processing and Integration Assays. Purified recombinant HIV-1 integrase wild-type and a deletion mutant containing amino acids 50–212 (IN^{50-212}) were from R. Craigie, Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases. The IN^{50-212} lacks the N-terminal zinc finger and C-terminal DNA-binding domains (23). The enzyme to be assayed was preincubated at a final concentration of 0.5 μM at 30°C with inhibitor in reaction buffer [50 mM NaCl/1 mM Hepes/50 μM EDTA/50 μM dithiothreitol/10% (wt/vol) glycerol/7.5 mM $MnCl_2$/bovine serum albumin at 0.1 mg/ml/10 mM 2-mercaptoethanol/10% (wt/vol) dimethyl sulfoxide/25 mM Mops, pH 7.2]. After 30 min, 0.3 pmol of the labeled cleavage/integration substrate was added, and the incubation was continued for an additional 60 min at 30°C. The final reaction volume was 16 μl. The reaction was quenched by the addition of an equal volume of Maxam–Gilbert loading dye, and an aliquot was electrophoresed on a denaturing 20% polyacrylamide gel. Gels were dried and subjected to autoradiography using Kodak XAR-2 film or exposed in a Molecular Dynamics PhosphorImager cassette.

Disintegration Assays. The disintegration reactions were done as above with the substrate shown in Fig. 1 (22).

Quantitation. Gels were analyzed by using a Betascope 603 blot analyzer (Betagen, Waltham, MA). Percent inhibition was calculated as described (8).

Integrase–DNA-Binding Assay. Reaction mixtures (10 μl) containing 0.5 μM integrase were incubated with various concentrations of AZT, AZTMP, or AZTTP in reaction buffer for 30 min at 30°C. One-third of a picomole of radiolabeled double-stranded oligonucleotide (21-mer) was then added to the reaction mixture, and incubation was continued for an additional 5 min. Eight microliters of the reaction mixture was then spotted onto a nitrocellulose filter that had been prewetted with 25 mM Mops, pH 7.2. The filters were washed under vacuum with 5 ml of the same Mops buffer, and the radioactivity retained on the filter was counted to measure DNA retention. Data were corrected for nonspecific binding of free DNA to the filter.

RESULTS

A schematic representation of the three assays used in this report is shown in Fig. 1. The 3'-processing (dinucleotide cleavage) and integration (strand transfer) activities can be quantitated using a 21-mer double-stranded oligonucleotide that represents the U5 end of the HIV long terminal repeat as the substrate (16). Once the dinucleotide GT is cleaved from the 3' end, a strand transfer is catalyzed by the integrase, in which the integrase-processed U5 oligonucleotide with the recessed 3' end is integrated into another U5 oligonucleotide that serves as the target DNA. To study inhibition of the

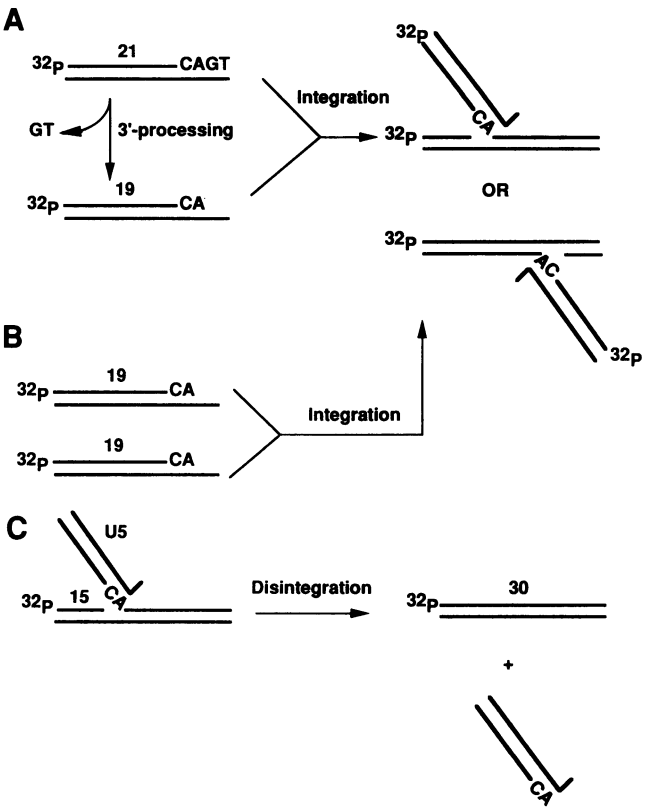

FIG. 1. Schematic representation of HIV-1 integrase assays: 3' processing (dinucleotide cleavage) and integration (strand transfer) with a double-stranded oligonucleotide (*A*), integration (strand transfer) with a "precleaved" or 3'-recessed double-stranded oligonucleotide (*B*), and disintegration with a "Y oligonucleotide" (*C*). The 5' end of the DNA that is radiolabeled is indicated. Size (in nucleotides) of each strand is indicated by the number above it.

strand-transfer reaction alone a "precleaved" 19-mer oligonucleotide is annealed to a 21-mer oligonucleotide. This substrate undergoes the strand-transfer reaction only. The reverse or "disintegration" activity can be studied by using a "Y oligonucleotide" in which the U5 end has been integrated into a target oligonucleotide (22). The integrase can excise this U5 oligonucleotide in a transesterification reaction to generate a radiolabeled 30-mer. An interesting difference between the three assays is that the disintegration assay and the effects of drugs in this assay can be used with integrase deletion mutants because such integrase mutants can still catalyze disintegration while being inactive for 3' processing and integration (23).

Inhibition of HIV-1 Integrase-Catalyzed Reactions. The effects of AZT and its metabolites (AZTMP, AZTDP, and AZTTP) on 3' processing (dinucleotide cleavage) and integration (strand transfer) catalyzed by HIV-1 integrase were quantitated. A typical set of experiments is shown in Fig. 2. AZT does not inhibit integrase at concentrations up to 1 mM.

In contrast, all the AZT nucleotide metabolites inhibit both 3' processing and integration with comparable potency (IC_{50} values between 100 and 150 μM) (Fig. 2). Therefore, AZTTP, which selectively inhibits HIV-1 reverse transcriptase with a K_i of 16 nM (5), inhibits HIV-1 integrase at a concentration four orders of magnitude higher. Interestingly, the integrase inhibition by AZTMP at 100–200 μM is clinically relevant because 4-fold greater concentrations have been found in cells treated with AZT (5).

Partial Reversal of HIV-1 Integrase Inhibition Due to AZTMP with Either TMP or 2',3'-Dideoxythymidine Monophosphate (ddTMP). Incubation of integrase with 162 μM AZTMP yields a 43% inhibition of the 3'-processing reac-

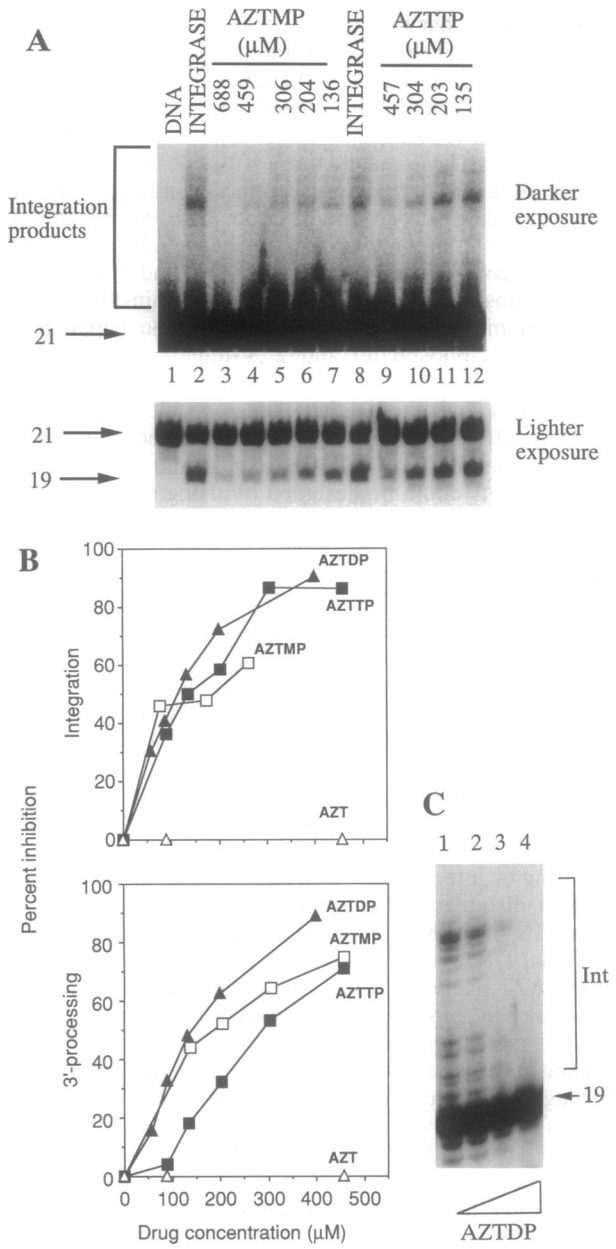

FIG. 2. Inhibition of HIV-1 integrase by AZT and the corresponding nucleotides. (*A*) The 3′-processing and integration reactions with AZTMP or AZTTP. Lanes: 1, labeled DNA alone; 2 and 8, integrase control; 3–7 and 9–12, integrase with the indicated concentrations of AZTMP and AZTTP, respectively. (*Upper*) Dark exposure to show DNA integration (strand transfer) products. (*Lower*) Lighter exposure to show DNA cleavage (3′ processing) product (19-mer, indicated by lower arrow) derived from the DNA substrate (21-mer, indicated by upper arrow). (*B*) Typical dose–response curves of inhibition of HIV-1 integrase by AZT and its three metabolites. Variability between experiments is ± 20%. (*C*) PhosphorImager picture of inhibition of integration (strand transfer) with the precleaved oligonucleotide (see Fig. 1*B*) in the presence of increased AZTDP concentrations. Lanes: 1, integrase control; 2–4, integrase with 100 μM (lane 2), 200 μM (lane 3), or 400 μM (lane 4) AZTDP.

tion, consistent with the dose–response curve in Fig. 2*B*. However, if either TMP or ddTMP are coincubated along with AZTMP, the integrase inhibition is partially reversed, suggesting that both TMP and ddTMP bind to the same site as AZTMP (Table 1). These data suggest that physiologically relevant concentrations of TMP may limit the integrase inhibition due to AZTMP. The reversal of the inhibition is not complete, however, even at very high concentrations of TMP and ddTMP.

Table 1. Reversal of the inhibition of 3′ processing catalyzed by HIV-1 integrase due to AZTMP (162 μM) in the presence of competing nucleotides

Concentration of competitor, μM	Inhibition of 3′ processing, %	
	TMP	ddTMP
0	42.6 ± 8.5	42.6 ± 8.5
10	11.8 ± 2.4	23.4 ± 4.7
50	7.6 ± 1.5	21.2 ± 4.2
500	21.4 ± 4.3	20.1 ± 4.0

AZT Nucleotides Inhibit HIV-1 Integrase Binding to DNA. To investigate the drug mechanisms of inhibition, AZTMP and AZTTP were incubated with integrase, and the formation of enzyme–DNA complexes was measured. In the absence of drug, integrase retained ≈70% of the DNA on the filter, whereas only 12% and 3% of the DNA were cleaved and integrated, respectively. As seen in Fig. 3, both of these AZT metabolites inhibit binding of the enzyme to DNA, whereas AZT itself does not. These results are consistent with the *in vitro* data showing inhibition of integrase by AZT metabolites but not by AZT.

AZT Nucleotides Act on the HIV-1 Integrase Core Region. In an attempt to define the nucleotide-binding site on HIV-1 integrase in greater detail, an integrase deletion mutant (IN^{50-212}) lacking the N-terminal zinc-finger region and the C-terminal DNA-binding domain was assayed for inhibition by the AZT metabolites. The finding that AZTMP (Fig. 4), AZTDP, and AZTTP (data not shown) are active against the IN^{50-212} mutant implies that the binding of the AZT nucleotides to the integrase core region is responsible for integrase inhibition.

DISCUSSION

HIV-1 Integrase Is Inhibited by Clinically Relevant Concentrations of AZTMP. The HIV-1 integrase inhibition by AZTMP (IC_{50} of ≈140 μM for integration) is significant. Because AZTMP accumulates in cells at concentrations up to 1 mM (5, 6), proviral integration can be inhibited by this

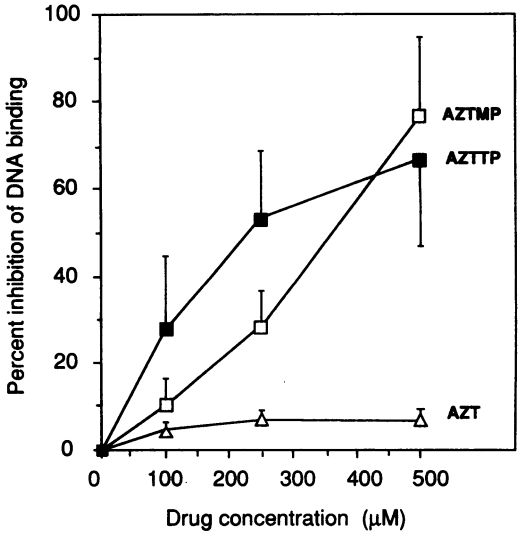

FIG. 3. Inhibition of HIV-1 integrase binding to the double-stranded substrate DNA after preincubation with either AZTMP or AZTTP. Binding was measured by using radiolabeled U5 DNA to nitrocellulose filters. Enzyme binding in the absence of drug was 63.3 ± 13% of total DNA. AZT, AZTMP, and AZTTP are depicted by △, □, and ■, respectively.

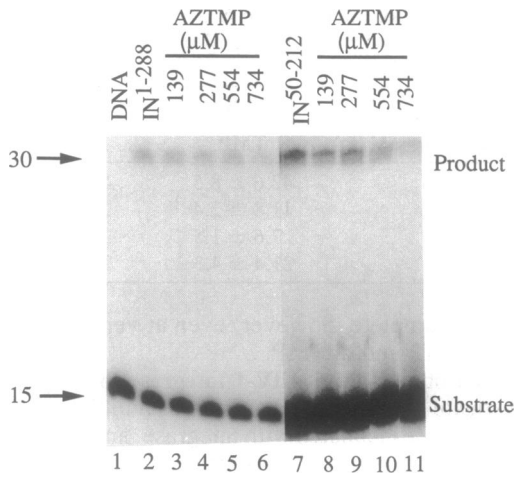

FIG. 4. Effect of AZTMP upon disintegration catalyzed by the full-size HIV-1 integrase (IN^{1-288}) and the deletion mutant IN^{50-212}. Lanes: 1, DNA alone; 2, IN^{1-288} control; 3–6, disintegration with IN^{1-288} and the indicated AZTM*P* concentrations; 7, IN^{50-212} control; 8–11, disintegration with IN^{50-212} and the indicated concentrations of AZTM*P*.

metabolite. This then demonstrates that an AZT metabolite can affect another virally encoded enzyme target.

AZTDP and AZTTP are also active against integrase-catalyzed reactions. However, because only concentrations of 5 and 2 μM, respectively, or lower are detected in cells exposed to AZT (3, 5), inhibition by these metabolites (IC_{50} of ≈120 and 150 μM, respectively, for integration) is probably not clinically relevant.

To better understand the significance of the inhibition by AZTMP, a comparison was made to the inhibition of other enzyme activities reported for this nucleotide. AZTMP has been reported (24) to inhibit the RNase H and DNA polymerase activities of HIV-1 reverse transcriptase with an IC_{50} of 50 and 5000 μM, respectively. The RNase H activity, although inhibited at pharmacologically relevant concentrations of AZTMP and distinct from the targeted DNA polymerase activity, still resides on the same enzyme, reverse transcriptase. Bebenek *et al.* (25) have reported an IC_{50} for (simian virus 40 origin-dependent) DNA replication in HeLa cell extracts of 5000 μM. Harrington *et al.* (26) have reported that AZTMP inhibited the hydrolysis of AZTMP-terminated DNA substrates catalyzed by a 3′-exonuclease (not associated with DNA polymerase δ, γ, or ε) with an IC_{50} of 250 μM. Bridges *et al.* (27) have demonstrated that AZTMP inhibits the 3′–5′ exonuclease activity of DNA polymerase δ with an IC_{50} between 100–250 μM. Taken together, these results suggest that the toxicity of AZT could be due to the incorporation of AZTT*P* into DNA coupled to a lack of removal of this nucleotide from DNA due to a block in the repair of AZTMP-terminated DNA. Interestingly, DNA polymerase activities, whether viral or human, are not very sensitive to this metabolite at clinically relevant concentrations. However, the RNase H activity of HIV-1 reverse transcriptase, integrase activities of HIV-1 integrase, and exonuclease activities are particularly sensitive. These latter activities all involve hydrolysis of a phosphodiester bond. However, HIV-1 integrase acts as an endonuclease because it liberates a dinucleotide, and it can use the 3′-hydroxyl end of its substrate as a nucleophile to attack the scissile phosphodiester bond as well.

Structure–Activity Relationships and Mechanism of HIV-1 Integrase Inhibition. The inhibition of integrase by AZT nucleotides and the fact that ddTMP and TMP both competed with AZTMP for inhibition of HIV-1 integrase (Table 1) suggest that there is a nucleotide-binding site in the integrase. It is interesting that the AZT nucleotides are active against HIV-1 integrase in light of the fact that the thymidine and 2′-deoxy- and 2′,3′-dideoxythymidine mono- and triphosphates show no inhibition against either 3′ processing or integration up to 0.8 mM (A.M. and Y.P., unpublished observations). These results suggest the possibility that the absence of a hydroxyl group at the 3′ position and the presence of an azido group are important determinants for activity against the integrase. Further evidence for this hypothesis comes from the fact that ATP and 2′-deoxyadenosine triphosphate (dATP) are not active against integration below 900 mM, whereas 3′-deoxyadenosine triphosphate (cordycepin triphosphate) and 2′,3′-dideoxyadenosine triphosphate (ddATP) are active (A.M. and Y.P., unpublished observations). Taken together, these results suggest that structure–activity relationships can be derived by using nucleotides as inhibitors of HIV-1 integrase and that additional antiviral agents could be designed by using nucleotide analogs and purified HIV-1 integrase.

Filter binding assays (Fig. 3) demonstrate that, if integrase is preincubated with an AZT metabolite, the enzyme binds substrate DNA less efficiently. One interpretation of these results is that the AZT metabolites bind to a site on the HIV-1 integrase, forming a binary complex that is then unable to catalyze subsequent 3′-processing and integration reactions after incubation with the substrate DNA, due to either distortion of enzyme–DNA contacts or destabilization of protein structure.

The disintegration assay (22) provides a convenient tool to measure the effects of drugs on integrase deletion mutants because these mutants can catalyze this reaction but not 3′ processing or integration. The AZT nucleotides were found to inhibit disintegration catalyzed by an integrase deletion mutant (IN^{50-212}) that lacks the N-terminal zinc finger and C-terminal DNA-binding domains (23). These results show that the AZT nucleotides bind to the enzyme catalytic core.

Relevance to Antiviral Drug Resistance. The emergence of AZT-resistant strains of HIV-1 has focused intense effort on mechanisms of viral resistance to AZT. A logical mechanism is through a mutation in the reverse transcriptase, the target of AZTTP. In fact, several isolates have been sequenced and found to have multiple mutations (28) in the reverse transcriptase domain involved in nucleotide recognition and enzyme function (29, 30). Four mutations at codons 67, 70, 215, and 219 appear common, with the most prevalent being Thr-215 → Tyr (31). However, purified reverse transcriptase from AZT-resistant isolates often shows no detectable drug resistance to AZTTP *in vitro* (28, 32, 33). The data in this report suggest that mutations may also exist in the integrase protein from these AZT-resistant isolates.

In summary, AZTMP, which accumulates in cells, has been found to inhibit the HIV-1 integrase. Therefore, the findings presented in this report suggest that the inhibition (by AZTTP) of the DNA polymerase activity of reverse transcriptase and the inhibition (by AZTMP) of the RNase H activity of reverse transcriptase and of viral DNA integration by HIV-1 integrase may contribute to the *in vivo* potency of AZT. New strategies for antiviral drug development could be based upon nucleotide analogs as inhibitors of the HIV-1 integrase.

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