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# Effects of Nucleotide Analogues on Human Immunodeficiency Virus Type 1 Integrase

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## SUMMARY

We extended our previous study with 3′-azido-3′-deoxythymidine nucleotides [*Proc. Natl. Acad. Sci. USA* **91**:5771-5775 (1994)] and examined the effects on human immunodeficiency virus type 1 (HIV-1) integrase of the nucleotides of three nucleoside analogues currently under evaluation in clinical trials: β-D-2′,3′-didehydro-3′-deoxythymidine, β-D-2′-*ara*-fluoro-2′,3′-dideoxyadenosine, and β-L-2′,3′-dideoxy-3′-thiacytidine. β-D-2′,3′-Didehydro-3′-deoxythymidine and β-D-2′-*ara*-fluoro-2′,3′-dideoxyadenosine nucleotides had $IC_{50}$ values for strand transfer of 100 and 200 μM, respectively, whereas the corresponding 2′,3′-dideoxynucleoside triphosphates, ddT triphosphate and ddA triphosphate, did not inhibit the integrase at 800 and 200 μM, respectively. β-L-2′,3′-Dideoxy-3′-thiacytidine triphosphate had no effect up to 500 μM. The L-enantiomers of 5-fluoro-2′,3′-dideoxycytidine monophosphate and triphosphate had $IC_{50}$ values of ~40 μM, whereas their D-enantiomer isomers showed no inhibition at 200 μM. NAD, pyridoxal phosphate, and coumermycin A1, which exhibit no antiviral activity but are typically used to probe nucleotide binding sites, were also tested. NAD was inactive, and its etheno derivative exhibited activity at 1 mM. In contrast, pyridoxal phosphate ($IC_{50}$ = 18 μM) and coumermycin A1 ($IC_{50}$ = 5 μM) were potent inhibitors. None of the coumermycin monomeric derivatives were active integrase inhibitors. The physiological ribonucleotides ATP and GTP inhibited HIV-1 integrase at or near cellular concentrations, suggesting that they may regulate HIV-1 integrase activity in cells. In general, the active nucleotides tested inhibited binding of HIV-1 integrase to its substrate DNA and inhibited an integrase deletion mutant containing only amino acids 50–212, indicating that nucleotides bind to the enzyme catalytic core. Consistently, the choice of nucleophile in the 3′-processing reaction was blocked to the same extent regardless of the nucleotide used (water, glycerol, or the viral DNA hydroxyl) by the enzyme. These observations suggest new strategies for antiviral drug development that could be based on nucleotide analogues as inhibitors of HIV-1 integrase.

Retroviruses encode the integrase protein at the 3′-end of the *pol* gene. This enzyme integrates a double-stranded DNA copy of the viral RNA genome, synthesized by RT, into a host chromosome. Integrase first catalyzes the excision of the last two nucleotides from each 3′-end of the linear viral DNA, leaving the terminal conserved dinucleotide CA-3′-OH at these recessed 3′ ends. This activity is referred to as the 3′ processing. After transport to the nucleus as a nucleoprotein complex, integrase catalyzes a DNA strand transfer reaction involving the nucleophilic attack of these ends on a host chromosome [for a review, see Katz and Skalka (1)]. Because of the requirement for a functional integrase in HIV-1 replication (2, 3), this enzyme is a suitable target for chemotherapeutic intervention (4, 5).

Previous studies have shown that a simple modification at the 2′ and 3′ positions to give a 2′,3′-dideoxy sugar in nucleosides can convert a normal substrate for nucleic acid synthesis into a potent inhibitor of HIV (6). Dideoxynucleosides are

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**ABBREVIATIONS:** HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; MP, monophosphate; TP, triphosphate; D4T, β-D-2′,3′-didehydro-3′-deoxythymidine; ddA, β-D-2′,3′-dideoxyadenosine; FddA, β-D-2′-*ara*-fluoro-2′,3′-dideoxyadenosine; FddC, β-L-5-fluoro-2′,3′-dideoxycytidine; 3TC, β-L-2′,3′-dideoxy-3′-thiacytidine; ddC, β-D-2′,3′-dideoxycytidine; ddI, β-D-2′,3′-dideoxyinosine; ddT, β-D-3′-deoxythymidine; FLT, β-D-3′-fluoro-3′-dideoxythymidine; ddG, β-D-2′,3′-dideoxyguanosine; AMT, β-D-3′-amino-3′-deoxythymidine; FDOC, β-L-5-fluoro-dioxolanylcytosine; AZT, 3′-azido-3′-deoxythymidine; SDS, sodium dodecyl sulfate.

phosphorylated in the cytoplasm of host cells to produce the corresponding 5′-triphosphate, which can then compete with normal nucleotides for RT and cause chain termination.

Several other nucleoside analogues (see Fig. 1) such as ddC ($IC_{50}$ = 0.2 μM for HIV-1 replication) (7–9), ddI ($IC_{50}$ = 3.5 μM) (10), FLT ($IC_{50}$ = 0.003 μM) (11), 3TC ($IC_{50}$ = 0.51 μM) (10), D4T ($IC_{50}$ = 0.62 μM) (10), β-D-2′,3′-dideoxyguanosine ($IC_{50}$ = 7.2 μM) (10), and FddA ($IC_{50}$ = 34 μM) (10) have been investigated as inhibitors of HIV-1 replication. Due to the toxicities of some of these nucleoside analogues (12) and to the appearance of drug-resistant viruses (13), combinations of antiretroviral drugs have generated much interest (14). Combinations of dideoxynucleosides have also been shown to be additive or synergistic (15, 16).

We previously demonstrated that AZT nucleotides inhibit HIV-1 integrase with an $IC_{50}$ value of 110–150 μM (17). Inhibition of an integrase deletion mutant containing only amino acids 50–212 suggested that these nucleotides bind in the catalytic core. Concentrations of ≤1 mM of AZTMP can accumulate intracellularly (18), indicating that integrase inhibition may contribute to the antiviral effects of AZT.

The present study is a further investigation of pharmacological and physiological mononucleotides as potential inhibitors of HIV-1 integrase. In addition, the antibiotic coumermycin A1, a *gyr*B inhibitor, was also found to inhibit *in vitro* HIV-1 integrase.

## Materials and Methods

**Nucleotides and analogues.** Dideoxynucleoside, deoxynucleoside, and ribodeoxynucleoside TPs were purchased from Boehringer Mannheim. NAD and analogues were purchased from Sigma. Coumermycin A1 and analogues were obtained from Dr. Anthony J. Razel (Bristol Myers Squibb, Wallingford, CT). FddATP was obtained from Dr. Victor Marquez (Laboratory of Medicinal Chemistry, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD). The nucleotides of β-D-thymidine and β-L-cytidine analogues were prepared according to a standard phosphorylation method (19) from their corresponding nucleosides. The 5′-monophosphate and 5′-triphosphate derivatives were fully characterized with the use of NMR ($^1H$ and $^{31}P$), fast atom bombardment mass spectroscopy, high performance liquid chromatography, and UV spectroscopy.

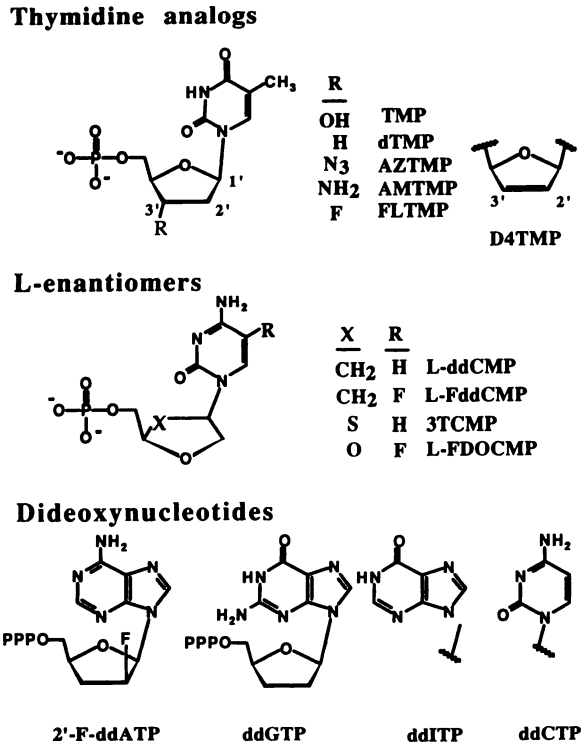


**Fig. 1.** Structures of the nucleotide analogues tested against HIV-1 integrase.

**Preparation of radiolabeled DNA substrates.** The following oligonucleotides were high performance liquid chromatography purified by and purchased from Midland Certified Reagent Company (Midland, TX): AE117, 5′-ACTGCTAGAGATTTTCCACAC-3′; AE118, 5′-GTGTGGAAAATCTCTAGCAGT-3′; AE157, 5′-GAAAGCGACCGCGCC-3′; AE146, 5′-GGACGCCATAGCCCCGGCGCGGTCGCTTTC-3′; AE156, 5′-GTGTGGAAAATCTCTAGCAGGGGCTATGGCGTCC-3′; AE118S, 5′-GTGTGGAAAATCTCTAGCA-3′; and RM22M, 5′-TACTGCTAGAGATTTTCCACAC-3′. The AE117, AE118, and the first 19 nucleotides of AE156 correspond to the U5 end of the HIV-1 long terminal repeat.

To analyze the extents of 3′ processing and strand transfer using 5′-end labeled substrates, AE118 was 5′-end labeled with $T_4$ polynucleotide kinase (GIBCO-BRL) and [γ-$^{32}$P]ATP (DuPont-NEN).The kinase was heat-inactivated, and AE117 was added to the same final concentration. The mixture was heated at 95°, allowed to cool slowly to room temperature, and run on a G-25 Sephadex quick spin column (Boehringer Mannheim, Indianapolis, IN) to separate annealed double-stranded oligonucleotide from unincorporated label.

To analyze the extents of 3′ processing and strand transfer using 3′-end labeled substrates, AE118 was 3′-end labeled with [α-$^{32}$P]cordycepin TP (DuPont-NEN) and terminal transferase (Boehringer Mannheim). The transferase was heat-inactivated, and RM22M was added to the same final concentration. The mixture was heated at 95°, allowed to cool slowly to room temperature, and run on a G-25 spin column as before.

To determine the extent of 30-mer target strand generation during disintegration, AE157 was 5′-end labeled, annealed to AE156, AE146, and AE117, and column purified as above.

**Integrase proteins.** Purified recombinant wild-type HIV-1 integrase and deletion mutant $IN^{50-212}$ were generous gifts of Drs. R. Craigie and A. Engelman (Laboratory of Molecular Biology, National Institute for Diabetes, Digestive, and Kidney Diseases, National Institutes of Health, Bethesda, MD).

**3′ Processing, strand transfer, and disintegration assays.** Integrase was incubated at a final concentration of 200 nM with inhibitor in reaction buffer [50 mM NaCl, 1 mM HEPES, pH 7.5, 50 μM EDTA, 50 μM dithiothreitol, 10% glycerol (w/v), 7.5 mM $MnCl_2$, 0.1 mg/ml bovine serum albumin, 10 mM 2-mercaptoethanol, 10% dimethylsulfoxide, and 25 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.2] at 30° for 30 min. The oligonucleotide substrate was then added and incubation was continued for an additional 60 min. The final reaction volume was 16 μl.

Disintegration reactions were performed as above with a Y oligonucleotide (i.e., the branched substrate in which the U5 end was "integrated" into target DNA).

**Electrophoresis and quantification.** Reactions were quenched by the addition of an equal volume (16 μl) of Maxam-Gilbert loading dye (98% deionized formamide, 10 mM EDTA, 0.025% xylene cyanol, 0.025% bromphenol blue). An aliquot (5 μl) was electrophoresed on a denaturing 20% polyacrylamide gel (0.09 M Tris-borate pH 8.3, 2 mM EDTA, 20% acrylamide, 8 M urea). Gels were dried and exposed in a Molecular Dynamics PhosphorImager cassette. Gels were analyzed with a Molecular Dynamics PhosphorImager (Sunnyvale, CA). Percent inhibition was calculated with the equation $100 \times [1-(D-C)/(N-C)]$ where $C$, $N$, and $D$ are the fractions of 21-mer substrate converted to 19 mer (3′ processing product) or strand transfer products for DNA alone, DNA plus integrase, and integrase plus drug, respectively. $IC_{50}$ was determined by plotting the drug concentration

versus percent inhibition and determining the concentration that produced 50% inhibition.

**UV cross-linking experiments.** Integrase was preincubated with inhibitor and then with substrate in reaction buffer as above for 5 min at 30°. Reactions were then irradiated with a UV transilluminator (254 nm wavelength) from 3 cm above (2.4 mW/cm$^2$) at room temperature for 10 min. An equal volume (16 μl) of 2× SDS-polyacrylamide gel electrophoresis buffer (100 mM Tris, pH 6.8, 4% 2-mercaptoethanol, 4% SDS, 0.2% bromphenol blue, 20% glycerol) was added to each reaction. Then, 20-μl aliquots were heated at 95° for 3 min before loading onto a 12% SDS-polyacrylamide gel. The gel was run at 120 V for 1.5 hr, dried, and exposed in a PhosphorImager cassette.

## Results

### Inhibition of HIV-1 Integrase by Nucleotide Analogs that Inhibit HIV-1 Replication

**The β-D-thymidine analogues.** D4T inhibits HIV-1 replication *in vitro* at concentrations of <0.01 μM. The 5′-triphosphate of FLT is a potent inhibitor of both hepatitis B virus DNA polymerase (20) and HIV-1 RT (11). Its corresponding nucleoside protects MT-4 cells against HIV at an $IC_{50}$ of 3 nM. The MP of FLT accumulates to a concentration of 198 pmol/$10^6$ cells (~132 μM) when H9 cells infected with HIV-1 are treated with 50 μM FLT (11).

The effects of the MP form of the two β-D-thymidine analogues, FLT and D4T (Fig. 1), on 3′ processing and strand transfer are shown in Fig. 2A, with the principle of the assays shown in Fig. 2C. The 3′ processing reaction (Fig. 2C, *step 1*) liberates a GT dinucleotide, resulting in the generation of a 19-mer oligonucleotide from a 21-mer substrate. The strand transfer reaction (Fig. 2C, *step 2*) results in the insertion of one 3′-processed oligonucleotide into another target DNA, yielding higher molecular mass species that migrate slower than the 21-mer substrate. Although TMP and ddTMP did not inhibit HIV-1 integrase even at 600 μM (*lanes 4 and 6*), D4TMP and FLTMP exhibited $IC_{50}$ values of ~100 μM (Table 1). To determine whether the strand transfer reaction was truly being inhibited or whether inhibition of the 3′ processing reaction caused the decrease in the subsequent strand transfer, a precleaved (19-mer) oligonucleotide substrate was used (Fig. 2C, *middle*). The effects of D4TMP and FLTMP in this assay are shown in Fig. 2B. Again, the strand transfer activity was inhibited, with $IC_{50}$ values of ~100 μM, implying that D4TMP and FLTMP inhibited both steps (3′ processing and strand transfer) of the integration reaction.

AMT is generated *in vivo* by reduction of AZT (21). The $IC_{50}$ values of AMTMP for strand transfer and 3′ processing (Table 1) are in the same range as those observed for AZTMP (17).

**The β-enantiomers of cytidine analogues.** ddC-5′-TP is a potent inhibitor of HIV-1 RT. Another dideoxycytidine analogue, 3TC, has also been shown to possess anti-HIV activity, with an $IC_{50}$ of 0.73 μM, while exhibiting less toxicity than AZT (22). Two ideas have resulted in the synthesis of nucleoside analogues such as 3TC in which carbon atoms in the deoxyribose are replaced by heteroatoms: 1) the high anti-HIV activity of dideoxynucleosides that have a strong electron-withdrawing group in the 3′ position and 2) the desire to provide nucleosides with a suitable measure of stability against rapid enzymatic and chemical degradation *in vivo* (23). Recently, the biochemical basis for the puzzling

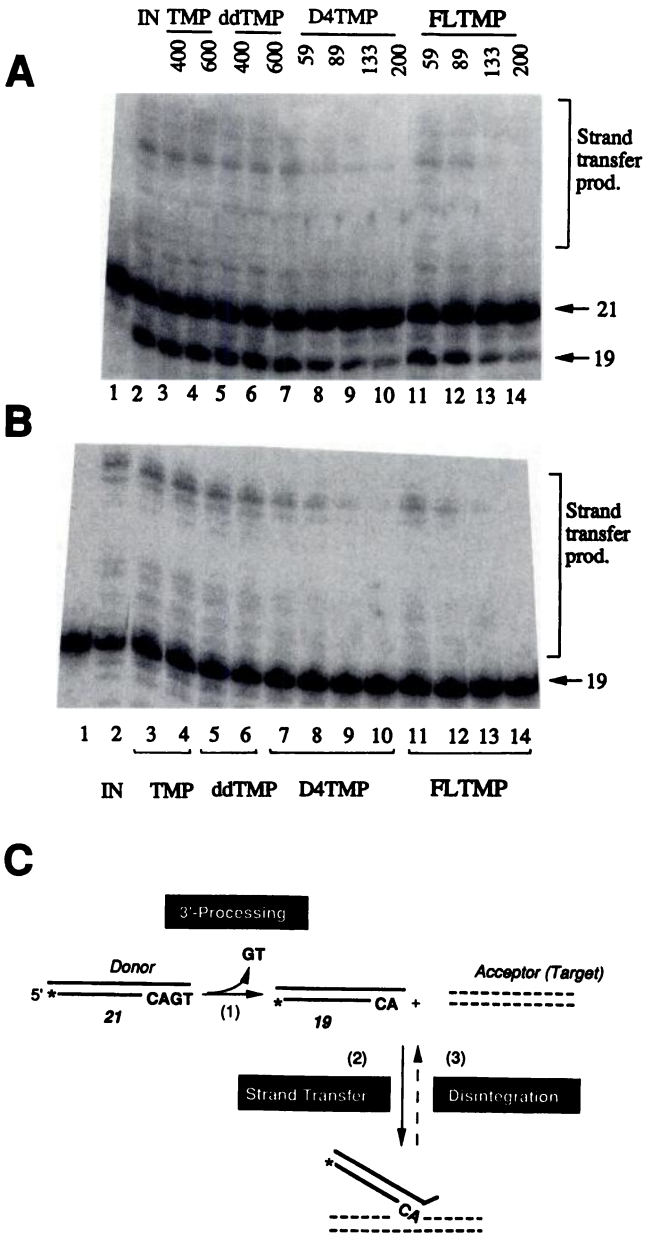


**Fig. 2.** Inhibition of HIV-1 integrase-catalyzed 3′ processing and strand transfer by thymidine analogues. *Lane 1*, DNA alone; *lane 2*, DNA plus integrase (*IN*) without nucleotide; *lanes 3 and 4*, DNA plus integrase in the presence of TMP; *lanes 5 and 6*, DNA plus integrase in the presence of ddTMP; *lanes 7–10*, DNA plus integrase in the presence of D4TMP; *lanes 11–14*, DNA plus integrase in the presence of FLTMP. Micromolar drug concentrations are indicated *above each lane*. *Bracket*, DNA strand transfer products. A, *top arrow*, DNA substrates (21 mer); *bottom arrow*, 3′ processing product (19 mer). B, *arrow*, DNA substrates (19 mer). A, PhosphorImager picture showing 3′ processing and strand transfer reactions. B, PhosphorImager picture showing inhibition of strand transfer using the precleaved oligonucleotide (19 mer substrate). C, Scheme showing principle of the dual 3′ processing and strand transfer, strand transfer, and disintegration assays.

difference in the anti-HIV activities of the L- and D-enantiomers of 2′,3′-dideoxy-3′-thiacytidine (24) has been elucidated. The differential *in vitro* antiviral activities of the two enantiomers has been suggested to result from different catabolic and metabolic pathways rather than from an enantioselectivity at the RT target (25). Another dideoxycytidine analogue, β-L-5-fluoro-dioxolanylcytosine, in which the 3′

TABLE 1

**Inhibition of 3′ processing and DNA strand transfer with nucleotide analogues**

| | $IC_{50}$[a] | |
|---|---|---|
| | 3′ Processing | Strand transfer |
| | μM | |
| D4TMP | 110 ± 20 | 95 ± 15 |
| FdTMP | 95 ± 15 | 70 ± 12 |
| AMTMP | 290 ± 60 | 195 ± 40 |
| ddATP | 250 ± 50 | 230 ± 60 |
| 3′dATP | 220 ± 50 | 185 ± 40 |
| FddATP | 220 ± 38 | 195 ± 32 |
| ddTMP | >1000 | >1000 |
| ddGTP | 660 ± 100 | 640 ± 100 |
| ddCTP | 550 ± 150 | 550 ± 125 |
| ddITP | >1000 | >1000 |
| L-ddATP | 160 ± 40 | 170 ± 38 |
| L-AZTTP | >225 | 145 ± 45 |
| L-ddCMP | 50 ± 8 | 45 ± 10 |
| L-5FddCMP | 46 ± 6 | 39 ± 4 |
| L-5FddCTP | 68 ± 2 | 48 ± 10 |
| ATP | 2400 ± 900 | 2700 ± 900 |
| ATP-γ-S | 1200 ± 300 | 1000 ± 200 |
| GTP | 2000 ± 400 | 1100 ± 200 |
| L-FDOCMP | >195 | >195 |
| TMP | >800 | >800 |
| ddTMP | >800 | >800 |
| 3TCMP | >200 | >200 |
| Amethopterin | 400 | 400 |
| Trimethoprim | >1000 | >1000 |
| Dipyridamole | >1000 | >1000 |

[a] The values reported are the mean of two or three independent experiments. The standard deviation between experiments is sometimes shown.

carbon has been replaced by an oxygen, has been developed for the same reasons as 3TC (9).

The effects of the nucleotide of these cytidine analogues on HIV-1 integrase were assessed (Fig. 3 and Table 1). Neither ddCTP nor β-D-FddCTP showed activity against HIV-1 integrase. However, the L-enantiomers of FddCTP and FddCMP were active against both 3′ processing and strand transfer. Therefore, the substitution of a L-deoxyribose for the normal D-sugar yields a marked increase in the potency, whereas a substitution on the heterocycle does not. The phosphorylation state of the cytidine analogue (i.e., whether MP or TP) does not seem to make a difference, which is consistent with our previous observations with AZT nucleotides (17). This marked enhancement in potency (Table 1) seen with the L-enantiomer of the deoxyribose moiety was not observed with other nucleotides. For example, the L-enantiomers of AZTTP, ddATP, and ddCTP showed only 1.5–2-fold increases in potency (Table 1).

**The purine 2′,3′-dideoxynucleosides.** ddI exerts its antiretroviral effect through inhibition of RT by its intracellular metabolite, ddATP (26). The ddATP $IC_{50}$ for strand transfer was 230 μM (Table 1). FddA is currently under investigation as an inhibitor of HIV-1 replication (10). Its pharmacologically active metabolite, FddATP (Fig. 1), was active against HIV-1 integrase in the same concentration range as ddATP (Table 1).

### Inhibition of HIV-1 Integrase by Physiological Deoxyribonucleotide and Ribonucleotide TPs

Deoxynucleoside TP levels in thymocytes, HL-60, H9, K-562, U937, and CEM cell lines have been reported in the range of 4–150 μM (27). Levels of ribonucleoside TPs (e.g., ATP and GTP) in CEM and WI-L2 human lymphoblasts cells have been found in the range of 160-4900 μM (27).

The finding that nucleotide analogues can inhibit HIV-1 integrase prompted us to investigate the effects of physiological nucleotides against HIV-1 integrase. GTP and ATP inhibited HIV-1 integrase in their biological concentration range (millimolar concentration) (Table 1 and Fig. 4). Interestingly, the nonhydrolyzable ATP analogue ATP-γ-S, in which a nonbridging oxygen in the γ-phosphate group of ATP is replaced by a sulfur, was ~3–4-fold more potent than ATP

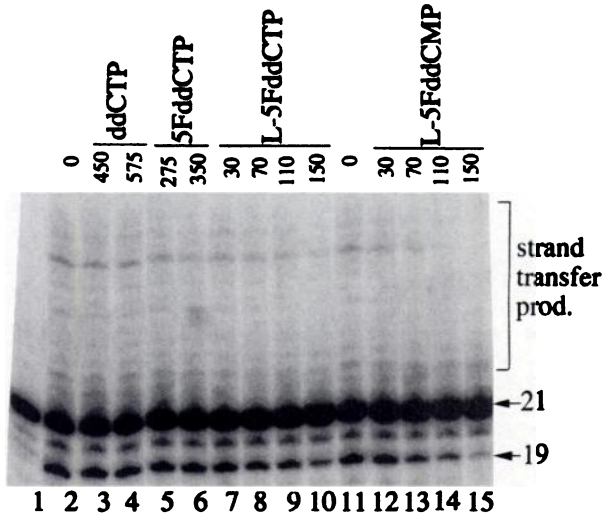


**Fig. 3.** Inhibition of HIV-1 integrase by L- and D-enantiomers of cytidine analogues. *Lane 1*, DNA alone; *lanes 2 and 11*, DNA plus integrase without nucleotide; *lanes 3 and 4*, DNA plus integrase in the presence of ddCTP; *lanes 5 and 6*, DNA plus integrase in the presence of 5-F-ddCTP; *lanes 7–10*, DNA plus integrase in the presence of L-5-F-ddCTP; *lanes 12–15*, DNA plus integrase in the presence of L-5-F-ddCMP. Micromolar drug concentrations are indicated *above each lane*. *Bracket*, DNA strand transfer products; *top arrow*, DNA substrate (21 mer); *bottom arrow*, 3′ processing product (19 mer).

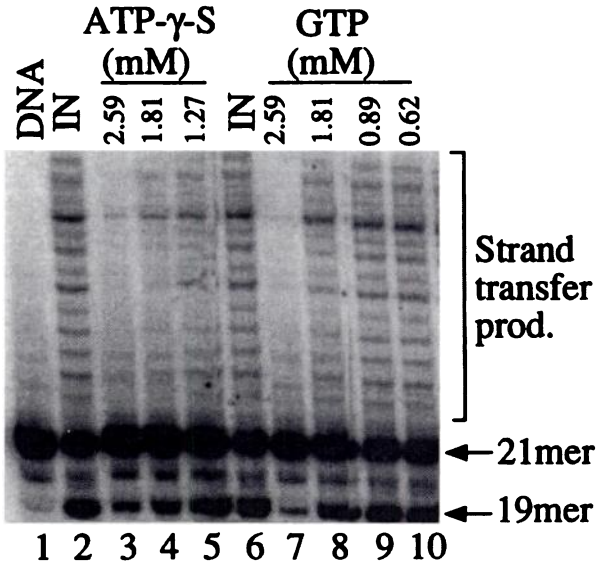


**Fig. 4.** Inhibition of HIV-1 integrase by ATP-γ-S and GTP. *Lane 1*, DNA alone; *lanes 2 and 6*, DNA plus integrase (*IN*) without nucleotide; *lanes 3–5*, DNA plus integrase in the presence of ATP-γ-S; *lanes 7–10*, DNA in the presence of GTP. Drug concentrations are indicated *above each lane*. *Bracket*, DNA strand transfer products; *top arrow*, DNA substrate (21 mer); *bottom arrow*, 3′ processing product (19 mer).

(Table 1 and Fig. 4). The deoxyribonucleotides, although active against integrase in the same concentration range as their ribonucleotide counterparts, did not show any activity at the lower concentrations in which they are present in cells (data not shown).

### Probing the Nucleotide Binding Site

**NAD and analogues.** NAD and several of its analogues (NMN, α-NAD, NADH, flavin adenine dinucleotide, NADP, NADPH, and nicotinamide hypoxanthine dinucleotide) were inactive at 1 mM. However, 3′-NAD phosphate and nicotinamide ethenoadenine dinucleotide had an $IC_{50}$ value of ~1 mM for both 3′ processing and strand transfer (data not shown).

**Coumermycin A1.** The coumarin family of antibiotics has been shown to target the DNA gyrase B protein (28) and topoisomerases I and II (29). These drugs inhibit the ATP hydrolysis activity of topoisomerase II enzymes and, therefore, are thought to bind to the same site as ATP (28). Coumermycin A1 inhibited both 3′ processing and strand transfer, with $IC_{50}$ values of ~10 μM, but all monomeric analogues tested were inactive (data not shown).

**Pyridoxal phosphate.** Pyridoxal phosphate has been used as a nucleotide binding probe for the DNA polymerases. Previously, it has been shown to completely inhibit HIV-1 integrase at 0.6 mM and to form a Schiff base with a nearby lysine ε-amino group (30). We investigated the concentration dependence of the inhibition of HIV-1 integrase by pyridoxal phosphate. Fig. 5 shows the significant inhibition of both 3′ processing and strand transfer at 50 μM (*lane 5*). The $IC_{50}$ values for 3′ processing and strand transfer were 25 and 18 μM, respectively.

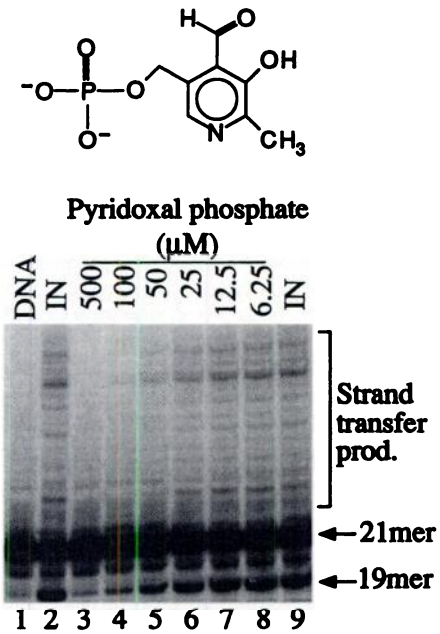


**Fig. 5.** Inhibition of HIV-1 integrase by pyridoxal phosphate. *Lane 1*, DNA alone; *lanes 2 and 9*, DNA plus integrase (*IN*) without drug; *lanes 3–8*, DNA plus integrase in the presence of pyridoxal phosphate. Drug concentrations are indicated above each lane. *Bracket*, DNA strand transfer products; *top arrow*, DNA substrate (21 mer); *bottom arrow*, 3′ processing product (19 mer).

### Effect of Nucleotides on the Choice of Nucleophile in the 3′ Processing Reaction

The mechanism of the inhibition of the 3′ processing reaction by nucleotides was probed by labeling the substrate DNA at the 3′-end (31, 32). Integrase can use glycerol, water, or the hydroxyl group of the viral DNA terminus as nucleophiles in the 3′ processing reaction, yielding a linear trinucleotide with a glycerol esterified to the 5′-phosphate (Fig. 6, *G*), a linear trinucleotide with a 5′-phosphate (*L*), or a circular trinucleotide (*C*), respectively. All of the nucleotides tested inhibited glycerolysis, hydrolysis, and circular nucleotide formation to the same extent (Fig. 6).

### Effect of Nucleotides on the HIV-1 Integrase Core Region

Integrase can also catalyze an apparent reversal of the DNA strand transfer reaction, called disintegration *in vitro* (Fig. 2C) (33). In contrast to the 3′ processing and strand transfer reactions, disintegration does not require either the amino-terminal zinc-finger region or the carboxyl-terminal DNA-binding domain (34). For this reason, an integrase deletion mutant $IN^{50-212}$ lacking both of these domains can be used in this assay (34) to define in more detail the nucleotide binding site on HIV-1 integrase. The finding that D4TMP and FLTMP are active against the $IN^{50-212}$ mutant (Fig. 7) implies that the binding of nucleotides to the integrase core region is responsible for integrase inhibition. These results are consistent with those obtained previously with AZT nucleotides (17).

### Inhibition of DNA Binding by HIV-1 Integrase

To determine whether DNA binding was affected by the nucleotides, UV cross-linking of integrase DNA reactions was performed. Cross-linking of substrate DNA to integrase followed by electrophoresis produces an enzyme/DNA complex with a molecular mass of ~39 kDa. As seen in Fig. 8, all of the nucleotides tested can inhibit binding of integrase to its DNA substrate. For example, significant inhibition of binding is seen in the presence of 2 mM ATP (*lane 4*), 1.2 mM ATP-γ-S (*lane 9*), and 250 μM ddATP (*lane 13*). These concentrations are in the same range as those that inhibit 3′ processing and strand transfer activities. Therefore, inhibition of HIV-1 integrase activity correlates with inhibition of DNA binding by the enzyme. These results are consistent with those obtained previously with AZT nucleotides with the use of filter binding assays (17).

## Discussion

**Nucleotide analogues as inhibitors of HIV-1 integrase.** The results of this study demonstrate the inhibition of HIV-1 integrase by nucleotide analogues at lower concentrations than those needed for inhibition by physiological nucleotides. $IC_{50}$ values for strand transfer ranged from 35 to 600 μM (Table 1). The base and the phosphorylation state of the nucleoside (i.e., MP, diphosphate, or TP) seem to be less important than modification to the sugar moiety.

Three examples point to the importance of deoxyribose modifications. First, unsaturation at the 2′ and 3′ positions converts dTMP, an inactive nucleotide, into D4TMP, a nucleotide with potency in the 100 μM range. Second, the presence of electron-withdrawing and electron-donating moieties at the 3′ position correlated with potency. Moreover, removal

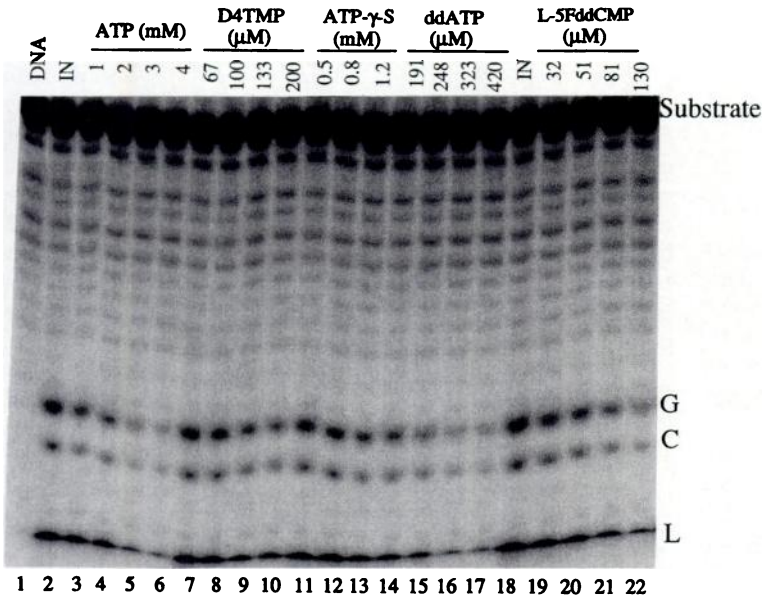


**Fig. 6.** Global inhibition of nucleophilic attack in the 3′ processing reaction by nucleotides. *Lane 1*, DNA alone; *lanes 2 and 18*, DNA plus integrase (*IN*) without nucleotide. *lanes 3–6*, DNA plus integrase in the presence of ATP; *lanes 7–10*, DNA plus integrase in the presence D4TMP; *lanes 11–13*, DNA plus integrase in the presence ATP-γ-S; *lanes 14–17*, DNA plus integrase in the presence ddATP; *lanes 19–22*, DNA plus integrase in the presence L-5-F-ddCMP. *G, C, and L*, 3′ processing products; *substrate*, DNA substrate.

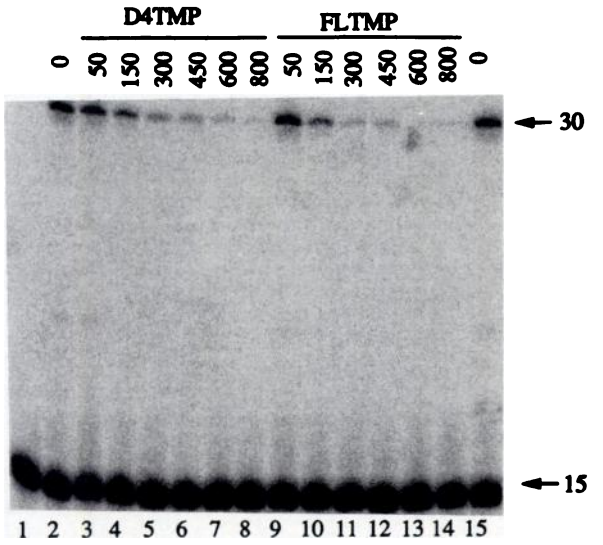


**Fig. 7.** Inhibition of disintegration catalyzed by the HIV-1 integrase deletion mutant $IN^{50-212}$. PhosphorImager picture showing disintegration reactions. *Lane 1*, substrate DNA alone (15 mer); *lanes 2 and 15*, DNA plus $IN^{50-212}$; *lanes 3–8*, $IN^{50-212}$ in the presence of the indicated micromolar concentrations of D4TMP; *lanes 9–14*, disintegration in the presence of the indicated micromolar concentrations of FLTMP. *Top arrow*, disintegration product (30 mer).

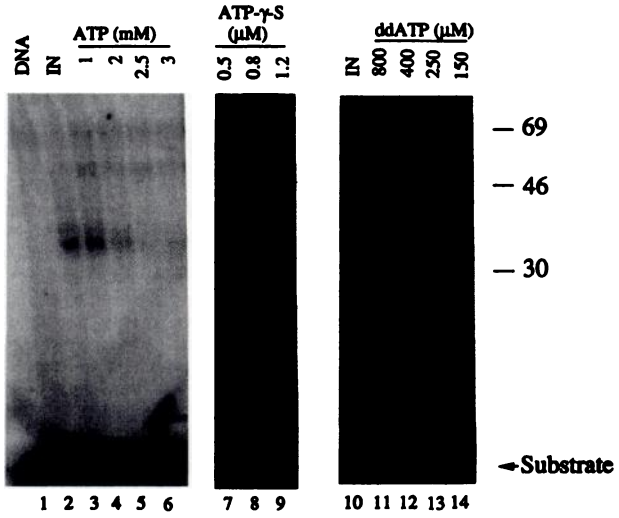


**Fig. 8.** Effects of nucleotide analogues on the extent of DNA binding of HIV-1 integrase. PhosphorImager picture showing UV crosslinking of wild-type integrase with duplex U5 oligonucleotide substrates in the presence of nucleotides. *Right*, migrations of proteins of known molecular mass. *Lane 1*, DNA alone; *lanes 2 and 10*, DNA plus integrase (*IN*) without nucleotide; *lanes 3–6*, DNA plus integrase in the presence of ATP, *lanes 7–9*, DNA plus integrase in the presence of ATP-γ-S; *lanes 11–14*, DNA plus integrase in the presence of ddATP.

of an hydroxyl group from the 3′ position in ATP, yielding cordycepin TP, enhanced potency against HIV-1 integrase. Third, substitution of a β-L-enantiomer for its β-D counterpart generally increased potency. These data suggest that interactions between HIV-1 integrase and the sugar moiety play a critical role in the binding of and inhibition by nucleotides. The possibility that HIV-1 integrase interacts with the sugar moiety is consistent with a recent investigation of nucleotide binding by HIV-1 integrase showing that oxidized ATP 1) inhibited 3′ processing at concentrations ≥30-fold lower than those required for inhibition by ATP and 2) covalently bound integrase (30).

Interestingly, although substitution or unsaturation (in the case of D4TMP) at the 3′ position enhanced potency, substitution at the 2′ position (compare 2′-FddATP with ddATP) did not seem to affect potency. The finding that 4′-azido-3′-deoxythymidine exhibits anti-HIV activity (35) suggests that future structure-activity studies could investigate the effect of substitutions at other positions on the deoxyribose.

**Relevance to antiviral drug mechanisms.** The $IC_{50}$ values for integrase inhibition by the nucleotide analogues tested were higher than the intracellular concentrations required for antiviral activity, confirming that integrase is not their primary target. For example, D4T, a thymidine analogue, has been shown to inhibit HIV-1 replication *in vitro* at concentrations of <0.01 μM, but its metabolites do not accumulate at >1 μM when cells are exposed to 10 μM D4T (36). Therefore, inhibition of HIV-1 integrase by D4TMP ($IC_{50}$ = 95 μM for strand transfer) may not play a role in its antiviral

activity. Similarly, ddCTP has been shown to accumulate to concentrations of ~30 μM when cells are exposed to 10 μM ddC (37). The significantly higher $IC_{50}$ value for inhibition of integrase (550 μM for strand transfer) demonstrates that RT is very likely the target of ddCTP.

For FLTMP (11), inhibition of HIV-1 integrase ($IC_{50}$ = 70 μM for strand transfer) may contribute to the antiviral activity of this thymidine analogue. This secondary mechanism of inhibition is analogous to the inhibition of HIV-1 integrase by AZTMP (17).

Although only AZT nucleotides inhibited integrase at physiological concentrations (17), the inhibition of other enzyme activities relevant to antiviral efficacy should be considered. For example, AZTMP can inhibit the RNase H and DNA polymerase activities of HIV-1 RT with $IC_{50}$ values of 50 and 5000 μM, respectively (38), and can inhibit (SV40 origin-dependent) DNA replication in HeLa cell extracts with an $IC_{50}$ of 5000 μM (39). AZTMP also inhibits the hydrolysis of AZTMP-terminated DNA substrates catalyzed by a 3′-exonuclease not associated with DNA polymerase δ, γ, or ϵ or associated with DNA polymerase δ, with an $IC_{50}$ value of 250 and 100–250 μM, respectively (40, 41). Taken together, these results suggest that the toxicity of AZT could be due to the incorporation of AZT into DNA coupled to a lack of removal of this nucleotide from DNA due a block in the repair of AZTMP-terminated DNA.

In summary, our data suggest that the phosphorylated derivatives of several nucleoside analogues are more potent inhibitors of HIV-1 integrase than AZT nucleotides. Further evidence for a nucleotide binding site on the integrase protein comes from the inhibition of integrase by pyridoxal phosphate (a nucleotide binding probe), by coumermycin A1 (which binds to the ATP binding site on the *gyr*B protein), and by the ribonucleotides ATP and GTP at or near their physiological concentrations.

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