Different Patterns of Inhibition of Avian Myeloblastosis Virus Reverse Transcriptase Activity by 3'-Azido-3'-Deoxythymidine 5'-Triphosphate and Its *threo* Isomer

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The two isomers 3'-azido-3'-deoxythymidine 5'-triphosphate (*erythro*-AZT-TP) and 1-(3'-azido-2',3'-dideoxy-β-D-xylofuranosyl)thymine 5'-triphosphate (*threo*-AZT-TP) were studied as inhibitors of the reverse transcriptase activity of avian myelobastosis virus. Kinetic analysis of the $(rA)_n \cdot (dT)_{12-18}$ (a standard template primer complex of polyriboadenylate and oligodeoxythymidylate of indicated length)-directed reaction revealed that *erythro*-AZT-TP was a competitive inhibitor with respect to dTTP, whereas *threo*-AZT-TP was a noncompetitive inhibitor. The apparent K_i values, as calculated from Dixon plots, were 0.48 and 5.5 μM, respectively, compared with a K_m value for dTTP of about 70 μM. These results indicate that *erythro*-AZT-TP had an approximately 150-times-higher affinity to the enzyme than dTTP had and that the avian myeloblastosis virus reverse transcriptase had different binding sites for the two isomers.

Reverse transcriptases are virus-coded enzymes which are obligatory and specific for the multiplication of retroviruses and therefore possible targets for antiviral chemotherapy. Several compounds inhibit the reverse transcriptase activity of various retroviruses (1–3, 9, 12). An effective inhibitor of reverse transcriptase may selectively block the formation of a viral DNA copy and thus possibly prevent the spread of retroviruses to new target cells. Only a few compounds have been shown effective in vivo, and a limited number of retrovirus inhibitors are under evaluation in clinical study programs as possible treatments for patients infected by the human immunodeficiency virus (HIV; 6, 9).

3'-Azido-3'-deoxythymidine (AZT; BW A509U) is an effective inhibitor of HIV multiplication in vitro (8). This thymidine analog is phosphorylated to a monophosphate derivative by the cellular thymidine kinase (5). The product is further phosphorylated by other cellular kinases to AZT-triphosphate (AZT-TP), which is a competitive inhibitor of HIV reverse transcriptase with respect to dTTP (5, 8; L. Vrang, H. Bazin, G. Remaud, J. Chattopadhyaya, and B. Öberg, Antiviral Res., in press). We studied the inhibitory mechanisms of AZT-TP and its *threo* isomer on avian myeloblastosis virus (AMV) reverse transcriptase activity.

MATERIALS AND METHODS

Template primers. $(rA)_n \cdot (dT)_{12-18}$ denotes a standard template primer complex of polyriboadenylate and oligodeoxythymidylate of indicated length. $(rC)_n \cdot (dG)_{12-18}$ and $(dC)_n \cdot (dG)_{12-18}$ denote complexes of polyribocytidylate and oligodeoxyguanylate, and polydeoxycytidylate and oligodeoxyguanylate, respectively. The synthetic template primers $(rA)_n \cdot (dT)_{12-18}$, $(rC)_n \cdot (dG)_{12-18}$, and $(dC)_n \cdot (dG)_{12-18}$ were purchased from Pharmacia, Uppsala, Sweden.

Enzymes and chemicals. AMV reverse transcriptase (lot MH 92107) was purchased from Pharmacia. [³H]dTTP (83 Ci/mmol) and [³H]dGTP (34.5 Ci/mmol) were from New

England Nuclear Corp., Boston, Mass., and unlabeled deoxynucleoside triphosphates (dNTPs) were obtained from Sigma Chemical Co., St. Louis, Mo. *erythro*-AZT-TP and *threo*-AZT-TP were synthesized as previously described (Vrang et al., in press). *threo*-AZT-TP contained about 0.5% *erythro*-AZT-TP. Foscarnet (trisodium phosphonoformate) was synthesized at Astra ALAB AB, Södertälje, Sweden.

Reverse transcriptase assay. Unless otherwise indicated in the figure legends, the standard reaction was performed in 100-μl mixtures containing 100 mM Tris hydrochloride (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 5 mM dithiothreitol, 400 μg of bovine serum albumin fraction V per ml, 0.05 A_{260} U (3.1 μg) of $(rA)_n \cdot (dT)_{12-18}$ per ml, 10.6 μM [³H]dTTP (specific activity, 1,047 cpm/pmol), and 1 U of AMV reverse transcriptase per ml. The reactions directed by $(rC)_n \cdot (dG)_{12-18}$ and $(dC)_n \cdot (dG)_{12-18}$ contained 0.39 A_{260} U (22.5 μg) and 0.35 A_{260} U (21.7 μg) of the template primer per ml, respectively, and 1.5 μM [³H]dGTP (specific activity, 7,650 cpm/pmol); KCl was omitted. The reactions were incubated at 37°C for 60 min. Samples of 40 μl were spotted onto paper disks (Munktell no. 5; Grycksbo Pappersbruk, Grycksbo, Sweden) and washed in 5% trichloroacetic acid–0.02 M

TABLE 1. Inhibition of AMV reverse transcriptase by foscarnet and the *erythro* and *threo* isomers of AZT-TP

Template primer	Concn (μM) giving 50% inhibition of AMV reverse transcriptase[a]		
	Foscarnet	*erythro*-AZT-TP	*threo*-AZT-TP
$(rA)_n \cdot (dT)_{12-18}$	5.4	0.58	5.0
$(rC)_n \cdot (dG)_{12-18}$	100[b]	100	100
$(dC)_n \cdot (dG)_{12-18}$	30[b]	100	100

[a] The $(rA)_n \cdot (dT)_{12-18}$-directed reaction incorporated 20.3 to 25.2 pmol of dTMP per 40 μl per 60 min, whereas reactions directed by $(rC)_n \cdot (dG)_{12-18}$ or $(dC)_n \cdot (dG)_{12-18}$ incorporated 3.3 or 2.3 pmol of dGMP per 40 μl per 20 min, respectively.
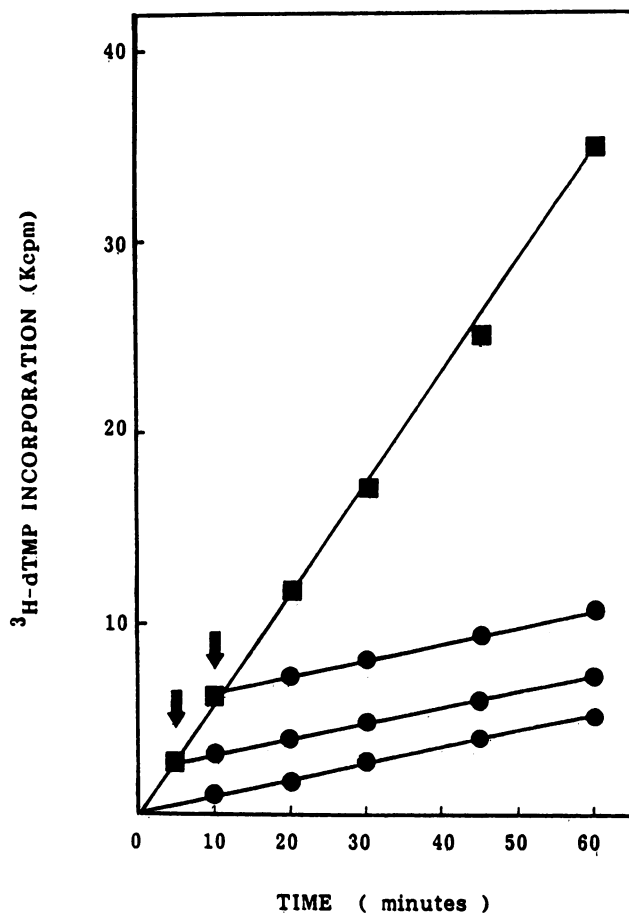[b] Data from Eriksson et al. (4).

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FIG. 1. Effect of erythro-AZT-TP added at a final concentration of 5 μM at zero time or at 5 or 10 min after initiation of the AMV reverse transcriptase reaction directed by $(rA)_n \cdot (dT)_{12-18}$ at a substrate (dTTP) concentration of 10.6 μM. Samples (40 μl) were removed from the different reaction mixtures at indicated incubation times, and acid-insoluble activity was determined as described in Materials and Methods. Mean values of triplicate reactions are presented. ■, Control reaction mixture without inhibitor. The mean deviation from the arithmetic mean to the outer range value was 5.9% (range, 1.7 to 12%) at the six indicated incubation times. A corresponding deviation was observed in all other experiments (data not shown).

sodium pyrophosphate and then ethanol as described previously (4). A minimum of two experiments were performed for each assay.

Enzyme kinetics. The experiments were performed in substrate intervals in which no deviation from linearity was observed. The amount of product formed was proportional to the incubation time in the substrate intervals studied. The kinetic constants K_m and K_i were determined graphically from Lineweaver-Burk and Dixon plots, respectively.

RESULTS

The inhibitory effects of foscarnet and the two isomers of AZT-TP on AMV reverse transcriptase activity are shown in Table 1. From dose-response curves, a 50% reduction of the $(rA)_n \cdot (dT)_{12-18}$-directed reaction was observed at 0.58 μM erythro-AZT-TP. About 10-times-higher concentrations of threo-AZT-TP or foscarnet were needed to give corresponding effects. At 10 μM, erythro-AZT-TP inhibited the reverse

transcriptase reaction by 90%, whereas threo-AZT-TP or foscarnet gave a 60% reduction. The AMV reverse transcriptase reaction directed by $(rC)_n \cdot (dG)_{12-18}$ or $(dC)_n \cdot (dG)_{12-18}$ was not affected by either isomer. Only foscarnet inhibited these reactions, although to a minor extent. The noncomplementary natural substrates dATP, dCTP, and dGTP did not inhibit the $(rA)_n \cdot (dT)_{12-18}$-directed reaction at concentrations up to 1 mM (data not shown).

We also studied the effect of erythro-AZT-TP added to the ongoing $(rA)_n \cdot (dT)_{12-18}$-directed reaction. The addition of 5 μM erythro-AZT-TP before or 5 or 10 min after the initiation of the reaction gave similar inhibition patterns (Fig. 1).

Kinetic analysis of the two isomers revealed that erythro-AZT-TP was a competitive inhibitor of AMV reverse transcriptase when dTTP was the varied substrate (Fig. 2). A K_i value of 0.48 μM was calculated from the Dixon plot shown in Fig. 3. In contrast, threo-AZT-TP was found to be a noncompetitive inhibitor with respect to dTTP (Fig. 4). The corresponding Dixon plot (Fig. 5) revealed an apparent K_i value of 5.5 μM. The K_m value for dTTP as calculated from Lineweaver-Burk plots was 68 μM.

In an attempt to characterize the binding sites present on the AMV reverse transcriptase for erythro-AZT-TP and threo-AZT-TP, it clearly emerged from inhibition data presented in a modified Scatchard plot (11, 13) that each compound reacted with a single binding site on the enzyme and no cooperative effects were involved (Fig. 6). Inhibition
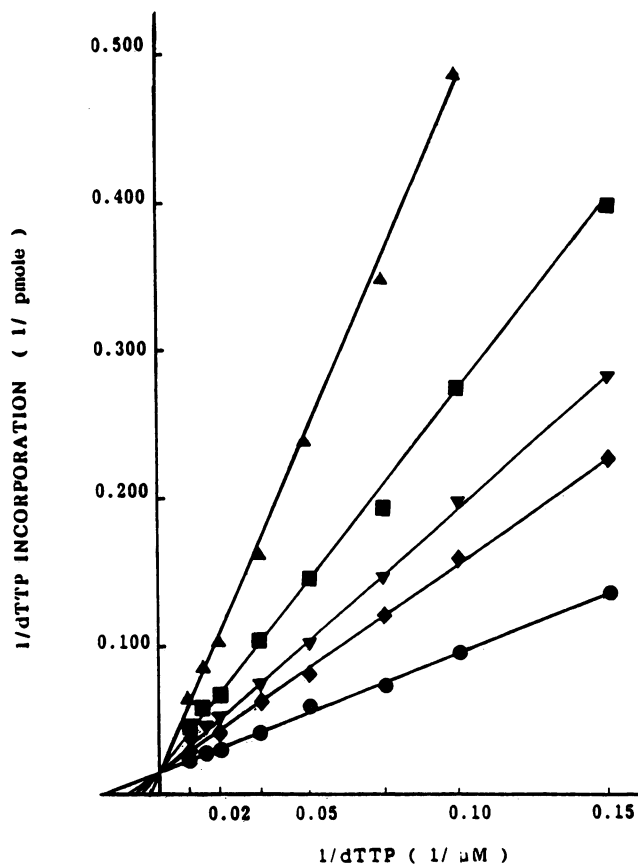

FIG. 2. Double-reciprocal plot of AMV reverse transcriptase activity directed by $(rA)_n \cdot (dT)_{12-18}$ in the presence of different concentrations of erythro-AZT-TP. The natural substrate dTTP was varied from 6.7 to 100 μM. erythro-AZT-TP concentrations (μM) were 0 (●), 0.3 (◆), 0.5 (▼), 1.0 (■), and 2.0 (▲).
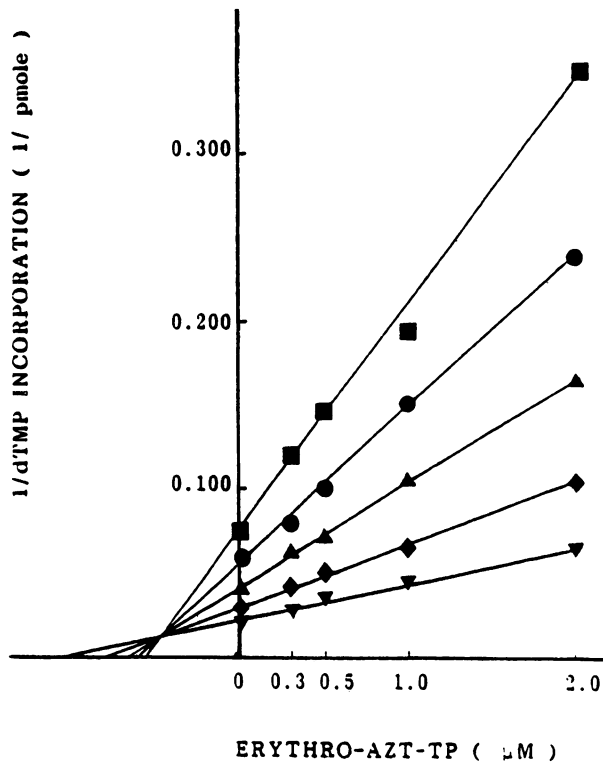
ERYTHRO-AZT-TP (μM)

FIG. 3. Dixon plot of data presented in Fig. 2. AMV reverse transcriptase reactions were performed in the presence of the indicated concentrations of *erythro*-AZT-TP at the following concentrations (μM) of dTTP: 13.3 (▼), 20 (◆), 30 (▲), 50 (●), and 100 (■).

THREO-AZT-TP (μM)

FIG. 5. Dixon plot of data presented in Fig. 4. AMV reverse transcriptase reactions were performed in the presence of the indicated concentrations of *threo*-AZT-TP at the following concentrations (μM) of dTTP; 10 (○), 13.3 (▼), 20 (●), 30 (◆), 50 (▲), and 100 (■).

data for foscarnet presented in the same manner resulted in a linear pattern almost identical to that observed for *erythro*-AZT-TP (data not shown).
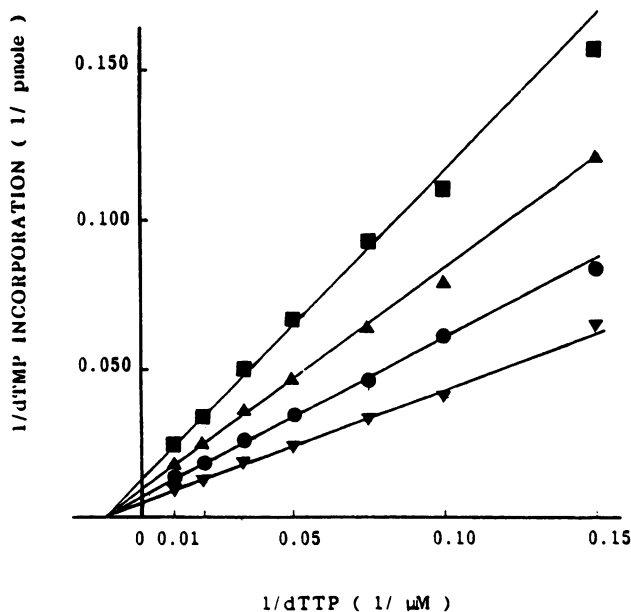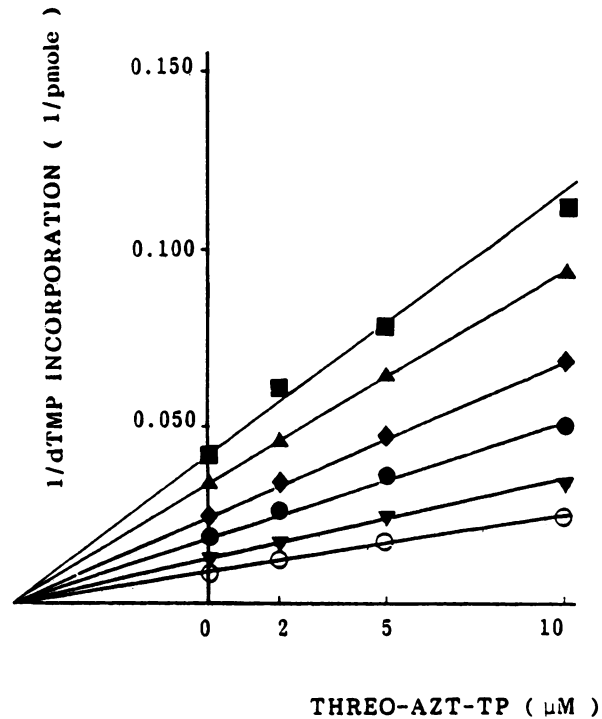

1/dTTP (1/ μM)

FIG. 4. Double-reciprocal plot of AMV reverse transcriptase activity directed by $(rA)_n \cdot (dT)_{12-18}$ in the presence of *threo*-AZT-TP. The dTTP concentration was varied from 6.7 to 100 μM. *threo*-AZT-TP concentrations (μM) were 0 (▼), 2.0 (●), 5.0 (▲), and 10 (■).

DISCUSSION

The search for new and, it is hoped, more effective inhibitors of reverse transcriptases should benefit from a better understanding of the structural requirements for inhibitors. Modifications of nucleoside analogs at the 3′ position have resulted in inhibitors such as AZT (8) and dideoxynucleosides (7). These compounds are expected to undergo a series of phosphorylation steps to their corresponding triphosphates before being active as inhibitors of reverse transcriptases. We analyzed the inhibitory properties of *threo*- and *erythro*-AZT-TP on the AMV reverse transcriptase activity. The 3′-OH group in these compounds is replaced by a 3′-azido group directed upwards in the *threo* isomer and downwards in the *erythro* isomer.

We found that *erythro*-AZT-TP was a better inhibitor of AMV reverse transcriptase activity than was *threo*-AZT-TP when the natural substrate dTTP was varied in the reaction directed by $(rA)_n \cdot (dT)_{12-18}$. This is in accordance with previous observations for HIV reverse transcriptase (Vrang et al., in press). Neither isomer inhibited the incorporation of dGMP in reverse transcriptase reactions directed by $(rC)_n \cdot (dG)_{12-18}$ or $(dC)_n \cdot (dG)_{12-18}$. These results imply that under these conditions, neither isomer has an affinity to the binding site for dGTP or to any other binding site. Observations by Vrang et al. (in press) showed that a 50% reduction of HIV reverse transcriptase activity was achieved at 0.04 μM *erythro*-AZT-TP when the reaction was directed by $(rA)_n \cdot (dT)_{12-18}$ and contained 10 μM dTTP. This shows that
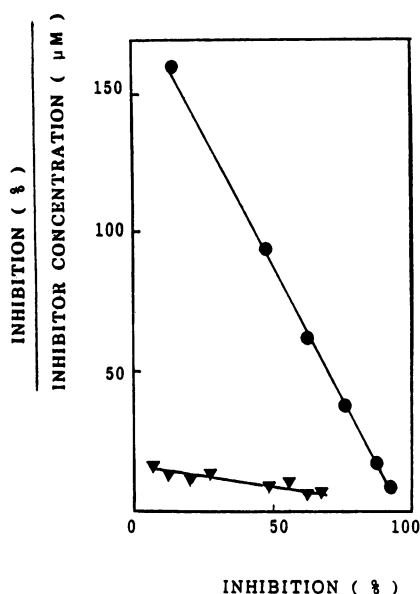
FIG. 6. Modified Scatchard plot of inhibition data of AMV reverse transcriptase by *erythro*-AZT-TP (●) and *threo*-AZT-TP (▼). The reactions were performed with $(rA)_n \cdot (dT)_{12-18}$ as the template primer as described in Materials and Methods at a dTTP concentration of 10.6 µM.

the AMV reverse transcriptase was less susceptible to inhibition by *erythro*-AZT-TP than was the HIV reverse transcriptase. A similar inhibition preference also held true for foscarnet (data not shown).

erythro-AZT-TP was found to be a competitive inhibitor of AMV reverse transcriptase when dTTP was the varied substrate. This corroborates previous observations for HIV reverse transcriptase (5; Vrang et al., in press). The calculated K_i values for *erythro*-AZT-TP and *threo*-AZT-TP (0.48 and 5.5 µM, respectively) and the K_m value for dTTP (68 µM) show that *erythro*-AZT-TP had an approximately 150-times-higher affinity to the natural substrate-binding site on the AMV enzyme than did dTTP. Also, *threo*-AZT-TP had a higher affinity (about 12 times) to its binding site than had dTTP to the substrate binding site. HIV reverse transcriptase has a considerably lower K_m value for dTTP (0.7 to 1.7 µM) and K_i value for *erythro*-AZT-TP (0.0022 µM), showing that the inhibitor has a greater than 300-times-higher affinity to the HIV reverse transcriptase than has dTTP (Vrang et al., in press).

threo-AZT-TP gave a noncompetitive inhibition pattern with AMV reverse transcriptase when dTTP was the varied substrate. The reason for the noncompetitive inhibition by *threo*-AZT-TP is unknown but may be explained either by an interaction between the azido group and proximal components in the base moiety of the nucleotide rendering the base unrecognizable as a thymine or by a specific reaction between the azido group in *threo*-AZT-TP (but not in *erythro*-AZT-TP) and a suitable reactive group of the enzyme molecule outside the dTTP-binding site. The noncompetitive inhibition pattern by *threo*-AZT-TP when AMV reverse transcriptase was used is not observed for HIV reverse transcriptase (Vrang et al., in press). This discrepancy may indicate the presence of structural differences at the catalytic sites of the two enzymes. An apparently low K_m value for $(rA)_n \cdot (dT)_{12-18}$ (about 0.0025 U/ml) made it difficult to determine whether *threo*-AZT-TP is a noncompetitive or

uncompetitive inhibitor of AMV reverse transcriptase with respect to the varied amounts of template primer. However, *threo*-AZT-TP did not compete with $(rA)_n \cdot (dT)_{12-18}$ for the template binding site (data not shown). In previous studies using foscarnet as an inhibitor, noncompetitive inhibition patterns were demonstrated with respect to both varied amounts of template primers and substrates in AMV and Visna virus reverse transcriptase reactions directed by different template primers (4, 14).

Since both isomers are dNTPs modified in the 3' position of the sugar moiety, it is expected that both compounds may serve as alternative substrates and on incorporation cause a termination of the growing DNA chain. The rapid termination of polymerase activity by *erythro*-AZT-TP added at different times of incubation (Fig. 1) is in agreement with a distributive synthesis and an inhibition of chain elongation. The kinetic data obtained in our experiments support the alternative-substrate model for *erythro*-AZT-TP but not for *threo*-AZT-TP. Further incorporation studies need to be performed to elucidate the mechanisms involved.

Inhibition by noncomplementary dNTPs was recently reported for DNA polymerase γ, purified from mouse myeloma MOPC 104E, in which dGTP and dATP were observed to compete with dTTP in an $(rA)_n \cdot (dT)_{12-18}$-directed reaction (10). When the same template primer was used, all three noncomplementary dNTPs (dATP, dCTP, and dGTP) were also reported to inhibit the activity of Rauscher murine leukemia virus reverse transcriptase in a noncompetitive manner, with apparent K_i values of 300 to 700 µM, indicating the existence of a second substrate-binding site (10). We were, however, unable to detect any inhibition caused by dATP, dCTP, or dGTP at concentrations up to 1 mM in reactions using $(rA)_n \cdot (dT)_{12-18}$ and the AMV reverse transcriptase.

Interactions between different inhibitors and reverse transcriptases, as well as other potential target enzymes, need to be further analyzed to improve our understanding of the structural requirements and to facilitate the design of more effective inhibitors, especially against the multiplication of HIV.

ACKNOWLEDGMENTS

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