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Interference with HIV-1 reverse transcriptase-catalyzed DNA chain elongation by the 5′-triphosphate of the carbocyclic analog of 2′-deoxyguanosine

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Summary

In an effort to better understand features in nucleotide analogs that result in the inhibition of HIV-1 reverse transcriptase, we have evaluated this enzyme with the 5′-triphosphate of the carbocyclic analog of 2′-deoxyguanosine (CdG-TP). CdG-TP was a reasonably potent competitive inhibitor of the incorporation of dGTP into DNA by HIV-1 reverse transcriptase using either a RNA or DNA template (K_i, 1 μM). CdG-TP was a good substrate for HIV-1 reverse transcriptase on both templates, but the DNA chain was poorly extended beyond the incorporation of CdG. These results indicate that substitution of ribose with a cyclopentane ring in nucleotides is not well tolerated by HIV-1 reverse transcriptase.

HIV-1; Reverse transcriptase; Carbocyclic nucleoside; DNA chain terminator

Carbovir (the carbocyclic analog of 2′,3′-dideoxy-2′,3′-didehydroguanosine, CBV) is a potent inhibitor of HIV-1 replication (Vince et al., 1988; Carter et al., 1990) due to its phosphorylation to the 5′-triphosphate (Bondoc et al., 1990) which selectively inhibits HIV-1 reverse transcriptase (HIV-RT; White et al., 1989; Parker et al., 1991). However, a closely related analog, CdG (the (+)enantiomer of the carbocyclic analog of 2′-deoxyguanosine), has no anti-HIV-1 activity in cell culture assays (Shannon, 1990) even though it is

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TABLE 1

Inhibition constants for (+)CdG-TP and (±)CBV-TP against HIV-RT

Compound	16S rRNA template			Gapped duplex DNA template		
	K_m (dGTP) (μM)	K_i (μM)	K_i/K_m	K_m (dGTP) (μM)	K_i (μM)	K_i/K_m
(+)CdG-TP	0.71 ± 0.09	1.2 ± 0.11	1.7	0.55 ± 0.05	1.0 ± 0.17	1.8
(±)CBV-TP*	0.96	0.09	0.09	0.74	0.05	0.07

HIV-RT was incubated with varying concentrations of [^{3}H]dGTP and (+)CdG-TP using either the rRNA or the DNA template as described (Parker et al., 1991). The incorporation of [^{3}H]dGTP into DNA was measured for each sample, and the kinetic constants were determined from Lineweaver-Burk plots. For both templates the lines converged at the Y-axis, indicating competitive inhibition. The K_i was calculated from a replot of the slope of each line versus the concentration of (+)CdG-TP. The K_m and K_i values shown for (+)CdG-TP represent the mean ± S.D. from three separate experiments.

*The values for (±)CBV-TP were taken from a previous publication (Parker et al., 1991) and are presented here for the purpose of comparison only.

phosphorylated to the 5′-triphosphate [(+)CdG-TP] in CEM cells to a similar degree as CBV (unpublished observation). Therefore, we have studied the inhibition of HIV-RT by (+)CdG-TP to aid in our understanding of the structural features in nucleotide analogs that are important to the inhibition of HIV-RT.

(+)CdG-TP was a competitive inhibitor of the incorporation of [^{3}H]dGTP into DNA by HIV-RT* using either a rRNA or DNA template (Table 1). The K_i for inhibition of HIV-RT by (+)CdG-TP (approximately 1 μM) was slightly greater than the K_m for dGTP as substrate for HIV-RT using either template. The (−)enantiomer of CdG-TP was also tested as an inhibitor of HIV-RT. Under identical assay conditions the concentrations required to inhibit HIV-RT activity by 50% using the DNA template were 2.5 and 160 μM for (+)CdG-TP and (−)CdG-TP, respectively (data not shown). These results indicated that HIV-RT was able to discriminate between the (+)and (−)enantiomers of CdG-TP.

We have utilized primer extension assays to determine the substrate characteristics of (+)CdG-TP with HIV-RT. In these experiments a [^{32}P]labeled primer annealed to either a rRNA or a DNA template was extended in the presence of varying concentrations of (+)CdG-TP. With both templates, dCMP was the first base in the template strand after the 3′ end of the primer so that a guanine nucleotide would be the first nucleotide added to the 3′

*HIV-RT was obtained from two sources. Most of the experiments were done using HIV-RT purified from HIV-1 virions obtained from the medium of cultures of infected CEM cells as described (Parker et al., 1991). However, because of low activity of this preparation of HIV-RT with the primer extension assay using rRNA template, these experiments (Table 2 and Fig. 3) were done with recombinant enzyme obtained from the University of Alabama at Birmingham, Center for AIDS Research, Gene Expression Core Facility (partially supported by P30 AI27767) that had been purified from *E. coli* expressing HIV-RT. Methodology for experiments is described in detail in our previous work (Parker et al., 1991).

TABLE 2

Kinetic constants for the incorporation of (+)CdG-TP and dGTP into the DNA by HIV-RT

Template	Nucleotide	K_m (μM)	V_{max} (%/min)
DNA	(+)CdG-TP	9.1 ± 3.3	0.27 ± 0.13
DNA	dGTP	2.7 ± 1.2	0.65 ± 0.28
16S rRNA	(+)CdG-TP	0.096 ± 0.026	1.1 ± 0.11
16S rRNA	dGTP	0.091 ± 0.016	0.75 ± 0.16

HIV-RT was incubated with [^{32}P]labeled primer annealed with either a DNA or rRNA template with varying concentrations of either (+)CdG-TP or dGTP as described (Parker et al., 1991). A guanine nucleotide was the first base added to the 3′ end of the primer. The bands on the gel representing primer and primer plus guanine nucleotide were quantitated with the aid of a densitometer. The extension of the primer is expressed as the percentage of the average concentration of substrate present throughout the experiment and was calculated as described previously (Mendelman et al., 1989; Parker et al., 1991). The kinetic constants were calculated from Lineweaver-Burk plots of the data. Each value represents the mean ± S.D. from three separate experiments.

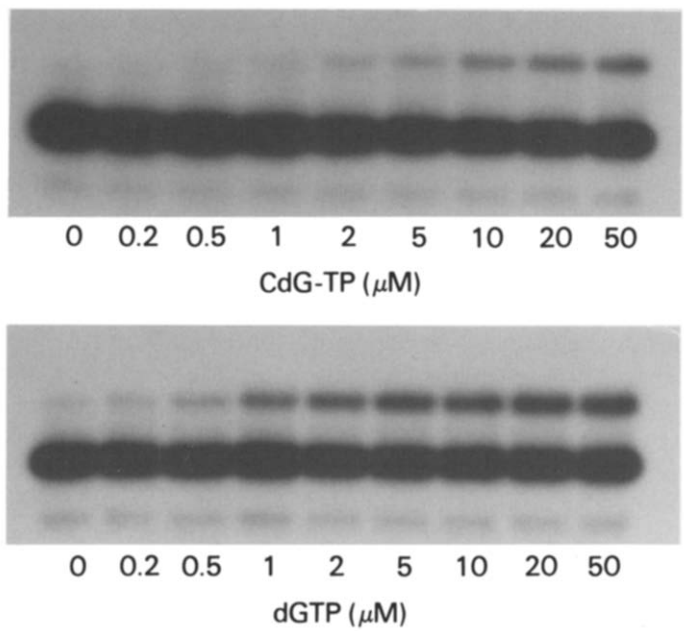

Fig. 1. Kinetic analysis of the incorporation of (+)CdG-TP into DNA by HIV-RT. HIV-RT was incubated in 10 μl vols. with a [^{32}P]labeled 15 base primer annealed to a 47 base DNA template and varying concentrations of either (+)CdG-TP or dGTP as previously described (Parker et al., 1991). The reaction was stopped by the addition of 25 μl of a 66% formamide, 33 mM EDTA solution containing bromophenol blue and was boiled for 5 min. Five μl of each sample were loaded onto a 15% polyacrylamide gel containing 7 M urea, the products of the reaction were separated from the primer by electrophoresis, and they were visualized by autoradiography.

end of the primer. An example of the procedure can be seen in Fig. 1. In these assays (+)CdG-TP was utilized as a substrate by HIV-RT using either the rRNA or the DNA template (Table 2) with kinetic constants similar to those seen with dGTP. In the evaluation of these data, it should be emphasized that the values of the kinetic constants for the incorporation of nucleotides is dependent on the sequence under evaluation (Mendelman et al., 1989). Therefore, the absolute value for these kinetic constants cannot be directly compared to the value obtained in the preceding competition studies which gives an average value for the entire sequence.

Experiments were done to determine the ability of the HIV-RT to extend the DNA chain after the incorporation of (+)CdG-MP into the DNA strand, because (+)CdG-TP contains a 3′-hydroxyl which could be extended by HIV-RT. For instance, (+)CdG-TP is used by the herpes simplex virus type 1 DNA polymerase as efficiently as dGTP (Parker et al., 1992). In these experiments HIV-RT was incubated with [^{32}P]labeled primer/template, 50 μM of either (+)CdG-TP or dGTP, and 50 μM each of dATP, dCTP, and TTP. When the [^{32}P]primer was annealed to the DNA template, (+)CdG-MP was incorporated into the chain and very little extension of the chain was observed beyond the incorporated (+)CdG-MP (Fig. 2). In an attempt to quantitate the effect of (+)CdG-MP incorporation on DNA chain elongation, a second experiment was designed to determine the concentration of TTP needed to see extension of the DNA chain past (+)CdG-MP (TTP is the base incorporated after (+)CdG-MP). No dATP or dCTP was included in this experiment, and the amount of enzyme was adjusted so that template excess allowed the extension products to accumulate linearly with respect to time. Under these conditions no more than 20% of the original primer was used in the reaction. No TMP was incorporated into the DNA chain after the incorporation of (+)CdG-MP even at concentrations of TTP as high as 1 mM. Only 10 μM of TTP was required to extend 50% of the DNA chains with TMP after an incorporated dGMP. In another experiment in which (+)CdG-MP was first incorporated four bases from the end of the primer, the incorporation of (+)CdG-MP still resulted in significant disruption of chain elongation, although a greater percentage of primers were extended beyond the (+)CdG-MP site (data not shown). Therefore, even though (+)CdG has a 3′-hydroxyl which could be used to add subsequent nucleotides, incorporation of (+)CdG-MP opposite a DNA template resulted in significant inhibition of chain elongation.

When the [^{32}P]labeled primer was annealed to the rRNA template, HIV-RT incorporated (+)CdG-MP into the growing DNA chain and was able to extend the chain beyond the incorporated (+)CdG-MP (Fig. 3). However, the primer was only extended about 10 bases. Under identical conditions with dGTP, HIV-RT was able to extend the primer by as many as 50 bases. These results indicated that even though (+)CdG-TP was used as a substrate for DNA synthesis by HIV-RT opposite the rRNA template, elongation after the incorporation of (+)CdG-MP was partially disrupted. The banding pattern representing the extension of the primer in the presence of (+)CdG-TP using

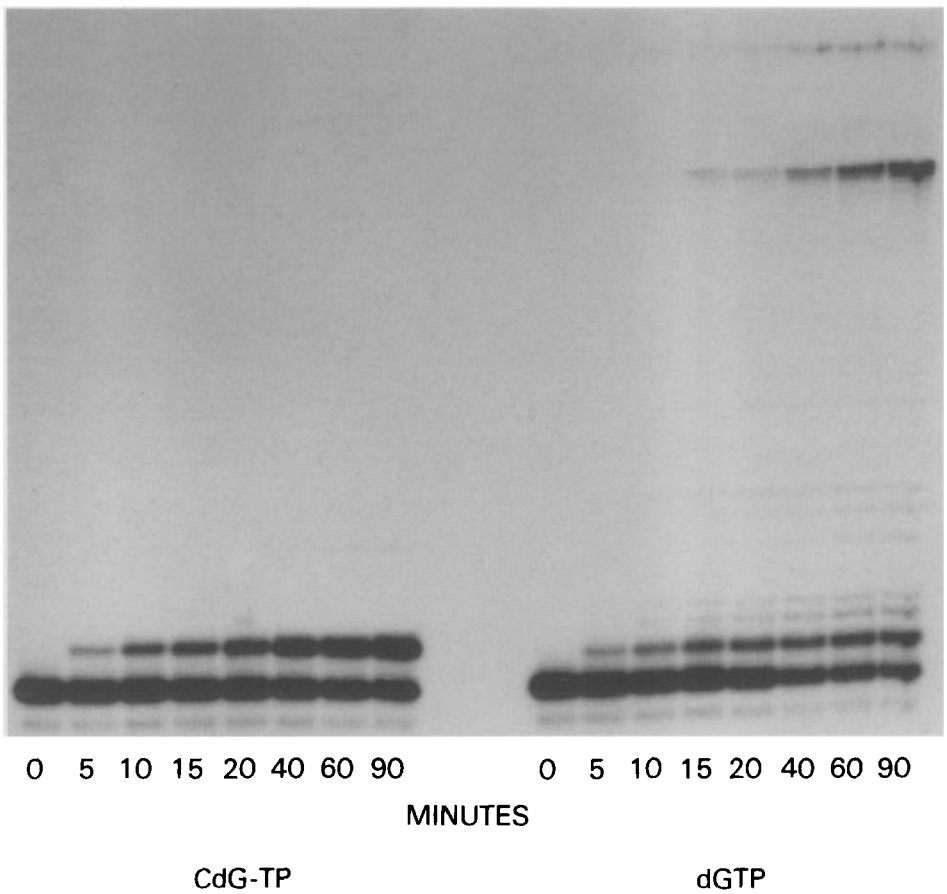

Fig. 2. Utilization of (+)CdG-TP for DNA synthesis by HIV-RT using a DNA template. HIV-RT was incubated in 100-μl solutions containing 50 mM Tris (pH 7.4), 125 mM KCl, 2 mM $MgCl_2$, 4 mM β-mercaptoethanol, 1 mg/ml of bovine serum albumin, 0.12 μg/ml of [^{32}P]labeled 15 base primer annealed to a 47 base DNA template (molar ratio 1 to 1), 50 μM of either dGTP or (+)CdG-TP, 50 μM each of dATP, dCTP, and TTP as described previously (Parker et al., 1991). The sequence of nucleotides added to the 3′-end of the primer was primer-GTC AGT CAG GTT CCA AGG GTT TCC CAA ATG AA-3′. At various times after the beginning of the assay, 10-μl aliquots were removed and the products were separated and visualized as described in the legend to Fig. 1.

the rRNA template was not similar to that observed when ddG-TP was used as the guanine nucleotide. These results indicated that the DNA chain was terminated at positions in addition to those where (+)CdG nucleotides were incorporated. The interpretation of the banding pattern is complicated by the mobility of (+)CdG-MP terminated primers which differ from that of primers terminated with either dGMP or ddG-MP. We compared the ability of HIV-RT to add the next nucleotide after the incorporation of either (+)CdG-MP or dGMP in assays in which the extension products accumulated linearly

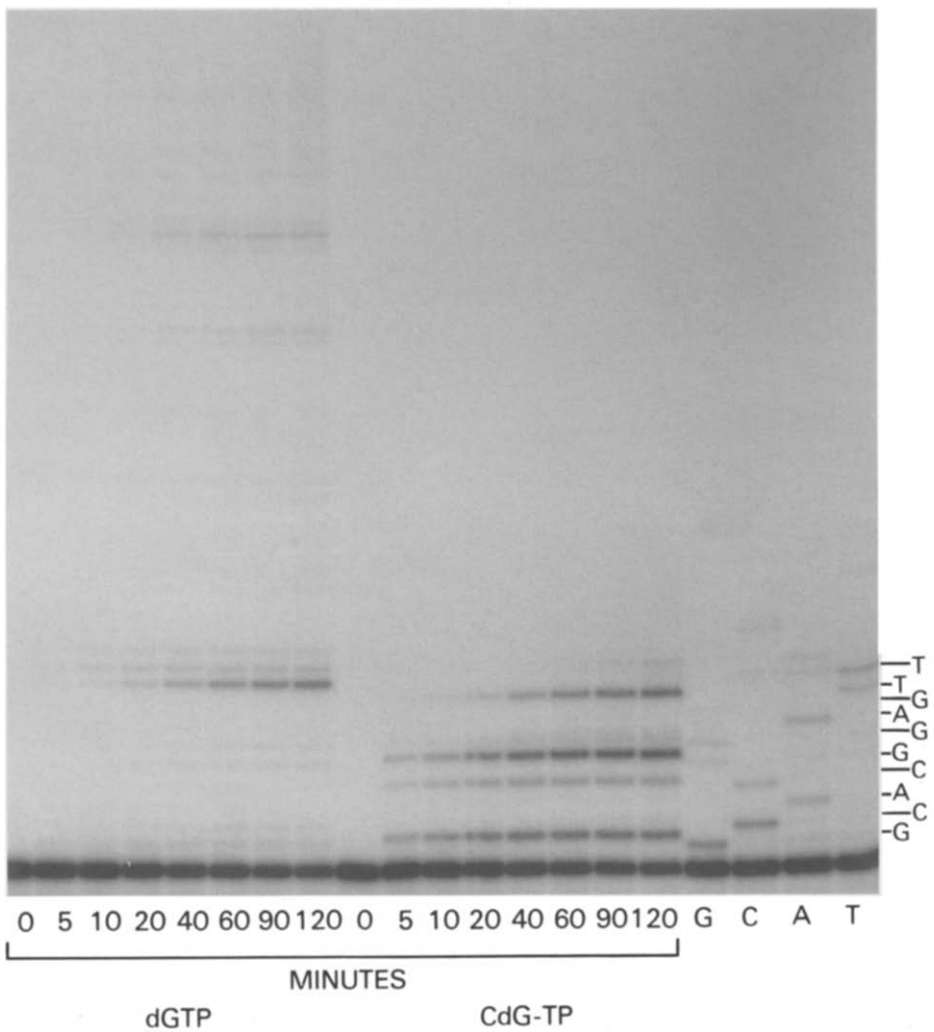

Fig. 3. Utilization of (+)CdG-TP for DNA synthesis by HIV-RT using a RNA template. HIV-RT was incubated in 100-μl solutions containing 50 mM Tris (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, 4 mM β-mercaptoethanol, 1 mg/ml bovine serum albumin, 750 μg/ml of [^{32}P]primer annealed to 16S rRNA (molar ratio of 1 to 1), 50 μM of either dGTP or (+)CdG-TP, and 50 μM each of dATP, dCTP, and TTP as described previously (Parker et al., 1991). At various times after the beginning of the assay, 10 μl aliquots were removed and the products were separated and visualized as described in the legend to Fig. 1. The four lanes on the right side of the figure are sequencing lanes done with the HIV-RT and ddGTP, ddCTP, ddATP, and ddTTP, respectively.

with respect to time and less than 20% of the original primer was used in the reaction. The concentration of dCTP needed to extend 50% of the primers by two bases was only slightly greater after (+)CdG-MP (25 μM) than after

dGMP (10 μM), indicating that insertion of the next base was only minimally inhibited by incorporation of one (+)CdG nucleotide. These data suggest that the incorporation of more than one (+)CdG nucleotide opposite a RNA template may be necessary to perturb the structure of the DNA enough to inhibit further DNA synthesis.

These results indicated that DNA synthesis by HIV-RT on both the rRNA and the DNA template was inhibited by (+)CdG-TP. However, the K_i/K_m ratios with (+)CdG-TP were 20-fold greater than those seen with (±)CBV-TP, indicating that much greater intracellular production of (+)CdG-TP would be needed to inhibit HIV replication to the same degree as (±)CBV-TP. In addition, when used by HIV-RT, (±)CBV-TP is an absolute chain terminator, whereas, with (+)CdG-TP some extension of the DNA chain does occur after the incorporation of (+)CdG-MP. Furthermore, if enough (+)CdG-TP were formed in cells to inhibit HIV-RT, then it should also be toxic to the cell due to the inhibition of DNA polymerase α. Our results presented here and elsewhere indicate that there is little selectivity in the inhibition of HIV-RT vs human DNA polymerase α by (+)CdG-TP (The K_i for (+)CdG-TP against DNA polymerase α is 0.95 μM, Parker et al., 1992). In contrast, (±)CBV-TP is a much more potent inhibitor of HIV-RT than DNA polymerase α (Parker et al., 1991). These differences in the inhibition of HIV-RT and DNA polymerase by these two carbocyclic nucleoside analogs account for their observed differences in anti-HIV activity.

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