Acyclic Guanosine Analogs Inhibit DNA Polymerases α, δ, and ϵ with Very Different Potencies and Have Unique Mechanisms of Action†

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ABSTRACT: Acyclovir triphosphate, ganciclovir triphosphate and penciclovir triphosphate inhibited DNA polymerases α, δ, and ϵ. Each triphosphate preferentially inhibited pol δ, although ganciclovir triphosphate was the most impressive of the three; the K_i for inhibition of pol δ was 2 μM (competitive with dGTP), while the K_is for inhibition of pol α and ϵ were 80 and 140 μM, respectively. Each of the compounds was polymerized by pol α, δ, and ϵ. Incorporation of acyclovir triphosphate resulted in immediate chain termination, whereas incorporation of ganciclovir triphosphate often allowed polymerization of additional dNTPs. Interestingly, chain termination most often occurred after polymerization of just one additional dNTP onto the ganciclovir monophosphate. All three compounds were very weak inhibitors of DNA primase. Acyclovir triphosphate, however, was a unique inhibitor of the pol α-catalyzed elongation of primase-synthesized primers. Immediately after DNA primase synthesized a primer, pol α frequently incorporated acyclovir triphosphate with consequent chain termination. If, however, pol α did not immediately polymerize acyclovir triphosphate onto the primase-synthesized primer, further dNTPs were readily added and acyclovir triphosphate was incorporated much less frequently.

Acyclic guanosine analogs are potent antiviral agents against members of the herpes virus family. Acyclovir and penciclovir (Figure 1) selectively inhibit herpes simplex virus replication (Elion et al., 1977; Vere Hodge, 1993), while ganciclovir potently inhibits cytomegalovirus replication (Field et al., 1983; Freitas et al., 1985). The active metabolites of these compounds are the corresponding triphosphates. Selective inhibition of viral replication by these compounds largely occurs because phosphorylation of the nucleoside to the nucleoside monophosphate is dependent upon a virally-encoded protein (Elion et al., 1977; Fyfe et al., 1978; Biron et al., 1985), with subsequent phosphorylation to the triphosphate catalyzed by cellular kinases (Miller & Miller, 1980, 1982). More potent inhibition of the viral polymerases compared to human polymerase α may also contribute to the selectivity of these compounds (Furman et al., 1980; Derse et al., 1981).

In addition to its antiviral properties, ganciclovir is quite cytotoxic and can have severe side effects due to its toxicity toward bone marrow cells (Faulds & Heel, 1990). The acyclic guanosine analogs are poor substrates for the human nucleoside kinases (Hovi & Field, 1988), but low levels of the triphosphates are none-the-less detected in uninfected cells (Biron et al., 1985; Vere Hodge, 1993). These low

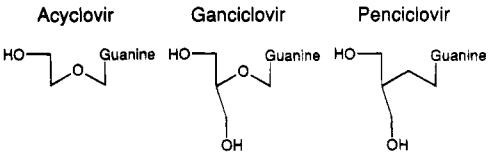

FIGURE 1: Structures of acyclovir, ganciclovir, and penciclovir.

levels of ganciclovir triphosphate (GCVTP)[1] are thought to cause the side effects associated with ganciclovir therapy. More recently, an experimental cancer chemotherapy that takes advantage of the activation of ganciclovir by viral kinases and the potent cytoxicity of the resultant GCVTP has been developed (Culver et al., 1992).

Previous studies have shown that ACVTP, GCVTP, and PCVTP are poor inhibitors of human pol α relative to the HSV DNA polymerase (Frank et al., 1984; Reardon, 1989; Reardon & Spector, 1989; Vere Hodge, 1993). However, these studies only included inhibition of pol α activity, and chromosomal DNA replication likely requires three DNA polymerases, pol α, δ, and ϵ. In addition, pol α copurifies as a complex with a second activity, DNA primase,[2] such that on single-stranded DNA, primase synthesizes short RNA primers which are then elongated by pol α (Grosse & Krauss,

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[1] Abbreviations: ACVTP, acyclovir triphosphate; BSA, bovine serum albumin; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid, sodium salt; GCVTP, ganciclovir triphosphate; HSV, herpes simplex virus; PCNA, proliferating cell nuclear antigen; PCVTP, penciclovir triphosphate; pol α, DNA polymerase δ; pol δ, DNA polymerase δ; pol ϵ, DNA polymerase ϵ; Tris, tris(hydroxymethyl)aminomethane, HCl salt.

[2] We shall use the terms "primase" and "pol α" to denote the primase and polymerase components of the pol α−primase complex. Unless noted otherwise, the calf thymus pol α−primase complex was used for all experiments.

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Table 1: Synthetic Oligodeoxynucleotides of Defined Sequence

oligodeoxynucleotide	sequence
DNA_G	TCCATATCACAT-3′ AGGTATAGTGTAGATCTTATCATCT
DNA_T	TCCATATCACATGCGCCG-3′ AGGTATAGTGTACGCGGCTTTGTGTAATAGTAAG
DNA_{Chad}	CAAACTGTGATACGATGGACTAGCTGAATC-3′ GTTTGACACTATGCTACCTGATCGACTTAGGAGCCTGCTATCGG
$d(TCC)_{20}$	5′-TCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCC
$d(TTC)_{13}$	5′-TTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTCTTC

1985; Hu et al., 1984). Effects of ACVTP, GCVTP, and PCVTP on these other enzymes have not been examined.

Using synthetic oligodeoxynucleotides of defined sequence, we investigated the effects of ACVTP, GCVTP, and PCVTP on DNA primase and pol α, δ, and ϵ. GCVTP selectively inhibited pol δ relative to the other replicative enzymes. ACVTP and PCVTP also targeted pol δ, but with decreased selectivity. Furthermore, we found that ACVTP has a unique mechanism for inhibiting pol α activity that only occurs when pol α elongated a primase-synthesized primer.

EXPERIMENTAL PROCEDURES

Materials

Unless noted, all materials were as described previously (Kuchta et al., 1992; Sheaff et al., 1991). Human pol α catalytic subunit was a generous gift from Dr. Bill Copeland at NIEHS, Research Triangle Park, NC, and human pol ϵ was kindly provided by Dr. Peng Huang and Dr. William Plunkett, M. D. Anderson Hospital, Houston, TX. Human pol δ, calf thymus pol α−primase, and PCNA were purified as described previously (Lee et al., 1989; Huang et al., 1991; Sheaff et al., 1991). A unit of activity is defined as polymerization of 1 nmol of dNTP h^{-1} under the previously described conditions for each enzyme (Lee et al., 1989; Sheaff et al., 1991). Synthetic primer-templates were synthesized and quantified as described previously (Sheaff et al., 1991; Sheaff & Kuchta, 1993). DNA_{Chad} was a generous gift of Chad Greef and Dr. Marvin Caruthers (University of Colorado). The concentrations of synthetic oligodeoxynucleotides are given in terms of 3′-termini.

PCVTP and GCVTP were enzymatically synthesized as described previously (Ertl et al., 1994). ACVTP was synthesized as described by Furman et al. (1979).

Methods

All assays were performed at 37 °C and contained 50 mM Tris, pH 7.5, and 5 mM $MgCl_2$. Reactions were initiated by addition of enzyme, and rates were measured under initial velocity conditions. All reactions were quenched by the addition of either 1.5 volumes of 50 mM EDTA, pH 7.5, or 1 volume of gel loading buffer (90% formamide). Radioactivity incorporated into products was measured either by a DE81 filter binding assay (Sheaff et al., 1991) or by polyacrylamide gel electrophoresis (18% acrylamide/8 M urea; Sheaff et al., 1991) followed by phosphorimagery on a Molecular Dynamics PhosphorImager.

Polymerase Activity. Assays (6−10 μL) contained 1 μM DNA, 5−20 μM dNTPs (with either [α-^{32}P]dATP, -dCTP, or -dTTP at a specific activity of ca. 1×10^5 cpm $pmol^{-1}$), 2 mM DTT, 0.05 mg mL^{-1} BSA, enzyme [1 unit of pol α, 0.4 unit of pol ϵ, or 0.1 unit of pol δ (16 ng) plus 35 ng of PCNA], and various concentrations of the acyclic analogs. For K_i and IC_{50} measurements, the amount of product was determined using the DE81 filter binding assay.

Primase and Primase-Coupled Pol α Activities. Primase activity was measured in assays (6−10 μL) containing 1 μM template, 50 μM [α-^{32}P]GTP (ca. 20 000 cpm $pmol^{-1}$), 50 μM ATP, 0.1 mg mL^{-1} BSA, 2 units of pol α-primase (measured as pol α activity), and various concentrations of the acyclic analogs. Reactions were quenched with gel loading buffer (90% formamide), and products were analyzed by gel electrophoresis and phosphorimagery. In those primase-coupled pol α activity assays where we wanted to visualize both primase and polymerase products, conditions were identical to primase assays except that they also contained 5 μM dATP and dGTP. Primase-coupled pol α activity assays in which only the polymerase products were visualized contained 1 μM template, 200 μM ATP and GTP, 0.1 mg mL^{-1} BSA, 2.5−20 μM dGTP, 2.5−20 μM [α-^{32}P]-dATP, 2 units of pol α−primase, and various concentrations of the acyclic analogs.

Polymerization of the Acyclic Analogs. Assays (6 μL) contained 1 μM DNA_G, 5 μM [α-^{32}P]dATP, dTTP, and dCTP, 2 mM DTT, 0.05 mg mL^{-1} BSA, enzyme (1 unit of pol α, 0.4 unit of pol ϵ, or 0.1 unit of pol δ plus 35 ng of PCNA), and either 5 μM dGTP or the indicated concentration of inhibitor. Reactions which contained pol α or δ were quenched after 5 min, while those containing pol ϵ were quenched after 16 min by the addition of an equal volume of gel loading buffer, and the products were analyzed by gel electrophoresis and phosphorimagery.

Elongation of Primase-Synthesized Primers by Klenow Fragment. Primase assays were performed as described above. Reactions were quenched after 30 min by heating to 65 °C for 5 min to denature pol α−primase, followed by a slow cool to room temperature to allow the primers to reanneal to the templates. $MgCl_2$ (2.5 mM), 625 μM of each dNTP, and 0.1 unit μL^{-1} Klenow fragment (exo$^-$) were now added (final concentrations), and the reactions were quenched after 15 min by the addition of 1 volume of gel loading buffer. Products were analyzed by gel electrophoresis and phosphorimagery.

RESULTS

Synthetic DNAs of defined sequence were used as substrates for these studies (e.g. DNA_G, Table 1). DNA_G is a good substrate for both pol α and pol ϵ (Sheaff et al., 1991; Cheng & Kuchta, 1993), and initial experiments demonstrated that in the presence of PCNA, DNA_G is also a good substrate for pol δ. Whereas pol δ exhibited little activity

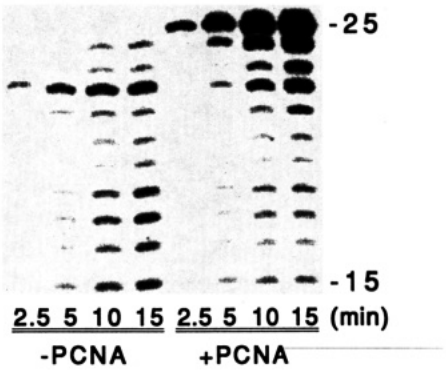

FIGURE 2: DNA_G is a good substrate for pol δ and PCNA. Assays contained 1 μM DNA_G, 6 μM [α-^{32}P]dATP, dCTP, dGTP, and dTTP, 2 mM DTT, 0.1 mg mL^{-1} BSA, and 16 ng of pol δ. At the indicated times, the reactions were stopped and analyzed by gel electrophoresis followed by phosphorimagery. The absence or presence of PCNA (35 ng) and the locations of the 15-mer and 25-mer products are noted on the gel.

Table 2: K_i and IC_{50} Values for the Acyclic Compounds

	K_i (μM)[a]		IC_{50} (μM)[b]		
	GCVTP	ACVTP	GCVTP	ACVTP	PCVTP
pol α	80	23	100 (125)	30 (90)	450
pol δ	2	2.7	3 (7)	4 (8)	120
pol ϵ	140	50	200 (250)	65 (125)	375

[a] K_i values for each polymerase were measured via Dixon plots using 1 μM DNA_G and varying all four dNTPs simultaneously, as described under Experimental Procedures. Values given are $\pm$20%. Calf thymus pol α was used for these experiments. [b] IC_{50} values were measured in assays that contained either 1 μM DNA_G or 1 μM DNA_{Chad}, and 6 μM dNTPs for pol α and δ, and 5 μM dNTPs for pol ϵ. The values in parentheses are the IC_{50} values when DNA_{Chad} was the substrate.

and very low processivity when PCNA was not present, Figure 2 shows that the addition of PCNA increased both the processivity and the rate of dNTP polymerization (12-fold), consistent with the previously reported effects of PCNA on pol δ (Prelich et al., 1987; Bambara & Jessee, 1991). In the presence of 10 μM dNTPs and PCNA, measuring the rate as a function of DNA_G concentration gave a V_{max} value of 1600 nmol of dNTP (30 min)$^{-1}$ mg^{-1}, and a $K_{M(DNA)}$ value of 4 μM. Thus, even these very short DNAs allow productive pol δ/PCNA interactions and can be used for inhibition studies. Furthermore, since PCNA appears to be an essential cofactor for pol δ, PCNA was included in all further studies of pol δ activity.

Inhibition of Pol α, δ and ϵ by ACVTP, GCVTP and PCVTP. Inhibition of pol α, δ, and ϵ by the acyclic analogs was measured using DNA_G as substrate (Table 2). In each case, inhibition by the compounds was competitive with respect to dGTP (data not shown).[3] Impressively, the K_i for inhibition of pol δ by GCVTP was 40- and 70-fold lower than for pol α and ϵ, respectively. Table 2 also shows the IC_{50} values for inhibition by GCVTP at a single dNTP concentration in order to allow a direct comparison of the relative potency of inhibition under identical conditions. Importantly, since inhibition was measured on the identical template with all three polymerases, differences in the potency of inhibition were not due to differences in the

[3] Consistent with GCVTP being a dGTP analog, GCVTP did not inhibit pol δ activity on DNA_T (Table 1), a DNA that lacks any deoxycytidylates in the single-stranded template.

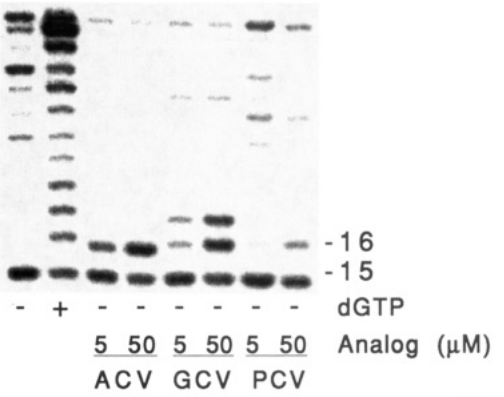

FIGURE 3: Polymerization of the acyclic analogs by pol δ. Assays contained 1 μM DNA_G and 5 μM dCTP, dTTP, and [α-^{32}P]dATP. The presence of 5 μM dGTP and the indicated concentrations of ACVTP (ACV), GCVTP (GCV), and PCVTP (PCV) are as noted. The first marked product (15-mer) represents polymerization of dCTP, dTTP, and [α-^{32}P]dATP onto the primer. The second product (16-mer) results from polymerization of dGTP or the acyclic analog. The small amount of 26-mer synthesized in each assay is probably an "end-addition" product due to polymerization of [α-^{32}P]dATP onto the 3'-end of the template strand.

template composition. To ensure that the selective inhibition of pol δ activity by GCVTP was not limited to this particular template, we next examined inhibition of each polymerase using a second DNA that had a different sequence and a longer duplex region (DNA_{Chad}, Table 1). Again, GCVTP preferentially inhibited pol δ (Table 2).

In addition to inhibiting calf thymus pol α, GCVTP also inhibited human pol α. Using DNA_G as substrate, the K_i for GCVTP was 75 μM, similar to the value obtained with the calf thymus enzyme. Thus, both the human and calf thymus enzymes interact with GCVTP equally well, and GCVTP differentially inhibits human pol α, δ, and ϵ.

ACVTP also inhibited pol δ more potently than pol α and ϵ, albeit with less selectivity (Tables 2 and 3). PCVTP, on the other hand, poorly inhibited all three polymerases such that we only determined IC_{50} values rather than K_is. Similar to ACVTP, PCVTP also showed slight specificity toward pol δ inhibition.

Pol α, δ, and ϵ Incorporate ACVTP, GCVTP, and PCVTP into DNA. Pol δ can use ACVTP, GCVTP, and PCVTP as substrates (Figure 3). The first lane shows the products synthesized by pol δ in an assay containing only DNA_G, [α-^{32}P]dATP, dCTP, and dTTP. The primary product that accumulated, a 15-mer, did so because the next required dNTP, dGTP, was omitted from the reaction.[4] If either ACVTP or GCVTP was now included in the assays, new products were synthesized. These new products were primarily 1−2 nucleotides longer than the major product synthesized in the absence of the analog, consistent with incorporation of the analogs. The presence of PCVTP results in the synthesis of at most small amounts of new products. The acyclic analogs appear to be poor substrates for pol δ, since even in the presence of 50 μM ACVTP, GCVTP, or PCVTP, pol δ incorporated the analogs into only a fraction of the DNA—the enzyme frequently dissociated rather than

[4] In the absence of dGTP, pol δ polymerized nucleotides past the template dC approximately 6% of the time. This surprisingly large amount of products longer than the 15-mer might be a function of the template sequence or size, or could be due to trace amounts of dGTP as an impurity in the other dNTPs.

incorporate the analog. When 50 μM ACVTP or 50 μM GCVTP was included in the assay, only 66% and 62%, respectively, of the 15-mer was elongated to a 16-mer (or longer product) via analog incorporation. In contrast, including only 5 μM dGTP resulted in elongation of 90% of the 15-mer into longer products. It should be noted, however, that these may be underestimates of the ability of pol δ to polymerize the analogs since the 3′ → 5′ exonuclease activity of pol δ may have excised some of the analog incorporated by the polymerase activity.

Consistent with ACVTP being a chain-terminator, incorporation of ACVTP inhibited any further polymerization by pol δ (Figure 3). Conversely, incorporation of GCVMP to generate the 16-mer did not necessarily result in chain termination; one additional dNMP was polymerized onto 33% and 28% of the 16-mer (5 or 50 μM GCVTP, respectively). Polymerization of this additional dNTP usually resulted in chain termination, as longer products were rarely observed. These data indicate that pol δ will recognize primers that contain a GCVMP at the 3′-terminus or one nucleotide removed from the 3′-terminus; however, they are utilized relatively inefficiently as substrates. PCVTP could also be polymerized by pol δ, albeit less efficiently. This is consistent with PCVTP being a weaker inhibitor of pol δ than GCVTP and ACVTP. Small amounts of products due to polymerization of multiple dNTPs were synthesized in assays containing GCVTP and PCVTP. This could result from either polymerization of multiple dNTPs onto the GCVMP or PCVMP or misincorporation opposite the template dC followed by polymerization of multiple dNTPs.

Pol α and pol ϵ could also incorporate ACVTP, GCVTP, and PCVTP into primer-templates, and the results were extremely similar to those with pol δ (data not shown). Incorporation of ACVTP resulted in immediate chain termination, whereas incorporation of GCVTP usually resulted in either immediate chain termination or polymerization of one additional dNTP followed by chain termination. PCVTP was also incorporated, albeit less efficiently than ACVTP or GCVTP. Polymerization of multiple dNTPs following incorporation of GCVTP or PCVTP rarely occurred.

Acyclic Analogs Are Poor Inhibitors of Primase Activity. Effects of ACVTP, GCVTP, and PCVTP on primase activity were examined using templates of defined sequence [$d(TCC)_{20}$ and $d(TTC)_{13}$, Table 1]. Once primase synthesizes a primer 7−10 nucleotides long (i.e., a unit-length primer), the newly generated primer-template is transferred intramolecularly from primase to pol α, whereupon further primase activity is negatively regulated until pol α elongates this primer-template (Sheaff et al., 1994). This negative regulation of primase activity after synthesis of a single unit-length primer results in a biphasic time-course of primase activity (Sheaff & Kuchta, 1993). Including either GCVTP (Figure 4) or ACVTP (data not shown) in primase assays had no effect on negative regulation, although the rate of primer synthesis decreased.

In order to precisely measure the effect of ACVTP, GCVTP, and PCVTP on primase activity, we examined steady-state rates of primer synthesis. The normal, biphasic time course of primer synthesis can be converted to a linear time course by preventing the newly generated primer-template from interacting with the pol α active site (Sheaff et al., 1994). This can be accomplished by including an

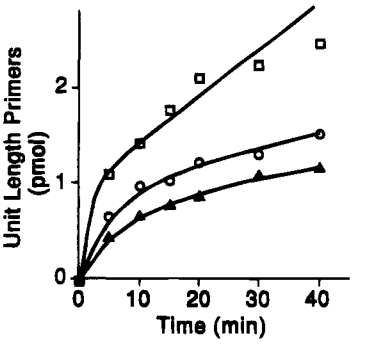

FIGURE 4: Time course of primase activity on $d(TCC)_{20}$ remains biphasic in the presence of GCVTP. Reactions contained 1 μM $d(TCC)_{20}$, 50 μM ATP, 50 μM [α-^{32}P]GTP, and 0 (□), 100 (○), or 180 μM (▲) GCVTP, and were performed as described under Experimental Procedures.

exogenously added primer-template (DNA_G) and aphidicolin in the assays. Under these conditions, a pol α·DNA_G· aphidicolin ternary complex forms and prevents primase-generated primer-templates from interacting with the pol α active site. This alleviates the negative regulation of primase activity that normally occurs after synthesis of a unit-length primer but does not otherwise affect primase activity (Sheaff et al., 1992, 1994). Under these steady-state conditions, ACVTP, GCVTP, and PCVTP weakly inhibited primase activity in assays containing $d(TCC)_{20}$ and 50 μM [α-^{32}P]-NTPs (IC_{50} = 100, 250, and 190 μM, respectively).

Pol α Incorporates ACVTP, GCVTP and PCVTP onto Primase Synthesized Primers. Figure 5 shows the effects of ACVTP, GCVTP and PCVTP on the products synthesized by DNA primase. The altered mobility of the longer primers suggested that the analogs were polymerized onto the primers. Klenow fragment was used to explicitly show that primers synthesized in the presence of ACVTP contained ACVMP at the 3′-terminus. Klenow fragment readily elongated primase-synthesized primers synthesized in the absence of ACVTP (Figure 5). However, if ACVTP was included during primer synthesis, Klenow fragment could no longer elongate the primers, indicating that the primers contained ACVMP at the 3′-terminus. Control experiments showed that the ACVTP did not inhibit Klenow fragment.[5]

To show that pol α was responsible for polymerizing the ACVTP, GCVTP, and PCVTP onto the primase-synthesized primers, aphidicolin and DNA_G were included in the assays in order to inhibit pol α (Figure 5). As expected, inhibition of pol α did not affect the pattern of products when only ATP and GTP were present. In contrast, inhibition of pol α eliminated the synthesis of products with altered mobility when the reactions contained either ACVTP, PCVTP, or GCVTP, and restored the pattern of products to that observed when only ATP and GTP were present. Thus, pol α polymerized the analogs onto the primase-synthesized primers.

Inhibition of Primase-Coupled Pol α Activity. On a single-stranded DNA, primase synthesizes primers that can be immediately elongated by pol α (i.e., primase-coupled pol

[5] Klenow fragment will slowly elongate primers containing a 3′-terminal GCVMP (Reardon, 1989) or a 3′-terminal PCVMP (data not shown). The primers of altered mobility that were synthesized in assays containing GCVTP or PCVTP were elongated by Klenow fragment more slowly than "normal" primers, suggesting that they contained GCVMP or PCVMP, respectively, at the 3′-terminus.

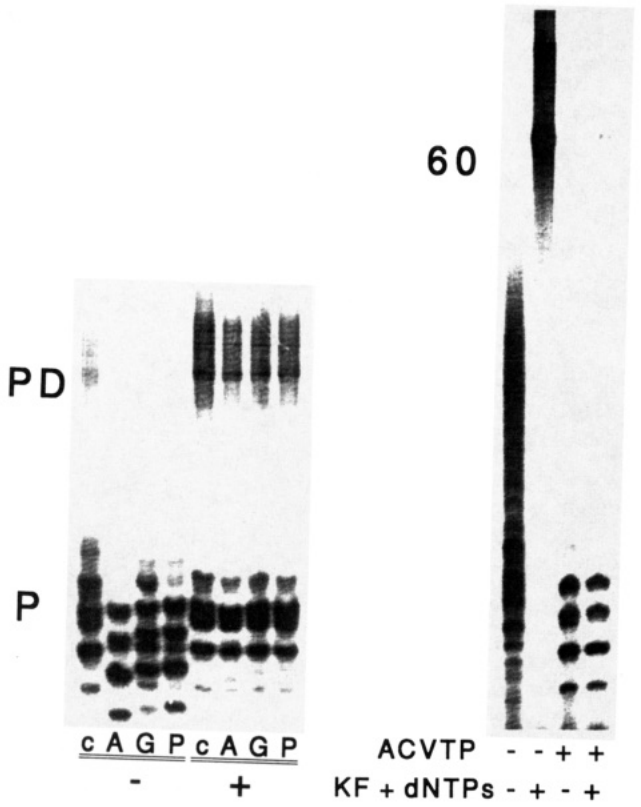

FIGURE 5: Pol α polymerizes ACVTP, GCVTP, and PCVTP onto primase-synthesized primers. Lanes marked c show the products from assays containing 1 μM $d(TCC)_{20}$, 0.1 mg mL^{-1} BSA, 50 μM ATP, and 50 μM [α-^{32}P]GTP. The presence of either 50 μM ACVTP (A), 50 μM GCVTP (G), or 50 μM PCVTP (P) in the assays is noted. Additionally, the assays marked with a "+" also contained 5 μM DNA_G and 50 μM aphidicolin to inhibit pol α, while the assays marked "−" lacked these compounds. The higher molecular weight products labeled PD are primer-dimers (Sheaff & Kuchta, 1993), products that are only formed in the absence of dNTPs. Primers synthesized in the absence (−) or presence (+) of ACVTP were then treated with Klenow fragment (KF) and dNTPs as described under Experimental Procedures. The location of a 60-nucleotide-long standard is shown.

Table 3: Inhibition of Primase-Coupled Pol α Activity by Acyclic Nucleotide Analogs[a]

	K_i (μM) for substrate	
analog	$d(TCC)_{20}$	$d(TTC)_{13}$
ACVTP	1	8
GCVTP	15	60
PCVTP	200	nd[b]

[a] Reactions contained 1 μM template, 200 μM GTP and ATP, 2.5−20 μM [α-^{32}P]dATP and dGTP, and varying concentrations of inhibitor. Apparent K_i values were determined via Dixon plots at three dNTP concentrations. [b] Not determined.

α activity). Inhibition of primase-coupled pol α activity by ACVTP, GCVTP, and PCVTP was measured in assays containing $d(TCC)_{20}$, NTPs, dGTP, and [α-^{32}P]dATP. Table 3 shows that each acyclic nucleotide inhibited [α-^{32}P]dATP polymerization. Inhibition was competitive with respect to dGTP, indicating that the reduced rate of [α-^{32}P]dNTP polymerization was due to inhibition of pol α. The more potent inhibition of primase-coupled pol α activity on $d(TCC)_{20}$ than on DNA_G is likely due to the higher percentage of template dC's in $d(TCC)_{20}$. Consistent with this idea, inhibition of primase-coupled pol α activity on the template $d(TTC)_{13}$ was substantially less potent than inhibition on $d(TCC)_{20}$ (Table 3).

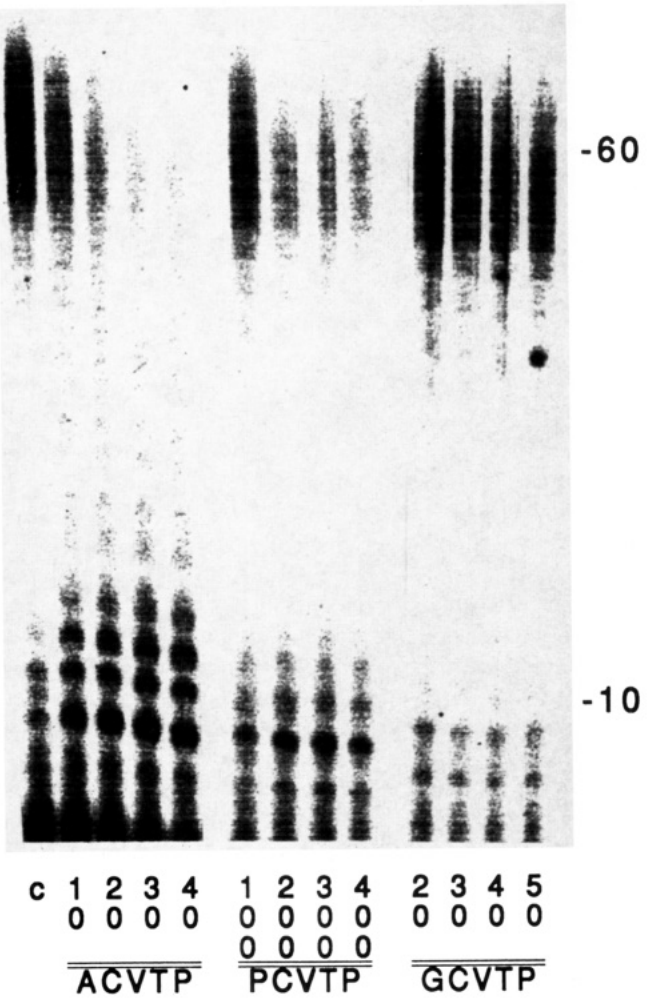

FIGURE 6: Inhibition of primase-coupled pol α activity by ACVTP, PCVTP, and GCVTP. Assays contained pol α−primase, 1 μM $d(TCC)_{20}$, 50 μM ATP, 50 μM[α-^{32}P]GTP, 5 μM dATP, 5 μM dGTP, and the indicated concentrations of ACVTP, PCVTP, or GCVTP. Lane c contained no inhibitor. The length of oligonucleotide standards is shown.

We then analyzed the effects of the acyclic nucleotides on the size distribution of products synthesized during the pol α-catalyzed elongation of primase-synthesized primers. Assays contained $d(TCC)_{20}$, [α-^{32}P]GTP, ATP, dGTP, and dATP; thus, all products would be labeled. In the absence of any inhibitor, pol α efficiently elongated the primase-synthesized primers into products 50−60 nucleotides long (Figure 6). GCVTP decreased the total amount of elongated products; however, there were no significant changes in the sizes of products. In contrast, ACVTP decreased the total amount and length of elongated product. Interestingly, large amounts of products ca. 10 nucleotides long accumulated at even low concentrations of ACVTP, but the amount of intermediate length products between ca. 12 and 45 nucleotides long increased much less. For example, addition of 20 μM ACVTP increased the amount of products ca. 10 nucleotides long by 130%, whereas the amounts of products from 12 to 45 nucleotides long increased by only 30% and products greater than 45 nucleotides long decreased by 65%. Addition of PCVTP affected primase-coupled pol α activity similarly to ACVTP, in that it also resulted in the accumulation of products ca. 10 nucleotides long.

Accumulation of products ca. 10 nucleotides long in the presence of ACVTP suggested that pol α had polymerized

ACVTP onto the primase-synthesized primers. This was examined using Klenow fragment as described earlier. Klenow fragment could not elongate the products ca. 10 nucleotides long that accumulated in the presence of ACVTP and dNTPs, whereas Klenow fragment readily elongated primers synthesized in the absence of ACVTP. Thus, these products ca. 10 nucleotides long contain ACVMP at the 3′-terminus.

The relative efficiency for polymerization of dGTP versus ACVTP during elongation of primase-synthesized primers was determined. Polymerization of ACVTP onto a primase-synthesized primer results in a product ca. 10 nucleotides long, whereas polymerization of dGTP results in products >10 nucleotides long. Thus, the relative frequency for dGTP versus ACVTP polymerization can be quantified as a function of [dGTP]/[ACVTP] in order to calculate $(k_{cat}/K_M)_{dGTP}/(k_{cat}/K_M)_{ACVTP}$ (Kuchta et al., 1992). During elongation of primase-synthesized primers, pol α preferred to polymerize dGTP rather than ACVTP by only a factor of 5.

DISCUSSION

The acyclic nucleotides ACVTP, GCVTP, and PCVTP inhibit DNA pol α, δ, and ϵ. All three compounds inhibit pol δ more potently than either α or ϵ, although GCVTP is the most impressive among them. A major question in DNA replication and repair is the respective roles of pol δ and ϵ, since both polymerases share several common features including accessory proteins and a 3′ → 5′ exonuclease. The ability of GCVTP to discriminate between pol δ and ϵ may be very useful for elucidating the roles of pol δ and ϵ. Additionally, the potent inhibition of pol δ by GCVTP suggests that inhibition of cellular DNA replication induced by treatment with ganciclovir is likely due to inhibition of pol δ.

Each DNA polymerase could incorporate all three compounds into DNA. Unlike ACVTP, however, GCVTP and PCVTP are not absolute chain terminators since they contain the equivalent of a 3′-hydroxyl. Interestingly, chain termination primarily occurred immediately after the polymerases had incorporated GCVTP as well as after polymerization of one additional dNTP onto the GCVMP. Reardon (1989) reported that pol α elongates primers containing a GCVMP either at the primer 3′-terminus or one nucleotide away from the primer 3′-terminus very inefficiently compared to normal primers containing only dNMPs, consistent with the chain termination we observed. HSV DNA polymerase has also been reported to incorporate GCVMP and one additional dNTP into DNA, but then have great difficulty in polymerizing further dNTPs (Reid et al., 1988). This phenomenon is not limited to acyclic nucleotides, since pol α and ϵ will polymerize 2′,2′-difluoro-2′-deoxycytidine triphosphate followed by one additional dNTP, and then terminate further dNTP polymerization (Huang et al., 1991). It should be noted, however, that termination after incorporation of a nucleotide analog followed by one additional dNTP is not a general result of 2′-modification of nucleotides, since araNTPs tend to be immediate chain terminators of DNA synthesis by purified polymerases (Huang et al., 1991; Townsend & Cheng, 1987).

The ability of DNA polymerases to incorporate GCVTP, and perhaps also PCVTP, and then polymerize additional dNTPs may have important *in vivo* consequences. PCVTP and GCVTP, both of which are mutagenic [Physicians Desk Reference (1994); SmithKline (1994)], could be incorporated into internucleotide linkages of cellular DNA during DNA replication or repair with consequent mutagenic and/or cytotoxic effects during the next S-phase when the cell attempts to replicate past the analog. Significantly, the effects of acyclovir, ganciclovir, and penciclovir on cellular DNA replication will be most severe in cells infected with a herpes virus. Whereas infected cells accumulate high levels of each triphosphate, uninfected cells accumulate only low levels.

Inhibition of Primase-Coupled Pol α Activity by ACVTP. ACVTP was a moderate inhibitor of pol α activity when an exogenously added primer-template was the substrate, consistent with previous studies on pol α inhibition (Reardon, 1989; Vere Hodge, 1993). However, ACVTP more potently inhibited primase-coupled pol α activity than pol α activity with exogenously added DNA primer-templates, and pol α readily polymerized ACVTP onto primase-synthesized primers. The facile polymerization of ACVTP onto primase-synthesized primers and consequent chain termination may cause the more potent inhibition of primase-coupled pol α activity compared to inhibition of dNTP polymerization onto an exogenously added primer-template. The relative paucity of products that are of intermediate length between fully elongated primase-synthesized primers (ca. 50−60 nucleotides long) and primase-synthesized primers containing an ACVMP at the 3′-terminus (ca. 10 nucleotides long) indicates that pol α either immediately polymerizes ACVTP onto the primase-synthesized primer or, failing this, polymerizes ACVTP into products much less readily. Consistent with this idea, pol α polymerizes dGTP 100-fold more readily than it polymerizes ACVTP when an exogenously added DNA primer-template is used as the substrate [V_{max}/K_M (Reardon, 1989)]. This may be equivalent to the situation if pol α fails to polymerize ACVTP onto the primase-synthesized primer. In contrast, pol α prefers to polymerize dGTP rather than ACVTP by a factor of 5 (V_{max}/K_M) during polymerization of the first nucleotide(s) onto the primase-synthesized primer. Interestingly, PCVTP appears similar to ACVTP regarding polymerization onto primase-synthesized primers and consequent accumulation of products ca. 10 nucleotides long (Figure 6), whereas GCVTP does not have this effect.

This reduced ability of pol α to discriminate against ACVTP could either be a property of polymerization onto an RNA primer or be a unique feature of primase-coupled pol α activity. We previously found that changing the primer from DNA to RNA could dramatically alter how pol α interacts with nucleotide analogs (Kuchta et al., 1992). For example, pol α discriminated against 2′,3′-dideoxy-NTP polymerization 85-fold less efficiently when the primer was RNA instead of DNA. More recently, we have found that primase-coupled pol α activity also has unique properties regarding the ability of araNTPs to act as chain terminators (H. Thompson and R. Kuchta, unpublished data).

Importantly, these data suggest that the addition of the first nucleotide(s) onto a primase-synthesized primer may be uniquely sensitive to inhibition by nucleotide analogs, and kinetic parameters for polymerization of these first few nucleotide(s) may be very different than the parameters for polymerization of nucleotides onto exogenously added primer-templates. Furthermore, when considering the effects

of a nucleotide analog on DNA replication, it may be more accurate to study primase-coupled pol α activity, as opposed to pol α activity on an exogenously added primer-template. Whereas pol α is almost certainly coupled to primase activity during DNA replication, it is unclear if pol α ever uses a "pre-existent" DNA primer-template during DNA replication.

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