

Analysis of inhibitors of bacteriophage T4 DNA polymerase

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ABSTRACT

Bacteriophage T4 DNA polymerase was inhibited by butylphenyl nucleotides, aphidicolin and pyrophosphate analogs, but with lower sensitivities than other members of the B family DNA polymerases. The nucleotides N²-(*p*-n-butylphenyl)dGTP (BuPdGTP) and 2-(*p*-n-butylanilino)dATP (BuAdATP) inhibited T4 DNA polymerase with competitive K_i values of 0.82 and 0.54 μM with respect to dGTP and dATP, respectively. The same compounds were more potent inhibitors in truncated assays lacking the competitor dNTP, displaying apparent K_i values of 0.001 and 0.0016 μM, respectively. BuPdGTP was a substrate for T4 DNA polymerase, and the resulting 3'-BuPdG-primer:template was bound strongly by the enzyme. Each of the non-substrate derivatives, BuPdGDP and BuPdGMPCH₂PP, inhibited T4 DNA polymerase with similar potencies in both the truncated and variable competitor assays. These results indicate that BuPdGTP inhibits T4 DNA polymerase by distinct mechanisms depending upon the assay conditions. Reversible competitive inhibition predominates in the presence of dGTP, and incorporation in the absence of dGTP leads to potent inhibition by the modified primer:template. The implications of these findings for the use of these inhibitors in the study of B family DNA polymerases is discussed.

INTRODUCTION

The DNA polymerase encoded by bacteriophage T4 (T4 pol) is a useful model for study of the structure and function of B family DNA polymerases. The enzyme contains 898 amino acids,¹ and possesses both DNA polymerase and 3'-5' exonuclease activities.² Isolation and sequence determinations of the wild type enzyme and numerous selected and engineered mutants have been described.^{1,3-5} T4 pol is a member of the B family of DNA polymerases, a family that includes eukaryotic enzymes such as animal cell DNA polymerases α, δ and ε and the corresponding enzymes from yeast, and the DNA polymerases from herpesviruses and other animal viruses and bacteriophages.^{1,6,7} Because of its high amino acid sequence homology with the B family enzymes and the availability of informative

mutants, the T4 enzyme represents a good model to study the structure and mechanism of the polymerase and exonuclease sites of these DNA polymerases. With respect to inhibitor profiles which are useful for classifying DNA polymerases, the T4 pol has been reported to be sensitive to inhibition by aphidicolin and the nucleotide analogs, N²-(*p*-n-butylphenyl)-2'-deoxyguanosine 5'-triphosphate (BuPdGTP) and 2-(*p*-n-butylanilino)-2'-deoxyadenosine 5'-triphosphate (BuAdATP), properties characteristic of B family DNA polymerases, but relatively insensitive to phonoacetic acid (PAA).⁸

We have studied in detail a unique series of synthetic nucleobases and nucleotides that are potent competitive inhibitors of certain replicative DNA polymerases. The purine dNTP analog BuPdGTP and its adenine counterpart BuAdATP inhibit animal cell DNA polymerase α (pol α)^{9,10} and the corresponding DNA polymerase I from yeast¹¹ with nanomolar potency. Other polymerases in the B family are inhibited by BuPdGTP and BuAdATP but with lower potencies. Animal cell DNA polymerases δ and ε are weakly inhibited (K_i ca. 100 μM),¹² but HSV1, φ29 and T4 DNA polymerases were reported to be inhibited in the 1-10 μM range.^{8,9} In contrast to many nucleotides that inhibit DNA polymerases largely as a consequence of incorporation into the primer:template (reviewed in ref. 13), BuPdGTP (and probably BuAdATP) do not appear to be substrates for pol α.¹⁴ Yet, T4 DNA polymerase which is less sensitive to BuPdGTP did incorporate this nucleotide (ref. 14 and *vide infra*). Significantly, the prototype family A DNA polymerase, *E. coli* DNA polymerase I (Klenow), was essentially insensitive to inhibition by BuPdGTP but incorporated the nucleotide with surprising efficiency.¹⁵

The finding that wild type T4 pol could use BuPdGTP as a substrate suggested that inhibitory mechanisms of this and related compounds could be complex and could differ between nominally homologous enzymes. For the purpose of comparisons with pol α, and to identify inhibitor probes of the active site of T4 pol, we have begun a systematic study of several classes of inhibitors, viz. butylphenyl compounds, aphidicolins and pyrophosphate analogs, against wild type and several mutant T4 DNA polymerases. We report in this paper that BuPdGTP, and probably BuAdATP, inhibit wild type T4 DNA polymerase by at least two distinct mechanisms depending on the assay conditions.

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MATERIALS AND METHODS

Materials

Inhibitors. Nucleotide analogs were synthesized as described: N²-(*p*-n-butylphenyl)guanine (BuPG), N²-(*p*-n-butylphenyl)-2'-deoxyguanosine 5'-diphosphate (BuPdGDP) and its 5'-triphosphate (BuPdGTP);¹⁶ 2-(*p*-n-butylanilino)adenine (BuAA) and 2-(*p*-n-butylanilino)-2'-deoxyadenosine 5'-triphosphate (BuAdATP);¹⁰ N²-(*p*-n-butylphenyl)-2'-deoxyguanosine 5'-(α , β -methylene)triphosphate (BuPdGMPCH₂PP) (Yanachkov and Wright, manuscript in press in *Nucleosides and Nucleotides*). Aphidicolin was a gift from the Natural Products Chemistry Branch, National Cancer Institute, and aphidicolin derivatives were synthesized as described.¹⁷ Phosphonoacetic acid (PAA) was obtained from Sigma and phosphonoformic acid (PFA) from Fluka; tetrasodium carbonyldiphosphonate (COMDP) was synthesized from tetraethyl methanediphosphonate as described.¹⁸

Enzyme. Wild type bacteriophage T4 DNA polymerase was isolated and purified as described,¹⁹ and was stored in aliquots of 4.2 mg protein/mL (23000 units/mL).

Oligonucleotides. The 17mer and 29mer oligodeoxyribonucleotides (see text for sequences) were obtained from Operon, and the 3'-BuPdG-18mer was synthesized as described.¹⁵ Conditions for 5'-³²P-labelling of primers, annealing of primer:templates, and conditions for denaturing polyacrylamide-urea gel electrophoretic (PAGE) analysis and x-radiography have been described.¹⁴

Enzyme assays

Assays of DNA polymerase activity were typically done in 25 μ L volumes containing 30 mM Tris-HCl (pH 7.5) and 20% glycerol, 400 μ g/mL activated calf thymus DNA,²⁰ 25 μ M each of dCTP, dATP and dGTP, 10 μ M [³H]dTTP (1250 cpm/pmol), 4 mM DTT, 10 mM Mg(OAc)₂, and 100 ng of enzyme. Reaction mixtures were incubated at 30°C for 10 minutes and stopped by the addition of 0.5 mL of ice cold 10% trichloroacetic acid/0.1 M sodium pyrophosphate. Acid-insoluble product was collected on GF/A filters which were washed three times with 0.1 M HCl/0.1 M sodium pyrophosphate and counted in 1 mL of Optifluor. Truncated assays lacking one dNTP were otherwise identical to the above assays. Assays of COMDP employed 30 mM HEPES, pH 7.5, in place of Tris buffer.

Assays with oligo dG:poly dC as primer:template were done in 25 μ L volumes containing 20 mM potassium phosphate (pH 7.5) and 20% glycerol, 0.26 mM oligo dG:poly dC (base ratio 1:10), 10 μ M [³H]dGTP (250 cpm/pmol), 250 μ g/mL BSA, 4 mM DTT, 0.1 mM EDTA, 10 mM MgCl₂, and 100 ng of enzyme. Incubations were done at 30°C for 10 min, and stopped and processed as described above.

Inhibitor assays. Compounds were tested in duplicate by the addition of twofold or threefold serial dilutions of inhibitors dissolved in buffer (nucleotides, pyrophosphate analogs) or Me₂SO (bases, nucleosides, aphidicolins) to enzyme assay reactions as appropriate. Control reactions for the latter inhibitors contained the same concentration of Me₂SO. For determinations of apparent K_i values in a truncated assay or IC₅₀ values in a full assay, 4–6 concentrations of inhibitor were used, and the results of at least two independent experiments were plotted as

% inhibition vs. log inhibitor concentration. Standard deviations of the values reported in Table 1 were typically within \pm 20%. For assays involving variable concentrations of a competitor dNTP, results were plotted (*SigmaPlot*) as 1/pmol dTMP incorporated vs. 1/substrate concentration at 3–4 concentrations of dNTP (Lineweaver–Burk plots, Figure 1). Regression lines were obtained using the program *Enzyme Kinetics* (D.G. Gilbert, Indiana University) which weighted the data points. K_i values are the average of K_i = 1/[K_m'/K_m]-1], where K_m' is the apparent K_m of the competitor dNTP in the presence of the inhibitor at concentration I. Reported values are the average of two experiments.

Primer extension reactions. These were done in 10 μ L reaction volumes in 30 mM Tris-HCl, pH 7.5, containing 5% glycerol, 4 mM DTT, 10 mM Mg(OAc)₂, 1.2 μ M of 5'-[³²P]-primer:template and 1 μ g of T4 DNA polymerase. Reactions were incubated for 5 min at 30°C, and terminated and processed for PAGE analysis as described.¹⁴

RESULTS

Inhibitor screening of wild type T4 DNA polymerase

The T4 DNA polymerase was assayed using activated DNA and a mixture of three dNTPs, including [³H]dTTP, but lacking the dNTP expected to be competitive with the inhibitor. (In assays of pyrophosphate analogs all dNTPs were used.) This 'truncated' assay without one dNTP represents a useful technique for detecting direct competitive inhibitors of DNA polymerases, because inhibition is amplified in the absence of the competitive substrate. We have shown that the apparent K_i values for reversible, competitive inhibitors derived from the truncated assay are identical to those obtained from classical variable substrate analyses.^{13,21} Most DNA polymerases will support synthesis in the absence of one substrate at a level sufficient to serve as 'control' incorporation. It is essential, however, that a balance of high specific activity of labelled dNTP and short assay times, where incorporation of label remains linear with time, be established for such assays to be useful.¹³ Incorporation of [³H]dTTP by T4 DNA polymerase under the truncated conditions used in this work was linear for up to 30 minutes, and all such inhibitor assays were done for 10 minutes. Results of the testing of base, nucleotide, aphidicolin and pyrophosphate analogs against wild type T4 polymerase are summarized in Table 1. The potent inhibition of the T4 enzyme by BuPdGTP and BuAdATP, i.e. apparent K_i values of about 1 nM, was similar to that reported for DNA polymerase α .^{9,10} However, the apparent K_i value of 1 nM in the 10 minute assay (Table 1) fell to 60 pM in a 30 minute assay. Considering that the T4 enzyme, but not pol α , was shown to incorporate BuPdGTP,¹⁴ we considered the possibility that incorporation of the modified nucleotides resulted in a product that was strongly inhibitory to the enzyme (see below). The corresponding nucleobases, BuPG and BuAA, although much weaker inhibitors than the nucleotides, had potencies similar to those reported for the bases against pol α ,^{10,22,23} and inhibition in each case was reversed specifically by the competitor nucleotide as expected (results not shown).

Aphidicolin showed significant inhibition of the enzyme, with an apparent K_i of 10 μ M in the absence of dCTP, although this is at least tenfold weaker than its potency against HSV1 pol²⁴ and animal cell pols α , δ and ϵ .^{12,25} Two analogs of aphidicolin were

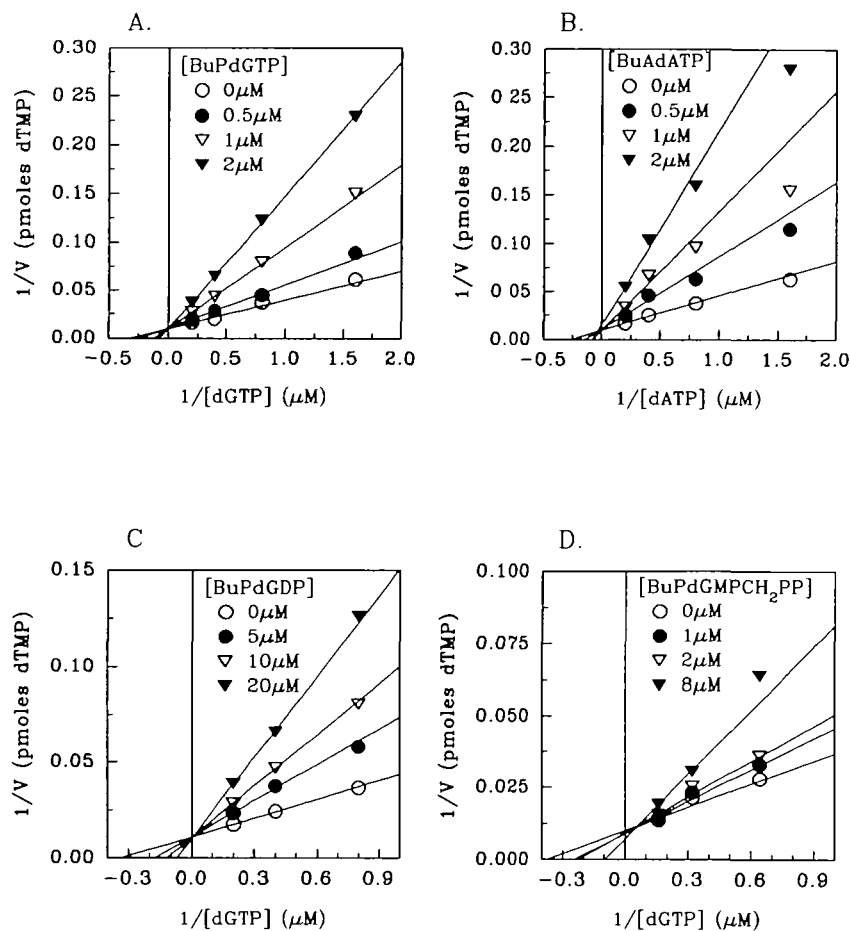


Figure 1. Kinetics of inhibition of T4 DNA polymerase by butylphenyl nucleotides. Reactions were done as described in Materials and Methods with activated DNA and with dGTP or dATP as variable substrate as indicated. Results are displayed as Lineweaver–Burk plots, from which competitive K_i values were obtained (see Materials and Methods). **A**, competition between BuPdGTP and dGTP. **B**, competition between BuAdATP and dATP. **C**, competition between BuPdGDP and dGTP. **D**, competition between BuPdGMPCH₂PP and dGTP. The K_m values for dGTP were 3.11 μM (panel A), 2.92 μM (panel C) and 2.65 μM (panel D); the K_m for dATP was 3.78 μM (panel B).

weaker inhibitors (Table 1), consistent with their relative activities reported for pol α inhibition.¹⁷

The pyrophosphate analogs phosphonoacetate (PAA) and phosphonoformate (PFA) were weak inhibitors of T4 pol under full assay conditions, with IC_{50} values of 120 and 540 μM , respectively (Table 1). These values are much higher than those reported for PAA and PFA inhibition of pol α (IC_{50} = 15 and 21 μM , respectively) and several herpesvirus DNA polymerases (IC_{50} s ca. 1 μM).²⁶ Carbonyldiphosphonate (COMDP), an inhibitor of DNA polymerases α and ϵ , but with tenfold selectivity for the latter enzyme,¹⁸ inhibited T4 pol weakly, with IC_{50} = 690 μM (Table 1). The weak response of the T4 enzyme is consistent with the reported lack of effect of COMDP at 100 μM on the herpesvirus DNA polymerases.²⁶ In general, although T4 DNA polymerase is less sensitive to aphidicolin and pyrophosphate analogs, its inhibitor profile (Table 1) is similar to that of the other B family DNA polymerases.¹³

Kinetics of competitive inhibitors of T4 pol

To determine the mechanisms of several inhibitors of T4 pol, we performed classical experiments using variable concentrations of competitive dNTP, and representative results are displayed

Table 1. Inhibitors of T4 DNA polymerase

Cpd	Condition ¹	apparent K_i (μM)
BuPdGTP	-dGTP	(0.001) ²
BuPdGDP	"	1.9
BuPdGMPCH ₂ PP	"	2.5
BuPG	"	35.5
BuAdATP	-dATP	(0.0016) ²
BuAA	"	185
Aphidicolin	-dCTP	10.0
16-Ketoaphidicolin	"	72
3-Epiaphidicolin	"	100
PFA	full assay	120
PAA	"	540
COMDP	"	690 ³

¹Assays were done as described for 10 minutes with activated DNA and four dNTPs, including [³H]dTTP (full assay), or with the truncated assay lacking the indicated dNTP. ²These values depended upon assay time (see text). ³Assay done with HEPES in place of Tris buffer.

in Figure 1 in the form of Lineweaver–Burk plots. The plots revealed that inhibition by BuPdGTP (Figure 1A), BuAdATP (Figure 1B) and aphidicolin (plot not shown) was competitive

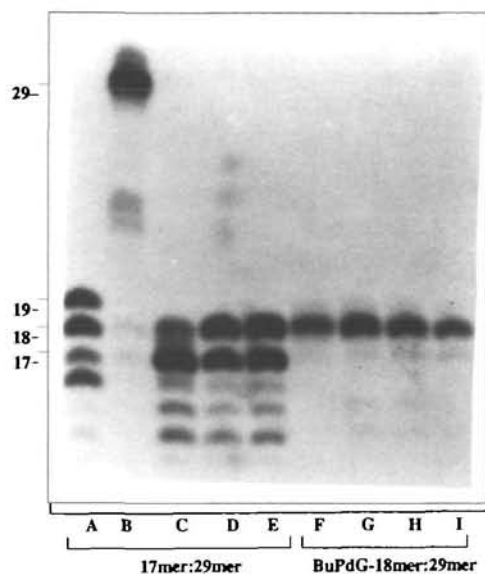


Figure 2. PAGE analysis of incorporation of BuPdGTP by T4 DNA polymerase. Assays described in Materials and Methods utilized a 5'-³²P-labelled 17mer:29mer duplex (lanes A–E), or a 5'-³²P-labelled 3'-BuPdG-18mer:29mer duplex (lanes F–I) as primer:templates with the indicated additions (see text for sequences). The products were separated on a 12% polyacrylamide gel by electrophoresis at 1500 volts for 4 h, and the dried gel was exposed to X-ray film as described (ref 14). Lane A, 25 μ M each dGTP and dTTP. Lane B, 25 μ M each dGTP, dTTP, dCTP and dATP. Lane C, 25 μ M dGTP. Lane D, 10 μ M BuPdGTP. Lane E, 10 μ M BuPdGTP + 100 μ M dTTP. Lane F, primer:template alone (no enzyme). Lane G, 100 μ M dTTP. Lane H, 25 μ M dTTP. Lane I, no additions (enzyme + primer:template).

with respect to dGTP, dATP and dCTP, respectively. The K_i value derived from the results for aphidicolin was $10.7 \pm 1.8 \mu$ M (K_m for dCTP = 2.88μ M), a value nearly identical to the K_i obtained in the assay lacking dCTP, as expected. However, the K_i values derived for BuPdGTP, $0.82 \pm 0.3 \mu$ M, and for BuAdATP, $0.54 \pm 0.13 \mu$ M, were much higher than those found in the truncated assay (Table 1), suggesting different mechanisms of enzyme inhibition under the two conditions.

The ability of BuPdGTP to interact with T4 pol specifically as a dGTP analog was confirmed in experiments measuring [³H]dGTP incorporation into oligo dG:poly dC. In the presence of variable concentrations of dGTP, BuPdGTP acted as a competitive inhibitor of the enzyme, with $K_i = 80 \pm 12$ nM, where the K_m for dGTP was $8 \pm 1.2 \mu$ M (data not shown). Furthermore, in separate experiments with oligo dG:poly dC, the inhibitory effect of 125 nM BuPdGTP was not reversed in the presence of 800 μ M of dATP, dCTP or dTTP.

Effect of non-substrate analogs of BuPdGTP

The possibility that inhibitor incorporation could explain the difference in apparent K_i s of butylphenyl nucleotides in truncated and variable substrate assays was first tested with non-substrate analogs of BuPdGTP. One such compound, the 5'-diphosphate BuPdGDP, was found previously to be nearly equipotent with BuPdGTP as an inhibitor of pol α .⁹ BuPdGDP displayed an apparent K_i of 1.9 μ M against T4 pol in the truncated assay lacking dGTP (Table 1), a value somewhat lower than the K_i of $5.5 \pm 1.2 \mu$ M derived from assays at variable dGTP concentrations (Figure 1C). The results of the latter assay clearly showed that BuPdGDP was competitive with dGTP as an inhibitor of T4 pol.

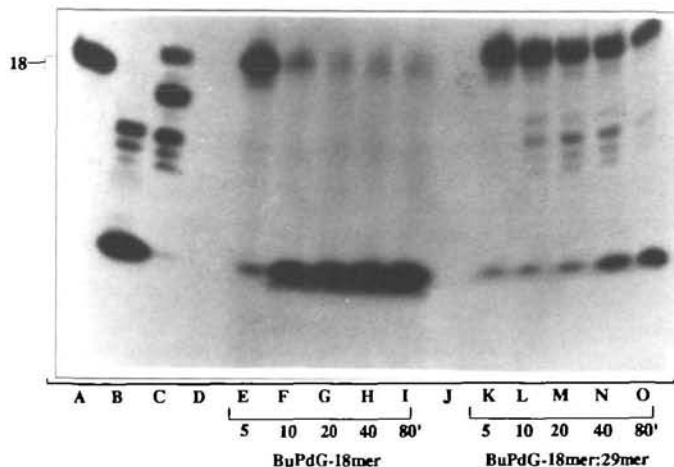


Figure 3. Exonucleolytic degradation of 3'-BuPdG-18mer by T4 DNA polymerase. Assays were done and analyzed by PAGE as described in Materials and Methods. Incubations with T4 pol were done in the absence of dNTPs, but in the presence of single stranded 5'-³²P-labelled 3'-BuPdG-18mer (lanes E–I) or double stranded, labelled 3'-BuPdG-18mer:29mer (lanes K–O). Lane A, 3'-BuPdG-18mer alone. Lane B, 17mer:29mer + enzyme (control). Lane C, 17mer:29mer + enzyme + 25 μ M dGTP. Lanes D and J, empty. Lanes E–I, incubations of 3'-BuPdG-18mer + enzyme for 5, 10, 20, 40 and 80 min, respectively. Lanes K–O, incubations of 3'-BuPdG-18mer:29mer + enzyme for 5, 10, 20, 40 and 80 min, respectively.

A derivative of BuPdGTP that would be a true triphosphate analog, but one that was expected to be non-incorporable by DNA polymerases, was sought. We, therefore, synthesized the α , β -methylene derivative BuPdGMPCH₂PP (manuscript in press) and tested its effects on the T4 DNA polymerase. BuPdGMPCH₂PP inhibited T4 pol with a K_i of 2.5 μ M in the assay lacking dGTP (Table 1). In the variable substrate assay, the Lineweaver–Burk plot indicated that this phosphonate was competitive with dGTP, with $K_i = 2.3 \pm 0.5 \mu$ M (Figure 1D). These results suggested that BuPdGMPCH₂PP was a non-substrate, competitive inhibitor of the enzyme in both truncated and variable competitor conditions. Indeed, neither BuPdGMPCH₂PP nor BuPdGDP at 10 μ M showed detectable incorporation in primer extension reactions with T4 pol under conditions where BuPdGTP itself completely converted primer to 3'-BuPdG-primer (see below).

Incorporation of BuPdGTP by T4 DNA polymerase

The greater apparent potencies of BuPdGTP and BuAdATP in truncated assays lacking the competitive substrates (Table 1) than in assays containing those substrates (Figure 1) suggested that the inhibitors are incorporated into primers at dGTP and dATP-requiring sites, respectively, in the absence of the competitive substrate, and that the resulting 3'-modified-primer:templates strongly bind the enzyme in an unproductive complex. Indeed, the degree of inhibition of T4 DNA polymerase by 1.25 μ M BuPdGTP ($83 \pm 3.5\%$) did not vary during the linear period (16 minutes) of an assay containing a limiting concentration of dGTP (4 μ M).

In an attempt to understand the molecular mechanism(s) of BuPdGTP-T4 pol interactions, we utilized synthetic primer:templates and denaturing polyacrylamide-urea gel electrophoretic (PAGE) analysis of products to study the effects of BuPdGTP at a single incorporation site. A 5'-³²P-labelled 17mer primer, 5'-d(GTAAAACGACGGCCAGT), was annealed

to a 29mer template, 3'-d(CATTTTGCTGCCGGTCA-CATGCCGATCCC), and used to compare the ability of T4 pol to incorporate dGTP or BuPdGTP to produce a normal 18mer product or the 3'-BuPdG-18mer [5'-d(GTAAAACGACGGCC-AGT^{BuP}G)], respectively. We also compared the latter product with 5'-³²P-labelled 3'-BuPdG-18mer, synthesized chemically as described,¹⁵ and asked if the 3'-BuPdG-primer:template was a terminator of DNA synthesis. Preliminary experiments established that 1 µg of T4 pol per assay tube (ca. 1 µM enzyme) gave full conversion of 17mer to 29mer in the presence of 25 µM dNTPs without substantial primer degradation due to 3' to 5' exonuclease activity.

The results of the primer extension assays are summarized in Figure 2. Both dGTP (lane C) and BuPdGTP (lane D) gave products migrating as 18mers with weak bands at lengths of 14–16 resulting from 3'–5' exonuclease degradation. However, when the next substrate dTTP was present in the reaction, extension to the 19mer only occurred when dGTP was the substrate (lane A); no extension of the putative BuPdG-18mer was detected in the presence of 100 µM dTTP (lane E). Comparison of this result with that from direct incubation of enzyme with synthetic 3'-BuPdG-18mer:29mer revealed that the modified primer was also not extended with dTTP (lanes G and H).

The results in lane I of Figure 2 suggested that 3'-BuPdG-modified primers were resistant to exonucleolytic cleavage by the T4 enzyme. A more complete study (Figure 3) indicated that this resistance is relative. Incubation of double stranded 3'-BuPdG-18mer:29mer with enzyme alone resulted in degradation of primer, but with a half life greater than 80 min (lanes K–O). In contrast, double stranded 17mer:29mer degraded to near completion in 10 min (lane B). Single stranded 3'-BuPdG-18mer was degraded rapidly by the enzyme (lanes E–I), however, with a half life of less than 10 min. Thus, in stark contrast to the complete resistance of single stranded 3'-BuPdG-primers to exonucleolytic degradation by *E. coli* pol I,¹⁵ the T4 polymerase is able to remove 3'-BuPdGMP, but at a considerably lower rate than removal of dGMP.

Does the 3'-BuPdG-18mer inhibit T4 DNA polymerase?

The question of whether 3'-BuPdG-primer:templates had unusual affinity for T4 DNA polymerase was addressed in a 'sequestration' experiment in which the effect of primer:template on incorporation of [³H]dGTP into oligo dG:poly dC by T4 pol was monitored. The 3'-BuPdG-18mer:29mer duplex, which could not itself incorporate label, inhibited this reaction (at 10 µM dGTP) with IC₅₀ = 73 nM (as 3'-OH groups), approximately the value of the K_i derived for inhibition of the oligo dG:poly dC reaction by BuPdGTP. (There is no strict single or double-stranded 'control' oligomer for comparison with this result, because these can be either degraded (single stranded, double stranded) or extended (double stranded) when added to the assay.) Neither assay time, up to 20 min, nor preincubation of 3'-BuPdG-18mer:29mer with enzyme, for 30 min at 0°C, affected inhibitory activity in this assay (data not shown). Thus, the interaction between polymerase and 3'-BuPdG-primer:template is reversible under the assay conditions employed.

In a similar experiment with the concentration of 3'-BuPdG-18mer:29mer fixed at 500 nM, addition of 100 µM dTTP (i.e. the next substrate) did not increase the degree of inhibition of T4 pol, suggesting that binding of the modified primer:template did not require the dNTP substrate. This

contrasts with the mechanism of typical chain terminating substrates in which terminated primer:templates complex strongly with DNA polymerases only in the presence of the next dNTP substrate.^{27–29} The possible lack of affinity of 3'-BuPdG-primer:templates for the next dNTP when bound to the T4 polymerase molecule may explain the inability of the modified primer to be extended by that substrate (Figure 2).

DISCUSSION

The response of T4 DNA polymerase to selected inhibitors was qualitatively similar to that of other B family enzymes, but there were some significant differences. For example, the T4 DNA polymerase was less sensitive than DNA polymerase α to aphidicolin and pyrophosphate analogs and to the butylphenyl nucleotides, BuPdGTP and BuAdATP; however, unlike pol α, the T4 enzyme incorporated the latter inhibitors. In addition, the butylphenyl nucleotide-terminated primer:template was a potent inhibitor of T4 DNA polymerase.

Evidence for this unique mechanism of T4 DNA polymerase inhibition by butylphenyl nucleotides was derived from several experiments. First, BuPdGDP and BuPdGMPCH₂PP, non-incorporable analogs of BuPdGTP, inhibited the T4 enzyme with similar potencies in the truncated (Table 1) and variable competitor assays (Figures 1C and 1D). Second, the enzyme utilized BuPdGTP to extend a primer:template at a site requiring dGTP incorporation but could not extend the modified primer (Figure 3). Third, a synthetic 3'-BuPdG-primer:template inhibited T4 pol in the reaction of [³H]dGTP with oligo dG:poly dC with a potency close to the K_i of BuPdGTP in the same reaction. The apparent 'nanomolar' potency of butylphenyl nucleotides in truncated assays of T4 pol with activated DNA is likely a direct result of their incorporation, in contrast to the 'micromolar' potency of the same compounds in full assays. DNA polymerase α, which has been shown not to incorporate BuPdGTP,¹⁴ thus remains a uniquely nanomolar-sensitive B family DNA polymerase to reversible, competitive inhibition by these compounds.

Incorporation of butylphenyl nucleotides *per se* would not necessarily lead to inhibition of T4 DNA polymerase activity. Indeed, *E. coli* DNA polymerase I (Klenow), an enzyme that is very resistant to BuPdGTP in full assay conditions, incorporated the nucleotide readily.¹⁵ The 3'-BuPdG-primers were resistant both to extension and to exonucleolytic degradation by pol I. BuPdGTP actually increased pol I-catalyzed incorporation of [³H]dTTP in a truncated assay lacking dGTP, likely as a result of protection of modified primers from exonuclease digestion.¹⁵ The latter result, in fact, implies that pol I does not appreciably bind the BuPdG-primer:templates. Indeed, the synthetic 3'-BuPdG-18mer:29mer did not inhibit pol I (Klenow) in sequestration experiments like those described above for T4 DNA polymerase (data not shown). Although T4 pol also incorporated BuPdGTP and could not extend the modified primers (see above), it is significantly different from pol I in its high affinity for the 3'-BuPdG-primer:templates and in its ability to remove the 3'-BuPdGMP residue.

Inhibition resulting from incorporation of BuPdGTP (and BuAdATP) by T4 DNA polymerase or other DNA polymerases confounds the measurement of the direct affinity of the inhibitors for the enzymes. This is especially important for comparison of sensitivity of different enzymes, and for interpretation of the effects of the nucleotides on mutant T4 pols (work in progress).

The similar potency of the α , β -methylene triphosphate derivative BuPdGMPCH₂PP in truncated and variable substrate assays is fully consistent with its inability to be a substrate for T4 DNA polymerase. Furthermore, its potency is only about threefold lower than that of BuPdGTP in the variable substrate assay, indicating a small effect of the methylene substitution on binding of the nucleotide. The finding that BuPdGMPCH₂PP inhibited calf thymus DNA polymerase α in the truncated assay with $K_i = 9.5$ nM, only fivefold lower than that of BuPdGTP (manuscript in press), indicates that this analog will be of significant use in studying affinities of butylphenyl nucleotides for B family DNA polymerases in the absence of incorporation.

Despite protein sequence similarities, B family DNA polymerases differ quantitatively and qualitatively in their response to the butylphenyl nucleotides. In addition to the T4 DNA polymerase described in this work, several of the polymerases with intermediate sensitivity to BuPdGTP and BuAdATP also incorporate the inhibitors, i.e. HSV1 pol (K.-W. Knopf, personal communication) and ϕ 29 pol (M. Salas, personal communication). Study of BuPdGTP/BuAdATP inhibitory potencies, incorporation kinetics and interaction of BuPdG/BuAdA-modified primer:templates with DNA polymerases, especially with mutant enzymes, may prove of value in analysis of the function of active site residues in B family DNA polymerases.

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