The molecular mechanism of inhibition of alpha-type DNA polymerases by N^2-(butylphenyl)dGTP and 2-(butylanilino)dATP: variation in susceptibility to polymerization

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ABSTRACT

Calf thymus DNA polymerase alpha (pol α) and bacteriophage T4 DNA polymerase (pol T4) were exploited as model enzymes to investigate the molecular mechanism of inhibitory action of N^2-(*p*-n-butylphenyl)dGTP (BuPdGTP) and 2-(*p*-n-butylanilino)dATP (BuAdATP) on the BuPdNTP-susceptible alpha polymerase family. Kinetic analysis of inhibition of pol α with mixtures of complementary and non-complementary template:primers indicated that both nucleotides induced the formation of a polymerase: inhibitor:primer-template complex. Primer extension experiments using the guanine form as the model analog indicated that pol α cannot utilize these nucleotides to extend primer termini. In contrast, pol T4 polymerized BuPdGTP, indicating that resistance to polymerization is not a common feature of the inhibitor mechanism among the broad membership of the alpha polymerase family.

INTRODUCTION

The dNTP analogs, N^2-(*p*-n-butylphenyl)dGTP (BuPdGTP), and 2-(p-n-butylanilino)dATP (BuAdATP), are potent and highly selective inhibitors of mammalian pol α and several other replication-specific polymerases of the pol α type (1–4). These BuPdNTPs have found wide use as pol α-specific inhibitor-probes and as tools for distinguishing enzymes of the pol α family from other mammalian DNA polymerases, including the aphidicolin-sensitive DNA polymerases, delta and epsilon (5).

To enhance the utility of the BuPdNTPs as probes of the structure of pol α and its dNTP-binding site, we seek to characterize precisely the molecular mechanism of the BuPdNTP-pol α interaction. Specifically, we aim to determine the extent to which the mechanism of these nucleotides mimics that of their pol α-specific base forms, N^2-(butylphenyl)guanine (BuPG) and 2-(butylanilino)adenine (BuAA; ref. 6), and that of 6-(*p*-hydroxyphenyl-hydrazino)uracil (H_2·HPUra), the prototypic, pol III-specific dGTP analog on which the design of this inhibitor class was originally based (7). In this paper, we address three fundamental questions with respect to the BuPdNTP-pol α interaction: (1) Do the BuPdNTPs induce the sequestration of pol α into a catalytically inactive DNA:inhibitor:protein complex? (2) Does the action of the BuPdNTP form on pol α involve polymerization of its dNMP moiety to the primer-terminus? (3) Does the basic mechanism discerned for *mammalian* pol α apply more broadly across the alpha-like family of BuPdNTP-sensitive DNA polymerases which display strong primary sequence homology with the mammalian enzyme?

MATERIALS AND METHODS

Nucleotides

Homopolymers and unlabeled forms of dATP, dCTP, dGTP, and dTTP were purchased from Pharmacia. BuAdATP and BuPdGTP were synthesized and purified as described in, respectively, (2) and (8).

Source and assay of DNA polymerases

Calf thymus DNA pol α was purified on an immunoaffinity matrix prepared from protein A-Sepharose 4B (Pharmacia) and an anti-calf thymus pol α monoclonal antibody provided by Dr. Lucy Chang (hybridoma 17; ref. 9). The immunoaffinity matrix was prepared and used to purify enzyme as described (9). Sodium dodecylsulfate-polyacrylamide gel (SDS-PAG) electrophoretic analysis and analysis of the polymerase activity of the immunopurified polymerase indicated a four peptide structure and a specific activity essentially identical to that described in (9). Pol α activity was assayed in the presence of activated calf thymus DNA or synthetic homopolymers as described in (1).

Poly(dA):oligo$(dT)_{12-18}$ and poly(dC):oligo$(dG)_{12-18}$ were prepared by annealing homopolymer and oligomer in the ratio of 10 A_{260} units of polymer: 0.5 A_{260} units of oligomer. Bacteriophage T4 DNA polmerase (pol T4) was an homogeneous

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preparation of recombinant enzyme prepared and assayed as described in ref. 15 and kindly provided by Dr. Linda Reha-Krantz.

Assay of primer extension

Primer extension was assessed by autoradiographic analysis of denaturing DNA sequencing gel electropherograms of the products resulting from the action of pol α on a 5′[^{32}P]-labeled 17 residue oligonucleotide primer annealed to a 29 residue template. The structure of the primer (M13 sequencing primer # 1211, Boehringer Mannheim) and template (Operon) is shown in the top panel of Fig. 1. The labeling of primer was based on the method described in (10); 0.125 OD_{260} U of primer was incubated with 500 μCi of [γ^{32}P]-ATP (3000Ci/mmol) and 5 units of polynucleotide kinase (New England Biolabs) at 35°C for 60 min in 50 μl of labeling buffer, (50 mM Tris·HCl (pH 7.6), 10 mM $MgCl_2$, 5 mM dithiothreitol, and 1 mM spermidine). The latter mixture was heated at 100° for 2 min and desalted on a 250 μl Sephadex G-25 column equilibrated with 50 mM Tris·HCl (pH 7.6). The desalted primer was mixed with an equal amount of the 29 residue template in labeling buffer minus spermidine, and the solution was incubated at 50°C for 5 min; to complete annealing, the solution was held at room temperature for 60 min and then quenched in ice.

The reaction conditions for primer extension by pol α and pol T4 were adapted from those described for assay of pol α in (11); 0.1−0.2 units of enzyme were incubated in the presence/absence of dGTP or BuPdGTP at 35° for 30 min in 12.5 μl of a solution containing 20 mM potassium phosphate buffer (pH 7.5), 8 mM $MgCl_2$, 4 mM dithiothreitol, 0.1 mM EDTA, and 50 μg/ml of labeled template:primer. The reaction was terminated by addition of 4 μl of stop buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% Xylene cyanol FF), and the mixture was boiled for 2 min and placed on ice. A 5 μl sample of the quenched mixture was applied to a 12% polyacrylamide DNA sequencing gel and electrophoresed for 4 hr at 1500 volts at constant power. The gel was dried and exposed to a 14″×17″ sheet of Fuji RX type X-ray film for 1−6 hr and developed.

Calf thymus terminal deoxyribonucleotidyl transferase (TdT; New England Nuclear) was used to obtain a sequencing ladder for identifying the length of specific products of primer extension with BuPdGTP; 0.25 units of TdT were incubated at 37°C with 1 μg of [^{32}P]-labeled primer in the presence of 50 μM BuPdGTP in 10 μl of a TdT assay buffer described in (12). After 30 min the incubation mixtures were quenched with stop buffer and subjected to gel analysis as described above for the products of pol α-catalyzed primer extension.

RESULTS

Kinetic analysis of inhibitor action

Table I summarizes relevant features of the interaction of immunopurified calf thymus pol α and the natural and N^2-substituted dNTPs (BuPdNTPs). In the presence of activated DNA the enzyme displayed a K_m of approximately 8 μM for the natural purine dNTPs and K_is for the BuPdNTPs in the nanomolar range. In contrast to human (HeLa cell) and Chinese hamster ovary cell pol alphas, both of which display essentially equal sensitivity to BuPdGTP and BuAdATP (1,2), the calf enzyme consistently displayed a sensitivity to BuPdGTP 3−4 times that which it displayed to BuAdATP. Differences in potency notwithstanding, the inhibitory effect of each BuPdNTP in the presence of activated DNA was, as expected, subject to competition with the analogous purine dNTP. The action of BuAdATP was not subject to detectable competition by dGTP, dCTP or dTTP, nor was the action of BuPdGTP antagonized detectably by dATP, dCTP, or dTTP.

The BuPdNTPs also were inhibitory in the presence of homopolymeric template:primers; the action of each inhibitor was subject to competition by the specific dNTP serving as the nucleotide substrate, and the potency of each was significantly influenced by its complementarity to the template. Two template primers were used, poly (dC):oligo(dG) and poly (dA):oligo(dT); unfortunately, the use of the BuAdATP-complementary template, poly(dT), was not possible, because it was not sufficiently effective in promoting oligo(dA)-driven synthesis. The

Table I. Calf Thymus Pol α vs Inhibitory and Natural dNTPs

	Nucleotide[a]					
Condition or Property	BuPdGTP	BuAdATP	dTTP	dCTP	dGTP	dATP
+DNA[b]	0.002μM	0.008μM	N.A.[c]	N.A.	8μM	8μM
Inhibitory action with DNA competively antagonized by:	dGTP only	dATP only	N.A.	N.A.	N.A.	N.A.
+poly(dC):oligo(dG)[d]	0.002μM	1.9μM	N.A.	N.A.	10μM	N.A.
Inhibitory action with poly(dC):oligo(dG) competitively antagonized by:	dGTP only	dGTP only	N.A.	N.A.	N.A.	N.A.
+poly(dA):oligo(dT)[d]	0.04μM	0.5μM	10μM	N.A.	N.A.	N.A.
Inhibitory action with poly(dA):oligo(dT) competitively antagonized by:	dTTP only	dTTP only	N.A.	N.A.	N.A.	N.A.

[a]K_m or K_i; the value represents an average of three determinations with a S.D. of ± 20%.
[b]Assayed in the presence of activated DNA as described in the methods section.
[c]N.A., not applicable.
[d]Assayed in the absence of activated DNA and in the presence of synthetic template:primer as described in the methods section.

poly(dC):oligo(dG)-dependent reaction displayed a K_m of 10 μM for dGTP and a K_i of 0.002 μM for BuPdGTP; both values closely approximated those determined with activated DNA. The poly(dC):oligo(dG)-driven reaction was, as expected, relatively resistant to the non-complementary BuAdATP; the latter displayed a K_i of 1.9 μM, a value nearly 250 times that observed with natural DNA as template primer. The poly(dA):oligo(dT)-driven reaction, in which the template was not complementary to either BuPdNTP, displayed resistance to both inhibitors relative to that displayed with activated DNA. The relative susceptibility of the enzyme to the guanine analog noted in the presence of activated DNA was even greater in the presence of the noncomplementary poly(dA) template; the K_i for BuPdGTP increased roughly 20-fold (0.002 μM to 0.04 μM) while that for BuAdATP increased approximately 60-fold (0.008 μM to 0.5 μM).

BuPdNTP action on pol α does not involve polymerization of the dNMP moiety to the primer terminus

In contrast to their base and lower nucleotide forms, the BuPdNTPs have the potential to serve as substrates for polymerization by the target enzyme. To assess the potential of the BuPdNTPs for incorporation by pol α, we exploited BuPdGTP as the model inhibitor and used sequencing gel analysis to assess its capacity to extend the 17 residue oligonucleotide primer shown in the upper panel of Figure 1. Specifically, we compared the capacity of dGTP and BuPdGTP to extend the primer by one nucleotide residue. The results are shown in the pol α middle panel of Fig. 1. The template:primer served as an effective DNA substrate. In the presence of a complete mixture of natural dNTPs, the bulk of the primer was extended to its full, 29 residue length (lane L). In the presence of dGTP at approximately 5, 10, and 20 times its K_m, the enzyme extended the primer by 1 residue (lanes D–F), as expected from the template structure. However, in the presence of BuPdGTP at approximately 50, 500 and 5000 times its K_i (lanes H, I, and J, respectively) no primer extension was detected, even upon prolonged autoradiographic exposure of the film.

Given a complementary template, BuPdNTPs act by sequestering pol α to template:primer

Paradigm for sequestration mechanism. The design of the butylphenyl purines and their respective dNTP forms (1,2) was based on the structure of an arylpurine prototype which we had found to be a selective inhibitor of bacterial DNA pol III (6,7). The pol III-specific prototype acts specifically by sequestering the target enzyme to template:primer (13,14). The mechanism of sequestration and the structural features essential to it are defined schematically in Fig. 2. As shown in the left panel, the inhibitor molecule has two essential domains, an aryl domain which binds the dNTP binding site of its target enzyme and a base pairing domain comprised of 3 substituents that hydrogen bond with the appropriate pyrimidine (cf., specific base pairs in the middle panel). Induction of complex formation by the inhibitor (cf. scheme in right panel) requires a template:primer with a base-paired 3′ OH primer terminus, and, just distal to this terminus, an unapposed template pyrimidine complementary to the base-pairing domain of the inhibitor. Given the template:primer structure, the inhibitor substitutes for the appropriate incoming purine dNTP and anchors the template pyrimidine to the active site of the enzyme, sequestering the latter in a catalytically inactive complex.

Analysis of complex formation. The results of the primer extension experiments (Fig. 1) and the results of the kinetic analysis of BuPdNTP action (Table I) were consistent with the conventional sequestration mechanism of inhibitor-induced complex formation. To determine specifically whether the latter mechanism actually

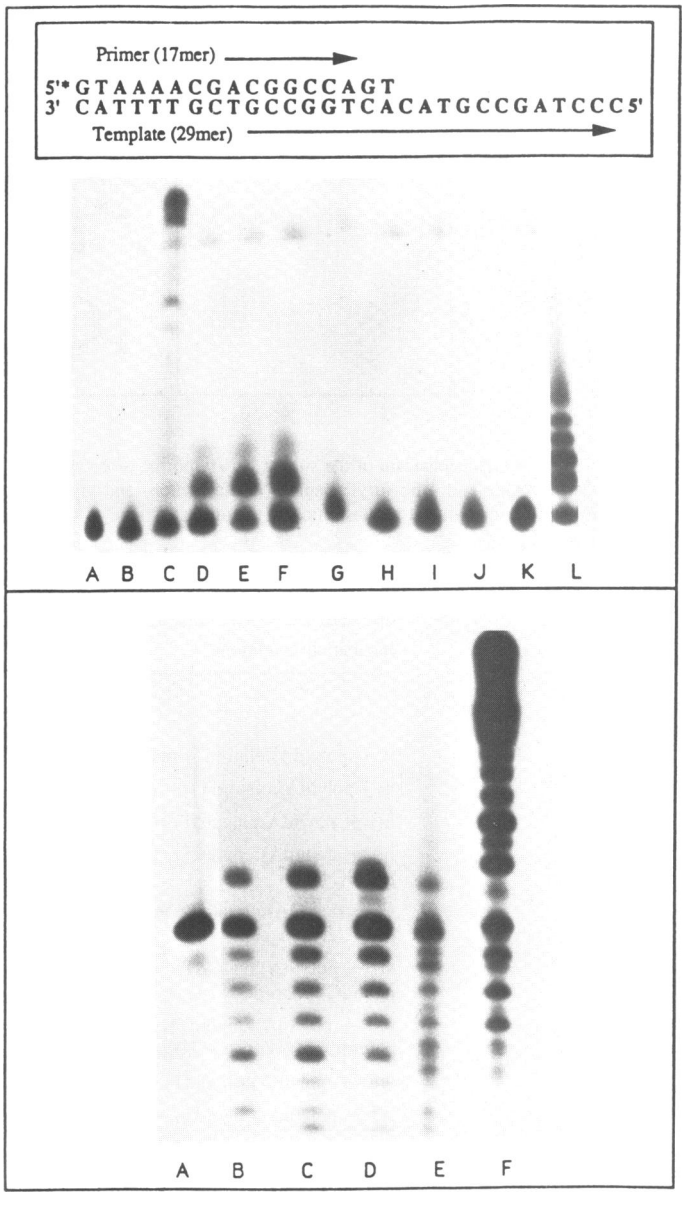

Figure 1. Sequencing gel analysis of primer extension in the presence of dGTP and BuPdGTP. *Upper panel*, structure of the template:primer. *Middle panel*, autoradiographic analysis of the products of pol α action on the above primer:template. 5′[^{32}P] labeling of the primer and the preparation and processing of the incubation mixtures are described in the methods section. Lane A, template:primer, no enzyme; Lanes B and G, template:primer plus enzyme, no dNTP; lanes D, E, and F:dGTP at, respectively, 50, 100, and 200 μM; lanes H, I, and J: template:primer plus enzyme with BuPdGTP at, respectively, 0.1, 1.0, and 10 μM. Lane C:dGTP, dCTP, dATP, and dTTP at 50 μM each; lane L, primer after treatment with TdT in the presence of 50 μM BuPdGTP; lane K, primer plus TdT treatment in the absence of BuPdGTP. Conditions for TdT treatment are described in the Methods section. *Lower panel*: Products of pol T4 action on the above template:primer. Lane A, template:primer, no enzyme; lane E, template:primer plus enzyme plus 25 μM dGTP; lane F, template:primer plus dATP, dGTP, dCTP, and dTTP at 25 mM each; lanes B, C, and D:template:primer plus BuPdGTP at, respectively, 10, 100 and 1000 nanomolar.

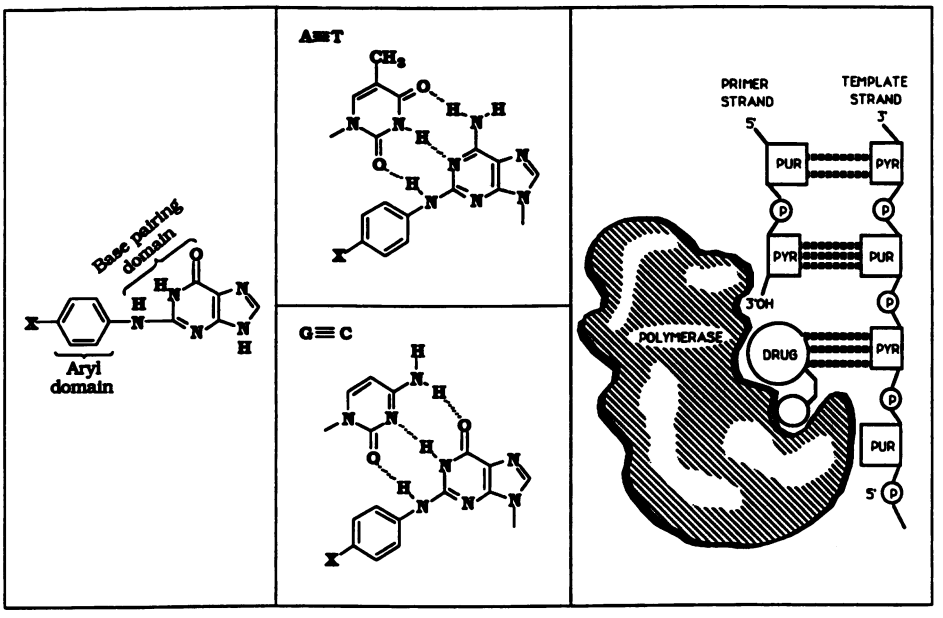

Figure 2. Structure and mechanism of the simple N^2-arylpurine prototype. *Left panel*: Structure, showing base pairing domain and aryl domain. *Middle panel*: Base pairs between cytosine and a N^2-(aryl)guanine (lower section) and thymine and a 2-anilinoadenine (upper section). *Right panel*: Schematic representation of the mechanism of inhibitor-induced sequestration of its target enzyme to template:primer.

Table II. Demonstration of BuPdNTP-Induced Sequestration of Pol α

Incubation conditions[a]	pmol dTMP polymerized		
	Control	+BuPdGTP (0.025μM)	+BuAdATP (0.1μM)
i) + poly(dA):oligo(dT) only	63	59	61
ii) - poly(dA):oligo(dT) + activated DNA[b]	<1	<1	<1
iii) + poly(dA):oligo(dT) + activated DNA[b]	58	5.3	5.2
iv) + poly(dA):oligo(dT) + activated DNA[b] + 800 μM dGTP	61	60	5.8
v) + poly(dA):oligo(dT) + activated DNA[b] + 800 μM dATP	62	5.4	63
vi) + poly(dA):oligo(dT) + poly(dC):oligo(dG)[c]	60	5.8	N.D.[d]
vii) + poly(dA):oligo(dT) + poly(dC):oligo(dG) + 800 μM dGTP	61	60	N.D
viii) + poly(dA):oligo(dT) + poly(dC)[e]	61	62	N.D.
ix) + poly(dA):oligo(dT) + oligo(dG)[f]	62	60	N.D.

[a]assay conditions were those described in the methods section
[b]activated DNA was present at 0.010 mg/ml, a concentration 200 times less than that used in conventional, DNA-directed enzyme assay
[c]present at a concentration of 0.002 μg/ml
[d]not determined
[e]poly(dC) was present at a concentration equivalent to that present as poly(dC):oligo(dG) in [c]
[f]oligo(dG) was present at a concentration equivalent to that present as poly(dC):oligo(dG)in [c]

obtained, we used an approach applied to demonstrate sequestration of bacterial pol III by the pol III-specific inhibitor H_2·HPUra (13,14). The approach exploits the assay of polymerase activity, and it depends on the relatively high level of resistance of pol α to BuPdNTPs when synthesis is driven by a template which is *not* complementary to the base-pairing domain of the inhibitor, (*i.e.* a template which cannot significantly support formation of an inhibitor:DNA:enzyme complex).

The approach exploits two different template:primers: a non-complementary homopolymer:oligomer on which incorporation of a radiolabelled dNMP is followed, and a sequestration-specific template:primer which provides an inhibitor-complementary pyrimidine in its template and the specific primer terminus structure required for inhibitor-induced sequestration (cf., Fig. 2, right panel). Specifically, oligo(dT) synthesis on poly(dA) is followed in the presence of a subsaturating concentration of the

polymerase and a concentration of inhibitor which: (i) generates little, if any, inhibition of dTMP incorporation and (ii) exceeds the lower K_i applicable in the presence of inhibitor-'complementary' natural DNA. The natural DNA is added at a concentration insufficient to support significant detectable dTMP incorporation *per se* but sufficient to serve as a trap for the bulk of the enzyme present. If the BuPdNTP induces sequestration of pol α on the natural DNA, it effectively pulls the enzyme away from the (dA):(dT) template:primer, and dTMP incorporation into oligo(dT) is reduced accordingly. Table II summarizes the results of applying this method to assess the capacity of BuAdATP and BuPdGTP to induce sequestration of pol α.

At a concentration approximately 12 times its K_i on complementary natural DNA, neither BuPdGTP nor BuAdATP significantly inhibited poly(dA)-driven oligo(dT) synthesis (row i). However, in the presence of a low concentration of complementary, activated DNA (a concentration which did not itself support significant dTMP incorporation; cf., row ii), each inhibitor reduced oligo(dT) synthesis by approximately 90% (row iii), suggesting inhibitor-induced sequestration of the enzyme by the natural template:primer. Supporting this suggestion was the observation that inhibitor/DNA-dependent suppression of oligo(dT) synthesis was prevented by a high concentration of the analogous dNTP; dGTP specifically antagonized the effect of BuPdGTP (row iv) and dATP specifically antagonized that of BuAdATP (row v). Additional support for the operation of the formal sequestration mechanism was obtained from examination of the effect of BuPdGTP on poly(dA)-driven oligo(dT) synthesis in the presence of the complementary homopolymeric template:primer, poly(dC):oligo(dG). Poly(dC):oligo(dG)-dependent inhibition (row vi) was prevented by dGTP (row vii), and this inhibition occurred only when the complementary poly(dC) was annealed to oligo(dG) to provide the specific primer:template structure required for sequestration (cf., Fig. 2, right panel). Neither poly(dC) alone (row viii) nor oligo(dG) alone (row ix) supported the BuPdGTP-induced inhibition of oligo(dT) synthesis.

Resistance of BuPdGTP to polymerization: a property of pol α not characteristic of the membership of the alpha-like DNA polymerase family

Mammalian pol α is the structural paradigm for the alpha-like family of viral and eukaryotic DNA polymerases—a family which displays significant α-like primary sequence (3, 15–17). Not surprisingly, several members of this family display a high level of sensitivity to BuPdGTP comparable to that of pol α. Among the most sensitive enzymes in this family is pol T4, the replication-specific DNA polymerase-exonuclease encoded by coliphage T4 (15); pol T4 is inhibited by BuPdGTP with K_i in the nanomolar range (3).

To determine whether BuPdGTP-induced inhibition of pol T4 also occurred in the absence of polymerization, we executed primer-extension experiments like those described for pol α. These experiments are summarized in the bottom panel of Fig. 1. Lane F displays an incubation mixture containing all 4 dNTPs; the broad array of product lengths reflects the contention of the respective polymerase and exonuclease activities of pol T4; the products ranged in size from ~n−9 to the expected full length product, n + 12. Lane E represents the product of incubation dGTP alone, and despite extensive exonuclease action, it clearly depicts a species expected for a primer extended by one dGMP residue. Replacementof dGTP by BuPdGTP at concentrations of 10, 100 and 1000 nanomolar (lanes B–D, respectively) clearly generated a product with a mobility expected for primer extended by one BuPdGMP residue.

DISCUSSION

The goal of this work was to characterize the precise molecular mechanism of action of the dNTP form of the N^2-substituted purine inhibitor prototype which we developed as pol α-specific agents (1,2). The work had three specific objectives—two major and one minor. The first was to determine whether the BuPdNTPs, unlike the corresponding, non-polymerizable base forms, are substrates for pol α, and the second was to determine if BuPdNTP-induced pol α inhibition resulted from the sequestration of the polymerase to DNA. The BuPdNTPs, indeed, inhibited pol α by sequestering it to the appropriate template pyrimidine (Table II), and, based on the model behavior of BuPdGTP, the dNTP structural format did not result in the polymerization of the dNMP moiety to an eligible primer terminus (cf. Fig. 1).

The third objective was to examine the generality of the pol α-specific mechanism of inhibition among the BuPdGTP-sensitive members of the structurally similar alpha family (16, 17). Using pol T4 as the model enzyme, we have demonstrated that rejection of a BuPdNTP as a polymerizable substrate is not characteristic of the entire BuPdNTP-sensitive alpha enzyme family. Indeed, we show that the substrate potential of BuPdNTPs and the inhibitory potential of BuPdNMP-terminated primers must be carefully considered in assessing the basis for the BuPdNTP sensitivity of a given enzyme—particularly when BuPdNTP sensitivity is to be exploited as a means of categorizing a given DNA polymerase and characterizing the basic structure of its dNTP binding site.

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