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This paper is dedicated, with great respect and affection, to Professor David Shugar on the occasion of his 80th birthday

Nucleotide probes of DNA polymerases*

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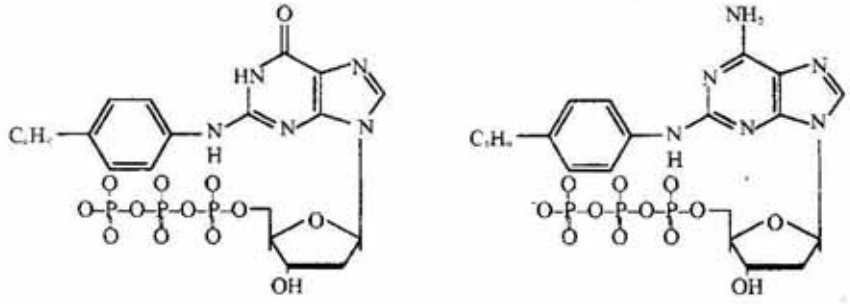
The modified nucleotides, N^2-(*p*-*n*-butylphenyl)dGTP and 2-(*p*-*n*-butylanilino) dATP and related compounds have been developed as inhibitor-probes of B family DNA polymerases. Synthetic approaches to these compounds are summarized. The nucleotides are potent, non-substrate inhibitors of DNA polymerase α. In contrast, they inhibit other members of the family with less potency but act as substrates for these enzymes. Modelling of the inhibitor: enzyme binding mechanism has been done based on the known structure of *E. coli* DNA polymerase I, and site-directed mutagenesis experiments to evaluate this mechanism are proposed.

The "butylphenyl" nucleotides BuPdGTP and BuAdATP (Scheme 1) are potent and selective inhibitors of eukaryotic DNA polymerase (pol) α. In contrast to many dNTP derivatives that inhibit DNA polymerases because they are enzyme substrates [1], the butylphenyl nucleotides are not incorporated into DNA by pol α [2]. However, although BuPdGTP is not an inhibitor of the unrelated *E. coli* pol I, the nucleotide is a terminating substrate for that enzyme [3]!

Pol α is a member of the "B family" of DNA polymerases, because it shares the high degree of primary amino-acid sequence homology with the 47 sequenced enzymes in this family [4]. Other members of this family are inhibited by the butylphenyl nucleotides, but weakly compared to the sensitivity of pol α (Table 1).



BuPdGTP BuAdATP *Scheme 1*

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Abbreviations: BuA, 6-(*p*-*n*-butylanilino); BuP, butylphenyl; CHO, Chinese hamster ovary; exo, exonuclease activity; HCMV, human cytomegalovirus; HSV, herpes simplex virus; IPTG, isopropyl β-D-thiogalactopyranoside; pol, DNA polymerase; VZV, varicella zoster virus.

Table 1
Spectrum of activity of butylphenyl nucleotides

Enzyme	Competitive K_i (μM)		Are they substrates?	Reference
	BuPdGTP	BuAdATP		
pol α	0.001–0.01	0.001–0.005	no	[9, 12, 13]
T4 pol	0.82	0.54	yes	[5]
φ29 pol	20	20	yes	[28]
HSV1 pol	52	55	yes	*
pol I	inact	inact	yes	[3]

*C. Knopf, personal communication.

Interestingly, all of these enzymes incorporate the inhibitors, and, in the case of bacteriophage T4 pol, the resulting primer:template potently inhibits the enzyme [5].

Because of the diverse responses of the B family DNA polymerases to butylphenyl nucleotides and the growing number of site-directed mutant enzymes available, we are interested in the use of these and related compounds as probes of the active site of these enzymes. In this report I propose to: 1) describe the basis for the design of butylphenyl nucleotides, including the chemical methods used to synthesize these compounds; 2) summarize the experimental studies of mechanism and selectivity of the compounds as inhibitors and substrates of DNA polymerases; and, 3) present a testable model of the inhibitor binding site that may both explain the high affinity of the inhibitors for pol α and help identify active site amino-acid residues responsible for binding and polymerization of substrates.

PROPERTIES OF BUTYLPHENYL NUCLEOTIDES

Design of butylphenyl nucleotides

In the study of simple but highly selective inhibitors of Gram+ bacterial DNA polymerase III, 6-anilinouracils and 6-anilinoisocytosines, we found that the potency of *p*-alkyl derivatives decreased with increasing length of the alkyl group. In contrast, DNA polymerase α from HeLa cells was sensitive to derivatives with longer alkyl groups. The optimal uracil analog, 6-(*p*-*n*-butylanilino)uracil (BuAU), was a moderately potent inhibitor of pol α (K_i = 60 μM), and its action was competitive specifically with dGTP [6]. (The action of the isocytosine analog was specifically competitive with dATP). The inhibitory mechanism, in the case of analogous inhibitors of DNA polymerase III, was a result of sequestration of the enzyme in a ternary complex of DNA, enzyme and inhibitor [7]. The inhibitors formed base pairs with cytosine (BuAU) or thymine (BuAIcyt) in the DNA template, explaining the competitive nature of inhi- bition, and the *p*-butylphenyl group interacted with a unique part of the active site in the enzyme, explaining the selectivity of the compounds for pol α.

The suggestion that BuAU and BuAIcyt occupied the active site of pol α required proof. This was sought by converting the molecules to a form resembling the authentic substrate, dGTP or dATP, with the expectation that potent inhibition and possible incorporation of such inhibitors would prove the location of the binding site. The sequence of syntheses for the dGTP analog is shown in Fig. 1. The first compound, N^2-(*p*-*n*-butylphenyl)guanine (BuPG), was easily prepared from 2-bromohypoxanthine and *p*-*n*-butylaniline. It retains the base pairing region and the butylphenyl group of BuAU and was found to be a more potent inhibitor of pol α (K_i = 12 μM), and it was competitive with dGTP as expected [8].

Conversion of the base BuPG to the desired deoxyribonucleoside 5′-triphosphate, BuPdGTP, was done in several steps. Glycosylation of the trimethylsilyl derivative of BuPG with 1-chloro-2-deoxy-3,5-di-(*p*-toluyl)-β-D-ribofuranose in the presence of a Lewis acid catalyst gave three isomeric nucleosides in low yields [9]. ^{1}H NMR

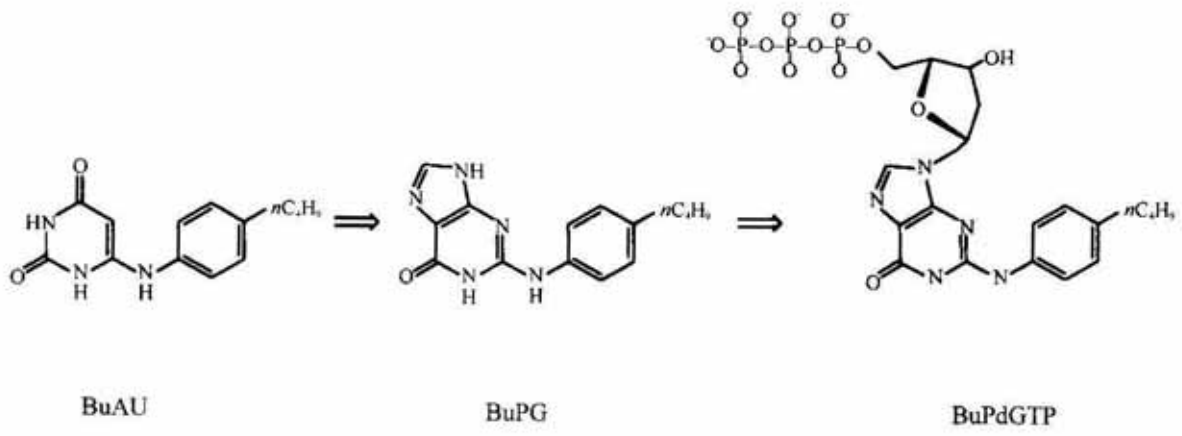

Fig. 1. The sequence of syntheses of BuPdGTP.

evidence suggested that the major product was the desired 9-β isomer, but this conclusion was based only on empirical rules. The structure of this compound, BuPdG, was proved indirectly. First, 2-bromohypoxanthine was converted to two isomeric 2-bromo-tri-*O*-acetylinosines with *tetra*-*O*-acetylribofuranose, and the isomer that gave authentic (9-β) guanosine on ammonolysis was reacted with *p*-*n*-butylaniline to give the 9-β isomer of N^2-(*p*-*n*-butylphenyl)guanosine. The latter compound was converted specifically to the 2′-deoxyribonucleoside by a multistep procedure [10], and this compound proved to be the major isomer obtained directly [9]. A simpler and more flexible synthetic method to prepare BuPdG and related nucleosides is the sodium salt glycosylation method. 2-(*p*-*n*-Butylanilino)-6-chloropurine, as the sodium salt, reacted rapidly with 1-α-chloro-2-deoxy-3,5-di-(*p*-toluyl)-β-D-ribofuranose in acetonitrile to give the corresponding blocked 9-β nucleoside in 64% yield [11]. Hydrolysis of this intermediate with a mixture of 2-mercaptoethanol and sodium methoxide in methanol gave BuPdG in 95% yield, and ammonolysis with methanolic ammonia at high temperature gave the deoxyadenosine derivative, BuAdA, in 81% yield [11].

Both BuPdG and BuAdA have been converted to the 5′-phosphates, BuPdGMP and BuAdAMP, with phosphoryl chloride [9, 12]. Condensation of the activated monophosphates with pyrophosphate yielded the desired 5′-triphosphates, BuPdGTP and BuAdATP, accompanied by small amounts of the 5′-diphosphates, BuPdGDP and BuAdADP [9, 12].

Inhibitory effects of butylphenyl nucleotides

The ultimate inhibitor/substrate analogs proved to be highly potent inhibitors of pol α. BuPdGTP had K_i = 9 nM against Chinese hamster ovary (CHO) pol α and was competitive with dGTP [13], and BuAdATP had K_i = 2.6 nM against CHO pol α and was competitive with dATP [12]. Neither compound inhibited the other eukaryotic DNA polymerases, pol β or pol γ, except at high concentrations. A defined oligodeoxyribonucleotide primer:template was used to determine that BuPdGTP is **not** a substrate for pol α. Incubation of a 17:29mer duplex, in which the next required substrate was dGTP, with calf thymus pol α did not show extension when BuPdGTP was the added nucleotide [2]. However, T4 pol, an enzyme with moderate sensitivity to BuPdGTP, did extend the primer:template with the inhibitor [2]. The latter experiment proved that BuPdGTP, and BuAdATP, interact at the active site of sensitive DNA polymerases in a manner consistent with their ability to be incorporated into DNA as dNTPs.

Potent inhibition of pol α requires a straight chain *p*-alkyl substituent on the phenyl ring of 2-anilinopurines [1]. That dependence for the dNTP analogs is emphasized in Table 2, where the only potent pol α inhibitors possess the *p*-*n*-butyl group or the *p*-*n*-octyl group. Even the sugar structure is less important as shown by potent inhibition of pol α by the arabinfuranosyl analogs, BuAaraATP and 2′-azido-BuAaraATP [14].

Table 2
Inhibition of pol α by N^2-substituted NTPs

	2-Substituent	K_i (μM)	Reference
dGTPs:			
	p-(*n*-butyl)phenyl	0.005	[9]
	p-(*n*-octyl)phenyl	0.046	[30]
	3-ethyl-4-methylphenyl	8	[29]
	phenyl	60	[29]
	n-hexyl	18	[29]
dATPs:			
	p-(*n*-butyl)anilino	0.008	[12]
	3,4-dichloroanilino	3	[29]
araATPs:			
	p-(*n*-butyl)anilino	0.017**	[14]
	p-(*n*-butyl)anilino (2′-azido)	0.038**	[14]

**IC_{50} values.

Anticipating the need to have available a non-substrate analog of BuPdGTP, we recently synthesized by two methods the α,β-methylene derivative BuPdGMPCPP [15], and showed that this compound is not a substrate for T4 pol [5] or *E. coli* pol I (unpublished observations). This compound was only fivefold less potent as an inhibitor of calf thymus pol α (K_i = 9.5 nM) than BuPdGTP [15].

Effect on other DNA polymerases

The most useful property of the butylphenyl nucleotides is their ability to discriminate between the eukaryotic DNA polymerases implicated in DNA replication, pols α, δ and ε. For example, IC_{50} values of BuPdGTP and BuAdATP against calf thymus pol α were 0.026 and 0.18 μM, but were 90–180 μM against calf thymus pols δ and ε [16]. This high degree of selectivity is surprising considering the similarity in primary structure of the enzymes (see below).

As illustrated in Table 1, members of the B family of DNA polymerases vary widely in sensitivity to the butylphenyl nucleotides. HSV1 and φ29 pols are inhibited weakly, but T4 pol has intermediate sensitivity. A detailed study has shown that T4 pol is inhibited by BuPdGTP and BuAdATP competitively with dGTP and dATP, respectively, with K_i values below 1 μM [5]. However, the apparent K_i values are much lower when the compounds are tested in the absence of the competitor nucleotides, in the "truncated assay". This phenomenon is a result of the ability of T4 pol to incorporate the nucleotides, and for the resulting modified primer:templates to strongly bind and inhibit the enzyme. Indeed, a chemically synthesized primer:template with a 3′-BuPdG residue in the primer strand [3] was a potent inhibitor of T4 pol with IC_{50} = 0.07 μM [5]. In contrast, the non-substrate derivative, BuPdGMPCPP, inhibited T4 pol with K_i = 2.3 μM, just threefold weaker than the competitive K_i of BuPdGTP itself [5].

The ability of T4 pol to both incorporate BuPdGTP and to bind the modified primer:template contrasts with the response of *E. coli* pol I, representing the A family of DNA polymerases, to the compound. While pol I is essentially insensitive to inhibition by BuPdGTP, it slowly incorporates the nucleotide, but then appears to dissociate from the modified primer:template [3]. The 3′-BuPdG-primer:template does not inhibit pol I activity, nor is the BuPdG residue removed by 3′ to 5′ exonuclease activity of pol I [3]. While K_m values for dGTP and BuPdGTP in primer extension assays were similar (0.8 and 2.0 μM, respectively [3]), turnover of the substrates was significantly different. BuPdGTP was incorporated by pol I into the 17:29mer at 37°C with k_{cat}

= 0.85 min^{-1} (unpublished observation), compared with a reported value of k_{cat} for incorporation of dATP/dTTP into poly-d(AT) of 498 min^{-1} [17].

THE NATURE OF DNA POLYMERASES

DNA-dependent DNA polymerases are functionally complex enzymes that bind multiple substrates and can possess multiple enzymatic activities. The best characterized enzyme, *E. coli* pol I, binds to partially double-stranded oligonucleotide primer:templates and 2′-deoxyribonucleoside 5′-triphosphates in a polymerization complex resulting in incorporation of the nucleotide. Pol I also possesses a 3′ to 5′ exonuclease (exo) activity that cleaves nucleoside 5′-monophosphates from double or single stranded DNA, and a 5′ to 3′ exonuclease activity. The X-ray crystal structure of a proteolyzed fragment (Klenow fragment) of pol I containing the pol and 3′ to 5′ exo activities [18] has revealed a structure, likened to a curled, open hand, that can accommodate double stranded DNA. Fig. 2A illustrates the overall backbone structure of pol I, emphasizing three regions of particular interest in the proposed active site. Genetic and chemical methods have suggested that amino-acid residues in the fingers and palm are involved in polymerization (Fig. 2B). (The 3′ to 5′ exo site, revealed by its binding to dTMP, is located about 30 Å from the pol site). Attempts to cocrystallize pol I (Klenow) with its substrates have given structures in which a primer:template was bound in an unusual conformation [19], and in which a dNTP substrate formed a complex that was unlikely to be catalytically relevant [20].

Recently, the X-ray crystal structure of eukaryotic enzyme, DNA polymerase β (pol β) as a complex with primer:template and a substrate analog was reported [21]. This structure represents the most detailed and catalytically relevant picture to date of a DNA polymerase. Although pol β is a small DNA polymerase (molecular mass 39 kDa) with only pol activity and little primary sequence homology to other DNA polymerases, a common polymerase mechanism has been suggested by comparison of structure motifs of pol β with those of pol I and reverse transcriptase [22].

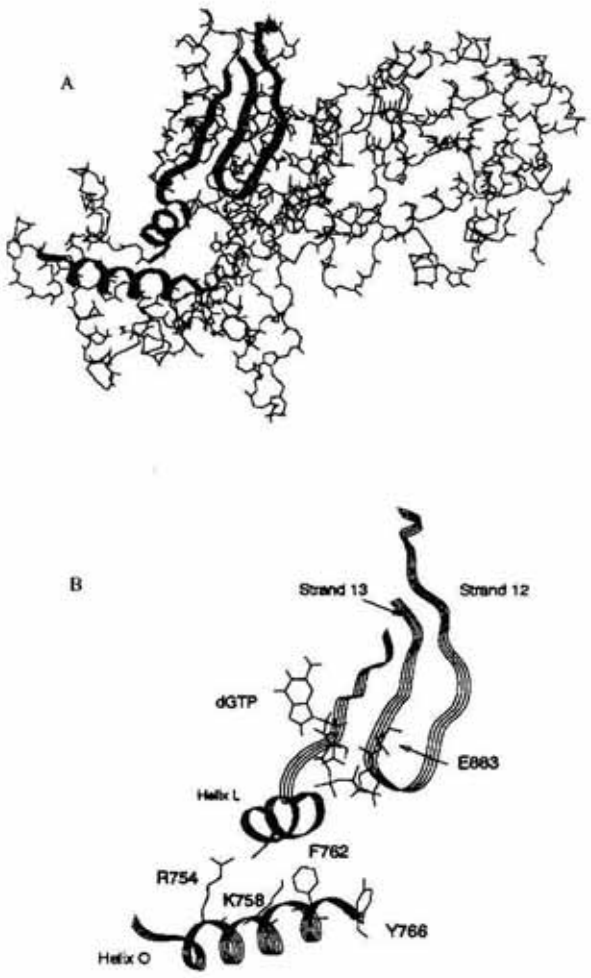

Fig. 2. Representations of the structure of E. coli DNA polymerase I.
A. Overall backbone structure emphasizing three regions in the palm and fingers of the pol site. B. Details of the three regions in the pol site and a dGTP molecule, emphasizing conserved and chemically modifiable amino acids. Coordinates and secondary structure nomenclature were derived from [18]. Figures were obtained using Insight software (Biosym Technologies) on an Iris Indigo computer.

DNA polymerases I and β are prototypes of two of the four families (A and D, respectively) of DNA polymerases [4]. The largest family (at least 47 members) is the B family consisting of animal cell replicative enzymes pols α, δ and ε, and the corresponding enzymes from yeast; herpesvirus DNA polymerases (HSV1, HSV2, HCMV, VZV); T-even bacteriophage (T4) and other phage (ϕ29, M2, PRD1) DNA polymerases. The B family enzymes contain up to seven regions of significant sequence homology, and many mutant enzymes involving these regions have been identified in drug resistant host organisms and in engineered pol genes.

Table 3
Homologous regions of B family DNA polymerases.
hu α, human polymerase α.

B family region I/pol I β strands 12, 13		
hu α	998	VI**Y**G**DT**D**S**IMINTNS
T4	614	IAAG**DTDS**VYVCVDK
φ29	452	II**YCDTDS**IHLTGTE
[pol I	878	MQVH**D**.E**L**VFE.VHK]
B family region II/pol I β strand 9, helix L		
hu α	855	FILLL**D**FN**S**L**Y**P**S**IIQEFNICFITVQ
T4	403	YIMSF**DLTS**L**YPSI**IRQVNISPETIR
φ29	244	EGMVF**D**VN**SLYP**AQMYSRLLPYGEPI
[pol I	700	VIVSA**D**Y.S**Q**I**E**LRIMAHLSRDKGLL]
B family region III/pol I helix O		
hu α	944	IRQKAL**K**LT**A**NSM..YGCLGFSYSRFYAKPLA
T4	551	TNQLNRKILINSL..**Y**GALGNIHFRYYDLRNA
φ29	378	IKQ.LAKLMLNSL..YGKFASNPDVTGKVPYL
[pol I	752	EQRRSAKAI.N**F**GL**I**YGMSAFGLARQLNIPRK]

MODELLING OF THE BUTYLPHENYL NUCLEOTIDE BINDING SITE

Sequences of several regions in relevant B family enzymes are shown in Table 3 together with corresponding sequences predicted to be functionally homologous in *E. coli* pol I. Alignment of pol I with the B family motifs was based on the modelling work of Lindborg [23] and on calculations with the Chou-Fasman algorithm (results not shown). Many of the conserved residues (Table 3, bold type) have been implicated in catalysis, specifically in binding of primer:template or dNTP, and those residues that have been altered by site-directed mutation are double underlined in Table 3. Although the sequences of pol I and B family enzymes have limited identity, the secondary structure of the active sites may be similar. For example, a triad of negatively charged residues spanning regions II and I of the B family and α helices 9, 12 and 13 of pol I is conserved in all DNA polymerases. These anchor points in the putative active sites of pol I and pol α/T4 pol were used to orient the nucleotide molecules in the models of Figs 2B and 3A and 3B (residues not shown).

Several B family DNA polymerase mutants have altered sensitivities to butylphenyl nucleotides, especially mutations involving aromatic residues. The pol α region II mutants, Y865S/F, have increased K_m for substrates and are resistant to BuPdGTP and aphidicolin, leading to the suggestion that this Tyr(865) binds dNTP bases and also is involved in aphidicolin binding [24]. The analogous change Y254F in φ29 pol gave a mutant with reduced affinity for Mg^{2+}·dNTPs but hypersensitivity to BuPdGTP [25]. Several pol α mutations in region I alter both aphidicolin and BuPdGTP sensitivity [26]. For example, the finding that the Y1000F mutant was highly resistant to BuPdGTP prompted Dr B. Lindborg to postulate that the inhibitor may bind B family enzymes with the butylphenyl ring sandwiched between the β sheets corresponding to pol I strands 12 and 13 (see Fig. 3A) [23]. Among φ29 mutant pols, the Y390S/F mutants in region III had greatly reduced pol activity but were hypersensitive (about 100-fold) to BuPdGTP and BuAdATP [27], although either affinity or incorporation rates, or both, may have changed. Interestingly, pol α mutants in the same region, K950A/N, are hypersensitive (50–100-fold) to BuPdGTP and the non-incorporable analog BuPdGMPCPP, but normally sensitive to

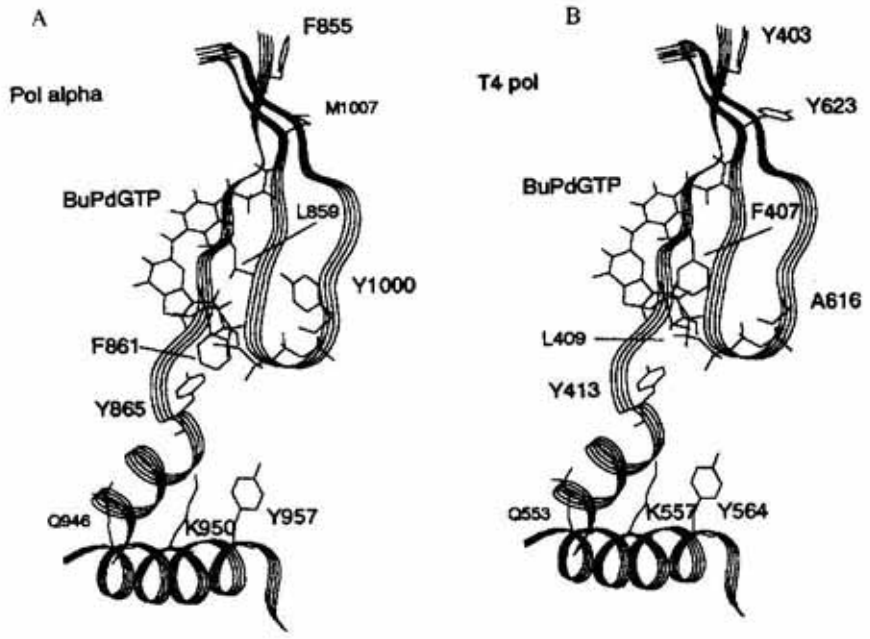

Fig. 3. Models of the BuPdGTP binding site of B family DNA polymerases.
A. BuPdGTP:pol α model. B. BuPdGTP:T4 pol model. Amino acids are superimposed on secondary structures derived from *E. coli* pol I (Fig. 2).

pyrophosphate (T.S.-F. Wang, personal communication).

Answers to two questions may reveal fundamental insight into the active site structure and mechanism of DNA polymerization. Why is pol α uniquely sensitive to the butylphenyl nucleotides compared with the other members of the B family, and why is pol α alone incapable of incorporating the nucleotides into DNA (Table 1)? Inspection of the sequence differences in several conserved regions among the 47 B family enzymes [4] and consideration of the effects of mutations on inhibitor sensitivity have enabled us to develop a model of the BuPdGTP/BuAdATP:polymerase binding site.

We have undertaken a study of wild type and mutant T4 DNA polymerases to clarify the specific step(s) in the polymerization reaction in which changes in sensitivity to butylphenyl inhibitors may be involved. BuPdGTP inhibits T4 pol as a reversible competitive inhibitor in the presence of dGTP (K_i = 0.8 μM), but it acts as a substrate in the absence of dGTP leading to 3′-BuPdG-terminated primer:templates that are potent inhibitors of the enzyme [5]. A synthetic primer:template containing a 3′-BuPdG residue, 3′-BuPdG-18:29mer, was both resistant to extension by T4 pol and strongly inhibitory to the enzyme. Non–substrate analogs of BuPdGTP, i.e. the diphosphate, BuPdGDP, and the α,β-methylenetriphosphate, BuPdGMPCPP, inhibited the enzyme with the same potencies in both truncated and variable substrate (dGTP) assays [5].

Working models of the BuPdGTP:pol binding site structure for human pol α and T4 pol are shown in Figs 3A and 3B, respectively, emphasizing the aromatic amino acids that are abundant in these conserved regions (see Table 3). In these models BuPdGTP replaces a substrate nucleotide maintaining the correct geometry of the Mg^{2+}-coordinated triphosphate group and conserved acidic residues (not shown). Because interaction of the butylphenyl group of BuPdGTP in the active site of sensitive enzymes likely involves aromatic and aliphatic hydrophobic amino acids, we sought differences in the pattern of these residues that might correlate with differences in sensitivity of the enzymes to BuPdGTP.

Several relevant mutants have already been studied by others at sites that are almost exclusively aromatic. The Y865S/F pol α mutants were BuPdGTP resistant [24], but that residue is invariant (47/47!) in all B family enzymes [4]. The Y1000F pol α mutant was 27-fold more resistant to BuPdGTP [26], but this residue is Ala in T4 pol and Tyr in HSV1 and ϕ29 pols, and is aromatic in 44/47 enzymes. Finally, Y390S/F mutants of ϕ29 pol (corresponding to Y957 of

pol α and Y564 of T4 pol) showed hypersensitivity to BuPdGTP [27, 28]; however, this residue is also nearly invariant (46/47!) in B family enzymes.

Inspection of the pattern of aromatic amino acids in the major B family polymerases has led us to design specific mutants of T4 pol to test the role of these residues in BuPdGTP binding. Given the unique sensitivity of human pol α, other higher eukaryotic pol α's, and yeast pol α to BuPdGTP, our intention is to create a T4 polymerase that has nanomolar affinity to the inhibitor and/or has lost the ability to incorporate it. The first mutant T4 pol, Y623M, has been expressed and purified, and preliminary data are available for it. This residue is aromatic in 26/47 B family enzymes, but is Met in human and yeast pol α. If this residue is directly involved in binding to the *p*-butyl group, we expect that the Y623M T4 pol will be hypersensitive to BuPdGTP. (In the T4 pol model of Fig. 3B the methyl group of the butyl group of the inhibitor is only 4.9 Å from the ring of Y623). If the Tyr in T4 pol facilitates the incorporation of BuPdGTP, either directly or indirectly, the mutant may lose the ability to incorporate the inhibitor.

Ms Shelli Stocki in Dr Linda Reha-Krantz' laboratory (personal communication) has constructed a T4 pol gene containing the change TAT· ATG yielding the Y623M amino acid replacement. (In addition, the gene was altered by several conservative codon changes to assure expression of only the full length protein). The mutated gene was inserted into phage under control of the *lac* promoter, resulting in a viable phage with growth properties and inhibitor sensitivity similar to wild type phage except that it had a weak antimutator phenotype. Sequencing of the plasmid confirmed that the expected mutations were present. The plasmid was transfected into *E. coli*, and expression of the mutated enzyme was induced by IPTG. The Y623M T4 pol was purified conventionally, and preliminary studies suggest that it has similar specific activity to the wild type enzyme. Inhibition studies of this enzyme are just beginning.

Preparation of other mutant T4 pols is proposed. **Y403F,P**: this residue is aromatic in only 6/39 B family polymerases, a group consisting of five α-like pols and T4 pol! (This residue is Pro in 16/39 enzymes, suggesting a structural role in these enzymes.) It may not be a coincidence that all the enzymes with an aromatic residue at this site are also those which possess highest sensitivity to, but inability to incorporate, BuPdGTP. If the Tyr in T4 pol is a major site of binding to the inhibitor phenyl ring, its conversion to Phe may make the mutant hypersensitive to BuPdGTP, i.e. pol α-like (F855, Fig. 3A), and its conversion to Pro may make the mutant BuPdGTP resistant, i.e. HSV1 or ϕ29 pol-like (see Table 1). Effects of the mutations on incorporation of BuPdGTP may parallel the affinity changes. Although the model (Fig. 3B) shows that the inhibitor methyl group is 7.1 Å from the β-CH_2 of Y403, rotation of the amino-acid side chain could clearly bring them closer. **F407L**: this residue is Leu in human pol α and Met in yeast pol α, both highly sensitive enzymes to BuPdGTP, but it is aromatic in 21/47 B family enzymes. If steric or other factors reduce inhibitor affinity to enzymes with an aromatic residue at this position, the conversion to Leu may make the mutant T4 pol hypersensitive to BuPdGTP and prevent incorporation of the inhibitor. In the model of Fig. 3B the phenyl rings of inhibitor and F407 are only 3.5 Å apart.

CONCLUSIONS

Butylphenyl nucleotides, among other DNA polymerase inhibitors, afford insight into the active sites and mechanisms of sensitive enzymes. The classical approach to chemical alteration of the inhibitor-probes is coupled with the molecular biological approach of biochemical alteration of the target enzymes. Changes in affinity and/or substrate properties of appropriate inhibitors has led to models of inhibitor : enzyme binding, as illustrated in Figs 3A and 3B. These models have suggested experiments to evaluate postulated roles of amino acids in inhibitor binding and incorporation, ultimately yielding information about the catalytic mechanism of polymerization.

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