Differential inhibition of human placental DNA polymerases δ and α by BuPdGTP and BuAdATP

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ABSTRACT

The *p*-*n*-butylphenyl- and *p*-*n*-butylanilino- substituted analogs of dGTP and dATP, respectively, were tested as inhibitors of purified human placental DNA polymerases α and δ . It was observed that DNA polymerase α activity was potently inhibited by these analogs with $I_{0.5}$ values as low as the nanomolar range, whereas DNA polymerase δ activity was poorly inhibited, with $I_{0.5}$ values of ca. 100 micromolar. These results argue for a distinct identity of these two enzymes, and demonstrate the usefulness of these analogs as probes of DNA polymerase structures. In addition, these analogs provide a rapid method for the discrimination of the two enzyme activities and a means for the selective assay of DNA polymerase δ . Aphidicolin inhibited both DNA polymerases.

INTRODUCTION

There are four types of DNA polymerase in mammalian cells, α , β , γ and δ (1,2). DNA polymerase α is generally regarded as the enzyme involved in replication, in contrast to polymerase β and γ . Evidence for the involvement of polymerase α in DNA replication has come from extensive studies of inhibitors of DNA synthesis, particularly by aphidicolin, which inhibits DNA synthesis *in vivo* and also selectively inhibits polymerase α activity (but not β and γ) *in vitro* (1-3), as well as demonstrations of the nuclear localization of the enzyme (4-6) and the inhibition of DNA synthesis in permeabilized cells by anti- α antibodies (7). More recently, a novel mammalian DNA polymerase, polymerase δ , has been described (8-13). This enzyme is unlike polymerase α in that it possesses an associated and apparently intrinsic 3' to 5' exonuclease activity which is not separated by any of the chromatographic and analytical procedures so far used. The significance of the 3' to 5' exonuclease activity is that it has the potential to provide a proof-reading or editing function. Like polymerase α , DNA polymerase δ is inhibited by aphidicolin (13,14), and is insensitive to dideoxynTP inhibition. Thus, these two inhibitors, which are useful in distinguishing polymerase α from polymerase β and γ , cannot distinguish polymerase α from polymerase δ . We have recently purified DNA polymerase δ and α from human placental tissue (14). The

polymerase activity of human DNA polymerase δ , like those of the reticulocyte and calf thymus enzymes (8-13) is associated with a 3' to 5' exonuclease activity throughout purification. Our studies are consistent with the view that polymerase δ is a distinct enzyme from α ; evidence for this includes the finding that inhibitory monoclonal antibodies to polymerase α do not inhibit placental polymerase δ (14). There nevertheless remain questions of whether polymerase δ can be simply regarded as a form of polymerase α , which is associated with a 3' to 5' exonuclease activity by virtue of its being either intrinsic or associated. In addition, polymerase δ activity cannot be readily distinguished in tissue extracts, since its identification largely depends on its complete separation from polymerase α and from other nonassociated exonuclease activities.

Wright and coworkers (15-20) have studied the inhibition of DNA polymerase activities from procaryotic and eucaryotic sources by 6-(arylamino) substituted uracils (15-17) and N^2 -(*p*-n-butylphenyl)guanine (18), and have shown that the potency and selectivity of these analogs are functions of the size and orientation of the substituents and are also dependent on the presence of a template containing complementary nucleotides. Of particular interest is the finding that BuPG [N^2 -(*p*-n-butyl phenyl) guanine] is a selective inhibitor of HeLa cell polymerase α (18). They have predicted and shown the corresponding triphosphate analog, BuPdGTP [N^2 -(*p*-n-butylphenyl)-9-(2-deoxy- β -D-ribofuranosyl)guanine 5'-triphosphate] to be an extremely potent inhibitor of eucaryotic DNA polymerase α , with K_i values in the nanomolar range, and also to selectively inhibit α -like polymerases from a variety of sources (19,20). Because of the obvious potential of these inhibitors both as chemotherapeutic drugs, as well as potential probes for the structural analysis of DNA polymerases, we report here the effects of two of these analogs, BuPdGTP [N^2 -(*p*-n-butylphenyl)-9-(2-deoxy- β -D-ribofuranosyl)guanine 5'-triphosphate] and BuAdATP [2-(*p*-n-butylanilino)-9-(2-deoxy- β -D-ribofuranosyl)adenine 5'-triphosphate] on purified human placental DNA polymerases α and δ , and show that they may be of significant utility in the identification of the two enzymes.

METHODS AND MATERIALS.

Materials. Radioactive materials were purchased from New England Nuclear. Poly(dA-dT) was obtained from Boehringer Mannheim. Poly(dG-dC), poly(dA) and oligo(dT)₁₂₋₁₈ were obtained from P-L Laboratories. Poly(dA)-oligo(dT) was annealed in the ratio of 20:1 at 60° C. BuPdGTP, BuAdATP, TMAU [6-(3,4-trimethylene-anilino)uracil], TMPG [N^2 -(3,4-trimethylene-phenyl)guanine], and the (ethyl, methyl) di-substituted derivatives, EMAU [6-(3-ethyl-4-methylanilino)uracil] and EMPG [N^2 -(3-ethyl-4-methylphenyl)guanine] were synthesized as described previously

(15,18,19). All other reagents were of the highest grade available.

Enzymes. DNA polymerase δ and polymerase α were purified from human placenta by procedures described elsewhere (14). The DNA polymerase δ preparation was near-homogeneous and had a specific activity of 48,000 units/mg assayed using poly(dA-dT) as the template primer. The polymerase α preparation used in these studies had a specific activity of 30,000 units/mg assayed using activated calf thymus DNA as the template primer. The polymerase α preparation was devoid of exonuclease activity.

Enzyme assays. DNA polymerase activity was assayed essentially as previously described (11-13). The standard assay for DNA polymerase using poly(dA-dT) alternating copolymer as the template primer contained 0.025 A_{260} O.D. units of activated poly(dA-dT), 40 mM TrisHCl (pH 7.5), 0.8 mM $MgCl_2$, 40 μ M dATP, 4 μ M [^3H]dTTP (500 - 1000 cpm/pmole), 5 μ g bovine serum albumin, 2% glycerol, 0.04% Triton X-100, 2 mM dithiothreitol, and 0.2 - 0.5 units of DNA polymerase δ in a total volume of 125 μ l. The assay mixture was incubated at 37° C for 15 minutes and terminated by the addition of 5% trichloroacetic acid containing 10 mM sodium pyrophosphate. The precipitate was collected on Whatman GF/C filters and counted. When activated poly(dG-dC) was used as the template primer, the reaction mixtures were the same except for the substitution of dCTP for dATP and [^3H]dGTP for dTTP. The standard assay when calf thymus DNA was used as the template primer contained 0.03 A_{260} O.D. units of activated calf thymus DNA, 40 mM HEPES (pH 7.5), 3 mM $MgCl_2$, 40 μ M each of dATP, dGTP, and dCTP, 4 μ M [^3H]-dTTP (500 - 1000 cpm/pmole), 5 μ g bovine serum albumin, 2% glycerol in a volume of 125 μ l.

Units of activity were expressed as nmoles of nucleotide incorporated per hr. Apparent $I_{0.5}$ values for the inhibition of DNA polymerase activities were determined under standard assay conditions and defined as the amount of analog which gave 50% inhibition of activity.

RESULTS

The effects of BuPdGTP and BuAdATP on DNA polymerases α and δ were examined in the standard assay systems using four different template primers - calf thymus DNA, poly(dA-dT), poly(dG-dC) and poly(dA)-oligo(dT)₁₂₋₁₈. BuPdGTP and BuAdATP exhibited very similar effects when calf thymus DNA was used the template primer (Fig. 1A and 1B, Table I): DNA polymerase α was potently inhibited by both analogs, while DNA polymerase δ activity was only inhibited at much higher concentrations. There was a very marked differential response of the two enzymes toward either analogs, with a difference of about 3 orders of magnitude in the $I_{0.5}$ values (Table I). With poly(dA-dT) as the template primer (Fig. 1C, 1D, Table I),

Figure 1. Inhibition of Human Placental DNA Polymerase α and δ by N^2 -(*p*-*n*-butylphenyl)dGTP and 2-(*p*-*n*-butylanilino)dATP.

The effects of BuPdGTP and BuAdATP on DNA polymerase α and δ were determined in the standard assay systems (Methods). The analogs were tested at concentrations of 1 nM, 10 nM, 100 nM, 1 μ M, 10 μ M, 100 μ M and 360 μ M. Data for the inhibition of DNA polymerase α are shown in the solid circles, broken lines, while data for DNA polymerase δ are shown in the solid triangles, solid lines. Panels A and B show data for BuPdGTP and BuAdATP, respectively, when calf thymus DNA was the template primer, and the incorporation of [^{3} H]dTTP was measured. Panels C and D show data for BuPdGTP and BuAdATP, respectively, when poly(dA-dT) was the template primer, and the incorporation of [^{3} H]dTTP was measured. Panels E and F show data for BuPdGTP and BuAdATP, respectively, when poly(dG-dC) was the template primer, and the incorporation of [^{3} H]dGTP was measured. Values for [BuPdGTP] and [BuAdATP] are in molar concentrations.

there was also a differential response although the difference was less marked. It should be noted that DNA polymerase α was more strongly inhibited by BuAdATP, a complementary nucleotide for this template primer, as compared to BuPdGTP. With poly(dG-dC) as the template primer (Fig. 1E, 1F, Table I), marked differential inhibition of polymerase α and δ activity was again observed, and a similar effect of greater inhibition of polymerase α by the analog (BuPdGTP) corresponding to the complementary nucleotide was observed. Data for the inhibition of DNA polymerase α and δ activities when poly(dA)-oligo(dT)₁₂₋₁₈ was the template primer (Table I) also show that polymerase α is more strongly inhibited than polymerase δ . As in the case of calf thymus DNA, BuPdGTP and BuAdATP gave very similar data. The inhibition of DNA polymerase δ by both analogs and with all four template primers was not markedly different, with IC_{50} values in the range of 50–100 μ M (Table I).

Table I. The Inhibition of Human Placental DNA Polymerases α and δ by BuPdGTP, BuAdATP and Aphidicolin.

	C.T.DNA		Poly(dG-dC)		Poly(dA-dT)		Poly(dA)-oligo(dT) _{20:1}	
	α	δ	α	δ	α	δ	α	δ
BuPdGTP	0.04	120	0.003	50	15	100	3	30
BuAdATP	0.02	50	1.1	56	0.3	50	7	105
Aphidicolin	5.0	0.6	6.7	0.9	6.7	0.55	1.1	1.1

Polymerases α and δ were assayed under standard conditions with varying amounts of BuPdGTP, BuAdATP or aphidicolin with the indicated primer-templates. Data are given as apparent $I_{0.5}$ values in μM inhibitor for BuPdGTP and BuAdATP, and as $\mu\text{g/ml}$ for aphidicolin.

The effects of aphidicolin, a widely used inhibitor of polymerase α , on the two human DNA polymerases are also shown in Table I. Aphidicolin, which is selective for the inhibition of DNA polymerase α (1-3) in that it does not inhibit polymerases β and γ , has also been shown to inhibit polymerase δ from the calf thymus(13) and rabbit reticulocyte (10). The data of Table I, however, provide the first comparison of the effects of aphidicolin on polymerase α and δ from the same tissue with different templates, and under comparable assay conditions, and show that polymerase δ is more sensitive than polymerase α to aphidicolin with 3 of the four template primers used.

We have also tested other base analogs which are potent inhibitors of *B. subtilis* polymerase III, but not of mammalian polymerase α (15,16). These included the trimethylenephényl substituted derivatives of the bases uracil and guanine, TMAU [6-(3,4-trimethylene-anilino)uracil] and TMPG [N^2 -(3,4-trimethylenephényl)-guanine], and the (ethyl, methyl) di-substituted derivatives, EMAU [6-(3-ethyl-4-methylanilino)uracil] and EMPG [N^2 -(3-ethyl-4-methylphenyl)guanine]. These analogs did not inhibit human DNA polymerase α or δ , even at concentrations as high as 600 μM .

DISCUSSION.

Our data on the effects of substituted nucleoside triphosphates, BuPdGTP and BuAdATP demonstrate that they are potent inhibitors of placental DNA polymerase α , results consistent with findings reported for the inhibition by BuPdGTP of polymerase α from a variety of sources including calf thymus, HeLa cells,

Drosophila and Xenopus (19,20). On the other hand, DNA polymerase δ is relatively insensitive to BuPdGTP and BuAdATP with $I_{0.5}$ values of ca. 100 μ M. The differential inhibition of polymerase α and δ ranged from a ratio of 1 to 4 orders of magnitude in the $I_{0.5}$ values, depending on the template-primer used (Table I).

Wright and co-workers have studied the mechanisms of action of p-butyl phenyl and p-butylanilino derivatives of uracil and guanine (15-20) and in the case of the analogs of the free bases, they have shown that the effects of these analogs are competitively overcome by dGTP. The potency of the inhibition was proposed to be due to the formation of an enzyme-primer/template-drug complex (20). The present data are consistent with a similar interpretation; however, because we are dealing with the nucleotide triphosphates, it is possible that these are incorporated, leading to the formation of primer termini which are no longer functional. If such were the case, it might explain the lack of response by polymerase δ , since this enzyme possesses a 3' to 5' exonuclease activity which could remove any misincorporated nucleotide. However, addition of 5' AMP, an inhibitor of the exonuclease activity of polymerase δ (21,22), did not enhance the inhibition (data not shown) as might be expected if misincorporation was involved. This type of effect has been observed in case of araATP which inhibits polymerase δ and is also incorporated into template primer, i.e. inhibition of the exonuclease activity by 5' AMP enhances both the incorporation and inhibitory effects of araATP (23). In addition, Khan et al. (20) have reported that their preliminary data do not support a misincorporation mechanism for the inhibition of polymerase α by BuPdGTP, since isolation of the template primer did not impair its reutilization as a template primer.

Inhibition of polymerase α by the free bases has been shown to be competitive, and to require the presence of a complementary template primer (17,18). This is not so for the triphosphate analogs, BuPdGTP and BuAdATP, although the inhibition of polymerase α was enhanced in the presence of the complementary template primer (cf. Fig. 1C, 1D, and Fig. 1E,1F). A similar finding of the nonrequirement of a complementary template primer has also been reported for the inhibition of polymerase α by BuPdGTP (20). These findings show that the potent inhibition of polymerase α by the nucleoside triphosphate analogs is more complex than that caused by the free bases, and it has been suggested that this may reflect the presence of discrete nucleoside triphosphate binding sites (20). The inhibition of polymerase δ also did not require the presence of a complementary template primer, and unlike polymerase α , inhibition was not enhanced in the presence of the complementary template primer. Polymerase β and γ are not inhibited by BuPdGTP, even at mM concentrations (19).

The inhibition of polymerases δ and α by aphidicolin was also examined (Table I). Aphidicolin has been extensively used to inhibit DNA synthesis *in vivo*, and has also been characterized as diagnostic for polymerase α as it does not inhibit polymerases β or γ (1-3). Our data confirm previous reports (10,13) that polymerase δ is, at the very least, as equally sensitive to aphidicolin as is polymerase α . This implies that aphidicolin inhibition is not absolutely diagnostic for polymerase α , unless polymerase δ is regarded as a form of α . The latter issue remains to be rigorously clarified by structural comparisons of the two proteins. However, the data of Table I also show that, with three of the template primers examined, polymerase δ in fact displays a significantly greater sensitivity (about 10-fold) than polymerase α to aphidicolin inhibition.

Studies of the uracil and guanine analogs have shown that a major determinant in their specificity is the nature of the alkyl substituents (15-18). The comparative insensitivity of DNA polymerase δ to inhibition by these analogs argues it has significant structural differences from polymerase α in the binding domains for these analogs, and provides further support for the view that it is a distinct enzyme. This issue is of importance at the present time, since the current enzymology of polymerases α and δ does not provide absolute evidence that they represent distinct gene products. For example, while most DNA polymerase α preparations have been reported to be devoid of 3' 5' exonuclease activity, there have been reports of DNA polymerase α preparations which do possess associated 3' 5' exonuclease activities (24,25). In practical terms it is readily seen that these analogs will provide a simple procedure which would provide for the selective assay of polymerase δ in the presence of α , and in fact provide a simple diagnostic assay for polymerase δ . This would resolve issues which may arise as to whether a polymerase α preparation which contains exonuclease activity is in fact a form of polymerase δ or is simply contaminated with a trace of nonassociated 3' to 5' exonuclease activity. Finally, these findings confirm and extend current evidence that these analogs may be useful probes for structural and functional differences between DNA polymerases.

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