Synthesis of Nucleotide Analogues That Potently and Selectively Inhibit Human DNA Primase†

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Received July 17, 2002; Revised Manuscript Received September 9, 2002

ABSTRACT: DNA primase synthesizes short RNA oligonucleotides that DNA polymerase α further elongates in order to initiate the synthesis of all new DNA strands during eukaryotic DNA replication. To develop potent and specific primase inhibitors, we combined 2′-modified sugars with bases incapable of normal Watson−Crick hydrogen bonding. The presence of a 2′-hydroxyl in either the ara or ribo configuration greatly enhances the ability of primase to polymerize a nucleotide. Further modifying the 2′-position by including both a hydroxyl and methyl group at this position greatly reduced the ability of primase to polymerize the resulting nucleotides. Replacing the base of the NTP with analogues incapable of normal Watson−Crick hydrogen bonding (benzimidazole, nitrobenzimidazole, and dichlorobenzimidazole) resulted in compounds that inhibited primase quite well and with similar potency. We synthesized arabinofuranosylbenzimidazole triphosphate (araBTP) and found that this sugar change increased inhibition by 2−4-fold relative to the ribofuranosyl analogue. AraBTP inhibited polymerization of both purines and pyrimidines, although primase polymerized only small amounts of the compound. Interestingly, even though araBTP was not readily polymerized by primase, it inhibited primase almost as potently as araATP, a compound that primase polymerizes extremely rapidly and that results in very strong chain termination. Importantly, this compound was a very weak inhibitor of and only slowly polymerized by DNA polymerase α, indicating that it is a specific primase inhibitor. The potential utility and mechanistic implications of these inhibitors are discussed.

In the eukaryotic nucleus, DNA polymerase α (pol α)[1] helps to initiate all new DNA strands during replication (*5*). Like all DNA polymerases, however, pol α lacks the ability to form phosphodiester bonds de novo and, therefore, requires a 3′-hydroxyl from an existing primer annealed to ssDNA. To provide this primer, pol α relies on an RNA polymerase, DNA primase, with which it is intimately associated. On ssDNA, primase produces a short (7−10 nucleotide) oligoribonucleotide onto which pol α polymerizes ca. 20 dNTPs (*6*). Upon dissociation of pol α, a more processive DNA polymerase (δ or ϵ) elongates this "DNA primer" (*7*). Eventually, the RNA portion is excised and the gap filled with DNA.

As an isolated enzyme, primase is a Mg^{2+}-dependent heterodimer of 49 and 58 kDa subunits (*8*−*10*). The p49 subunit contains phosphodiester bond formation activity, and the p58 subunit greatly enhances primer synthesis (*11*−*14*). Additionally, p58 serves to tether primase to pol α and likely helps to transfer the newly generated primer from the primase active site to the pol α active site (*15*). A minimal kinetic model provides for three phases of primer formation: initiation (formation of dinucleotide), elongation (conversion of the dinucleotide into a unit length primer 7−10 nucleotides long), and termination (passing the 3′ terminus of the unit length primer to the pol α active site) (*16*, *17*). Premature dissociation of the growing primer prior to completion of a unit length results in an abortive primer of 2−6 nucleotides that pol α cannot readily utilize. When such primer dissociation occurs, primase rapidly reinitiates synthesis without dissociation from the template. If primase succeeds in synthesizing a unit length primer, further primase activity is negatively regulated until pol α elongates this primer. In the absence of pol α activity, primase can reinitiate primer synthesis on the just synthesized unit length primer, polymerizing another 7−10 nucleotides onto it.

With an error rate of >1 in 30, primase is easily the most inaccurate replicative polymerase in eukaryotes (*18*). Furthermore, primase will polymerize multiple consecutive noncognate NTPs. While this tremendous infidelity seems astonishing, the transient nature of the RNA primer means errors are not retained in the genome. Misincorporation occurs most frequently after formation of the dinucleotide, suggesting proper base pairing is most important when the primer length equals 1. Despite studies showing that pol α requires correctly base-paired primers for efficient elongation of an exogenously added primer-template, pol α readily elongates primase-generated primer-templates, even when they contain mismatches at or near the 3′ terminus (*19*). The mechanistic basis for this altered tolerance of mismatches

† This work was supported by National Institutes of Health Grant GM54194 to R.D.K.

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[1] Abbreviations: BSA, *N*,*O*-bis(trimethylsilyl)acetomide; BTP, benzimidazole ribofuranosyl triphosphate; DCBTP, dichlorobenzimidazole ribofuranosyl triphosphate; 5(6)-NBTP, a 50:50 mixture of 5- and 6-nitrobenzimidazole ribofuranosyl triphosphates; pol α, DNA polymerase α; TEAB, triethylammonium bicarbonate; TMSOTf, trimethylsilyl trifluoromethanesulfonate.

10.1021/bi026468r CCC: $22.00
Published on Web 10/22/2002

Table 1: Synthetic DNAs of Defined Sequence Used in These Studies

dC_{40}	5'-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC
$d(TC)_{30}$	5'-TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC
$d(ATC)_{20}$	5'-ATCATCATCATCATCATCATCATCATCATCATCATCATCATCATCATCATCATCATC
DNA_A	5' TCCATATCACAT 3' AGGTATAGTGTA**A**TACTTATCATCT
DNA_C	5' TCCATATCACAT 3' AGGTATAGTGTA**C**ATCTTATCATCT
DNA_G	5' TCCATATCACAT 3' AGGTATAGTGTA**G**ATCTTATCATCT
DNA_T	5' TCCATATCACAT 3' AGGTATAGTGTA**T**ATCTTATCATCT

during primase-coupled pol α activity versus uncoupled activity remains obscure.

While a variety of compounds have been shown to inhibit DNA primase, none of them are highly selective. For example, actinomycin D intercalates into DNA and potently inhibits primase; however, it also inhibits other enzymes involved in DNA/RNA metabolism (*20*, *21*). Sphingosine molecules likewise constitute a unique class of primase inhibitors that function by competing for DNA binding (*22*). However, the presence of many other enzymes that interact with sphingosine results in this molecule having multiple biological effects. Last, araNTPs, an important class of cancer chemotherapeutics, potently inhibit DNA primase and result in chain termination upon incorporation by primase (*23*, *24*). However, araNTPs also potently inhibit DNA polymerases such that the concentrations needed to inhibit primase also result in potent inhibition of DNA polymerases.

To develop compounds that potently inhibit DNA primase with minimal effects on DNA polymerases, we have taken advantage of primase's remarkable infidelity along with the generally high fidelity of DNA polymerases.[2] Nucleoside triphosphates were synthesized that consisted of primase's preferred sugar, arabinose, and a "base" incapable of Watson−Crick hydrogen bonding (e.g., benzimidazole). These compounds potently inhibited primase but had minimal effects on pol α. The biological and mechanistic implications of these compounds are discussed.

EXPERIMENTAL PROCEDURES

Materials

Primase was overexpressed in *Escherichia coli* JM105 (DE3) deficient in exonuclease I and purified via Ni-NTA (Qiagen) chromatography as previously described (*14*). Human pol α and pol α-primase were immunopurified from baculovirus-infected insect cells as previously described (*25*). All chemicals used were of the highest commercial grade. When necessary, anhydrous solvents were purchased in sure-seal bottles from Aldrich (Milwaukee, WI). Synthetic oligonucleotides of defined sequence were from Oligos Etc., Inc. (Table 1). The DCBTP was generously provided by ISIS Pharmaceuticals (Carlsbad, CA).

Methods

NMR. Compounds were characterized by ^{1}H NMR, ^{31}P NMR, HRMS, and comparison with literature values where available. Deuterated solvents for NMR were purchased from Cambridge Isotope Laboratories (Boston, MA).

Liquid Chromatography. HPLC was performed on a Beckman Coulter System Gold instrument fitted with preparative heads and running 32Karat software. Reverse-phase separations were performed on a Vydac 5 μm C-18 analytical column (4.6 × 250 mm) and Beckman Ultrasphere C-18 semipreparative column (10 × 250 mm) using the following methods: method A, analytical column with a 25 mL gradient elution from 10 mM tetraethylammonium hydrogen sulfate to 20% AcN in buffer A; method B, semipreparative column with a 140 mL gradient elution from 20 mM triethylammonium bicarbonate (TEAB) to 50% AcN in buffer A. Strong anion-exchange separation was performed using a Whatman Partisil 10 SAX column using a linear gradient of 10 mM NaH_2PO_4 (pH 4) to 1 M NaH_2PO_4 (pH 4). Purification via anion-exchange chromatography was performed on DEAE-cellulose−bicarbonate (DE-52, Whatman) eluting with a linear gradient of 0.10 M TEAB to 1 M TEAB, pH 7.4. Desalting was performed through size-exclusion chromatography using Bio-Gel P-2 gel, fine (Bio-Rad).

Synthesis of Arainosine Triphosphate. Arainosine (25 mg, 93 μmol) was dissolved in 0.9 mL of freshly distilled trimethyl phosphate and cooled to 0 °C under argon. The solution was treated with 13 μL of fresh $POCl_3$ (1.5 equiv, 139 μmol) and stirred at 4 °C overnight (*26*). The triphosphate was generated by adding 5 equiv of 0.5 M tributylammonium pyrophosphate in anhydrous DMF followed by 5 equiv of tributylamine. The reaction was quenched by adding 2 mL of 1 M TEAB, diluted to 50 mL with water, and purified by anion-exchange column chromatography. Fractions were checked using HPLC-SAX. The yield was 30% based on UV activity: ^{31}P NMR (202 MHz, D_2O) −9.62 (d), −10.20 (d), −22.95 (dd); HRMS (FAB^+−glycerol) 552.9524 (calcd 552.9515, $MH_3Na_2^+$).

Synthesis of 2′-β-Methylinosine Triphosphate. Hypoxanthine (160 mg, 1.2 mmol) in 5 mL of anhydrous AcN and

[2] An important exception to this statement is the recently discovered "error-prone" DNA polymerases that may play important roles in lesion bypass and generation of immune system diversity (*1*).

0.58 mL of *N*,*O*-bis(trimethylsilyl)acetomide (BSA) (2.4 mmol) were refluxed for 1 h under argon. The reaction mixture was cooled to room temperature, and 341.6 mg of 1,2,3,5-tetra-*O*-benzoyl-2-*C*-methyl-*β*-D-ribofuranose (*27*) (588 *μ*mol) in 5 mL of anhydrous AcN was added to the solution followed by 0.16 mL of trimethylsilyl trifluoromethanesulfonate (882 *μ*mol) (*28*). The reaction was again brought to reflux for 16 h. The reaction mixture was cooled to room temperature and partitioned between methylene chloride and saturated $NaHCO_3$. The organic phase was dried over $MgSO_4$ and concentrated. The crude reaction product was chromatographed on silica using 1:1 EtOAc/hexanes to yield 200 mg of protected nucleoside (60%). The protected nucleoside was deprotected with methanolic ammonia overnight and purified by silica gel chromatography using 15% EtOH in methylene chloride to recover 47 mg (175 *μ*mol, 30% yield over two reactions): ^{1}H NMR (500 MHz, D_2O) δ 0.76 (3H, s), 3.74 (1H, dd, J = 10 and 3 Hz), 3.94 (4H, m), 5.94 (1H, s), 8.0 (1H, s), 8.20 (1H, s). 2′-*C*-*β*-Methylinosine was phosphorylated as described above: ^{31}P NMR (202 MHz, D_2O) −9.21 (d), −10.68 (d), −22.08 (dd); HRMS (FAB^+−glycerol) 523.0031(calcd 523.0032, MH_5^+).

Synthesis of 2′-α-Methylarainosine Triphosphate (29, 30). 2,3,5-Tri-*O*-acetyl-6-chloropurine riboside (3.2 g, 7.75 mmol) in 10 mL of absolute EtOH and 6 mL of 20% NaOEt were refluxed for 2 h. The reaction mixture was titrated to pH 4 with 1 N HCl and concentrated. The residue was warmed in hot EtOAc, filtered to remove NaCl, and concentrated to a smaller volume. 6-Ethoxypurine nucleoside was precipitated from solution with hexanes to yield 1.7 g (5.74 mmol, 74% yield): ^{1}H NMR (500 MHz, DMSO) δ 1.40 (3H, t J = 7 Hz), 3.56 (1H, m), 3.67 (1H, m), 3.95 (1H, m), 4.16 (1H, br), 4.58 (3H, m), 5.15 (1H, dd, J = 5 and 5.5 Hz), 5.28 (1H, br), 5.56 (1H, br), 5.97 (1H, d, J = 5.5 Hz), 8.52 (1H, s), 8.61 (1H, s). 6-Ethoxypurine riboside (1 g, 3.4 mmol) was protected with TIPDS−Cl_2 (1.1 g, 3.5 mmol) in 20 mL of DMF containing 0.8 g of DMAP as a catalyst. After 2 h the reaction was partitioned between water and EtOAc. The aqueous layer was extracted with EtOAc (three times). The organic layers were combined, washed with brine, dried over $MgSO_4$, and concentrated. The crude mixture was chromatographed on silica using 9:1 followed by 3:2 hexanes/EtOAc to yield 700 mg of product (40% yield): ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.04 (27H, m), 1.47 (3H, t, J = 6.5 Hz), 3.61 (1H, d, J = 1.5 Hz), 4.01 (1H, m), 4.12 (2H, m), 4.52 (1H, d, J = 5.5 Hz), 4.61 (2H, q, J = 6.5 Hz), 5.01 (1H, dd, J = 5.5 and 5 Hz), 6.02 (1H, d, J = 1 Hz), 8.09 (1H, s), 8.44 (1H, s). Protected 6-ethoxypurine riboside (700 mg, 1.3 mmol) was oxidized with 3 equiv of Dess−Martin periodiane (1.65 g, 3.9 mmol) overnight in 20 mL of methylene chloride. The reaction was concentrated and washed with saturated $NaHCO_3$ containing 3 g of NaS_2O_3. The organic layer was collected and dried over $MgSO_4$ overnight, filtered, and concentrated to yield 467 mg (67% yield) of 2′-ketonucleoside. The recovered ketone was used directly in the next reaction. 2′-Ketonucleoside was dissolved in 10 mL of anhydrous THF and cooled to −78 °C under argon. MeMgBr (0.9 mL of 3.0 M in Et_2O, 2.6 mmol) was added slowly over 15 min. The reaction was allowed to stir for 3 h under argon and come to room temperature. The solution was neutralized with 1 N NH_4Cl and partitioned between EtOAc and water. The organic phase was dried over Na_2SO_4 and concentrated. A single product was eluted from silica using 20% EtOAc in hexanes to yield 288 mg of product (522 *μ*mol, 60% yield). 2′-*C*-Methylinosine arabinoside was obtained by refluxing the protected nucleoside in 0.5 mL of 0.1 M NaOH in THF for 4 h. The stereochemistry was confirmed by comparing the 2′-methyl group chemical shift with the previous methylinosine of known stereochemistry, as well as by 2-D NOE spectroscopy: ^{1}H NMR (500 MHz, D_2O) δ 1.22 (3H, s), 3.78 (2H, m), 3.93 (1H, m), 4.02 (1H, d, J = 5 Hz), 5.99 (1H, s), 8.04 (1H, s), 8.24 (1H, s). Phosphorylation proceeded as described above: ^{31}P NMR (202 MHz, D_2O) −9.35 (d), −10.23 (d), −21.65 (dd); HRMS (FAB^+−glycerol) 566.9699 (calcd 566.9671, $MH_3Na_2^+$).

Synthesis of Benzimidazole Ribofuranosyl Triphosphate. Benzimidazole (236 mg, 2 mmol) and BSA (1 mL, 4 mmol) were refluxed in 4 mL of anhydrous 1,2-dichloroethane for 1 h under argon (*2*). The reaction was allowed to cool, and 1,2,3,5-tetra-*O*-acetylribofuranose (318 mg, 1 mmol) in 4 mL of 1,2-dichloroethane was added followed by TMSOTf (0.38 mL, 2.1 mmol). The reaction was again brought to reflux. After 2 h, the reaction mixture was allowed to cool to room temperature and partitioned between methylene chloride and saturated $NaHCO_3$. The organic layer was washed with saturated $NaHCO_3$ (twice) and brine (twice), dried over Na_2SO_4, and concentrated. The resulting foam was dissolved in 7:3 EtOAc/hexanes and separated on silica using the same solvent mixture. Fractions containing the protected nucleoside were collected and concentrated to yield 140 mg (371 *μ*mol, 37%) (*31*). Deprotection with methanolic ammonia overnight yielded the benzimidazole ribonucleoside after silica gel chromatography using 10% MeOH in EtOAc: ^{1}H NMR (500 MHz, DMSO) δ 3.63 (2H, m, 5′H), 3.97 (1H, ddd, 4′H) 4.11 (1H, br ddd, 3′H), 4.37 (1H, ddd, 2′H), 5.15 (1H, br dd, 5′OH), 5.25 (1H, br d, 3′OH), 5.50 (1H, br d, 2′OH), 5.87 (1H, d, 1′H), 7.25 (2H, m, ArH), 7.67 (1H, m, ArH), 7.74 (1H, m, ArH), 8.46 (1H, s, C2H); MS (FAB^+) 250.3 (M^+ 250); ϵ_{246} (H_2O) = 7300 M^{-1} cm^{-1} (reference 7400 M^{-1} cm^{-1}). Phosphorylation proceeded as described above: ^{31}P NMR (161 MHz, D_2O) −10.10 (d), −10.73 (d), −22.63 (dd); HRMS (FAB^+−glycerol) 491.0031 (calcd 491.0022, MH_5^+).[3]

Synthesis of 5- and 6-Nitrobenzimidazole Ribofuranosyl Triphosphate (4). 5-Nitrobenzimidazole (1.2 g, 7.3 mmol) in 10 mL of anhydrous AcN was refluxed with 2 mL of BSA (8.1 mmol) for 1 h under argon. The reaction was allowed to cool to room temperature after which 2.3 g of 1,2,3,5-tetra-*O*-acetylribofuranose (7.3 mmol) in 10 mL of AcN and 1.3 mL of TMSOTf (7.3 mmol) were added to the solution. The reaction was again brought to reflux for 3 h. The solution was cooled to room temperature and partitioned between methylene chloride and saturated $NaHCO_3$. The organic phase was washed with saturated $NaHCO_3$ (twice) and brine, dried over $MgSO_4$, and concentrated. The mixture of 5- and 6-nitrobenzimidazole nucleoside was purified by silica chromatography using 2:1 EtOAc/hexanes which

[3] Nucleoside synthesis with benzimidazole using the Vorbruggen glycosylation method as previously reported produced very little nucleoside product (~5%) (*2*). The electron-donating benzyl ring of the benzimidazole stabilizes the formation of a diglycosylated cation to the exclusion of monoglycosylated nucleoside, similar to a report by Al Mourabit et al. for imidazole glycosylation (*3*). Modification of the synthesis by utilizing 2 equiv of benzimidazole/equiv of sugar increased the yield of the nucleoside to 80−90%.

yielded 2.6 g of protected nucleoside (85%). Because of the difficulty in isolating the two isomers, the mixture was used. Deprotection with Na_2CO_3 in aqueous MeOH followed by silica chromatography with 10% MeOH in EtOAc afforded a 1 to 1 mixture of 5- and 6-nitrobenzimidazole ribonucleoside: ^{1}H NMR (500 MHz, DMSO) δ 3.66 (4H, m,5′H), 4.02 (2H, m, 4′H), 4.13 (2H, m, 3′H), 4.37 (2H, m, 2′H), 5.18 (1H, dd, 5′OH), 5.22 (1H, dd, 5′OH), 5.27 (2H, m, 3′OH), 5.56 (1H, d, 2′OH), 5.58 (1H, d, 2′OH), 5.97 (1H, d, 1′H 5-nitro), 6.02 (1H, d, 1′H 6-nitro), 7.87 (1H, d, 4H 6-nitro), 8.01 (1H, d, 7H 5-nitro), 8.13 (1H, dd, 5H 6-nitro), 8.17 (1H, dd, 6H 5-nitro), 8.57 (1H, d, 4H 5-nitro), 8.77 (1H, s, 2H 5-nitro), 8.83 (1H, s, 2H 6-nitro), 8.86 (1H, d, 7H 6-nitro). Phosphorylation proceeded as described above: ^{31}P NMR (161 MHz, D_2O) −8.45 (d), −8.87 (m), −11.03 (d), −22.39 (m); HRMS (FAB^+−glycerol) 535.9864 (calcd 535.9873, MH_5^+).

Synthesis of Benzimidazole Arabinofuranosyl Triphosphate. To a stirring suspension of 224 mg of benzimidazole (1.9 mmol) in 5 mL of anhydrous AcN under argon was added 84 mg of NaH (2.1 mmol, 60% suspension in mineral oil) to generate the sodium salt of benzimidazole (*32*). After 30 min, a solution of 1-chloro-2,3,5-tribenzylarabinose (*33*) (834 mg, 1.9 mmol) in 5 mL of AcN was added, and the reaction was allowed to stir. After 4 h the reaction was quenched by diluting with methylene chloride and washing with saturated $NaCO_3$ (twice) and brine (twice). The organic layer was dried using $MgSO_4$, filtered, and concentrated. Protected nucleoside was purified by column chromatography on silica using 1% MeOH in methylene chloride: ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.74 (2H, m), 4.15 (2H, dd), 4.20 (1H, m), 4.28 (2H, m), 4.60 (2H, m), 4.63 (2H, m), 6.26 (1H, d, J = 4.4 Hz), 6.90 (2H, d, J = 7 Hz), 7.21 (3H, m), 7.37 (14H, m), 7.88 (1H, d, J = 7 Hz), 8.32 (1H, s). Benzimidazole arabinoside (200 mg, 380 μmol) was deprotected with 6 mL of 1 M BCl_3 in CH_2Cl_2 at −78 °C for 2 h. After 2 h, the solvent was removed under reduced pressure and the reaction neutralized with saturated $NaHCO_3$. The aqueous layer was extracted with methylene chloride and then concentrated. The nucleoside was adsorbed onto silica and chromatographed with 10% MeOH in EtOAc: ^{1}H NMR (500 MHz, DMSO) δ 3.66 (1H, br m, 5′H), 3.70 (1H, br m, 5′H), 3.78 (1H, ddd, 4′H), 4.10 (1H, ddd, 3′H), 4.19 (1H, ddd, 2′H), 5.09 (1H, br, 5′-OH), 5.53 (2H, m, 2′-OH and 3′-OH), 6.36 (1H, d, 1′H), 7.22 (2H, m, ArH), 7.63 (2H, m, ArH), 8.39 (1H, s, C2H). The nucleoside was phosphorylated as described above: ^{31}P NMR (202 MHz, D_2O) −8.85 (d), −10.03 (d), −21.88 (dd); HRMS (FAB^+−glycerol) 490.9996 (calcd 491.0022, MH_5^+).

Primase Assay. Reactions (10 μL) typically contained 50 mM tris(hydroxylmethyl)aminomethane, pH 7.9 (HCl salt), 5 mM $MgCl_2$, 60 μM ssDNA template (total nucleotide), 0.05 mg mL^{-1} bovine serum albumin,1 mM dithiothreitol, and 100−800 μM [α-^{32}P]NTPs. Reactions were initiated by adding enzyme and incubating at 37 °C for 1 h. Assays were quenched by adding 2.5 volumes of gel loading buffer (90% formamide) and the products separated by denaturing polyacrylamide gel electrophoresis (20% polyacrylamide, 8 M urea) and analyzed by phosphorimagery (Molecular Dynamics).

Pol α *Assay*. Except as noted, reaction conditions were similar to those used for measuring primase activity. Pol α activity was measured on poly(dT)−$(rA)_{12-18}$ and on DNA_A, DNA_C, DNA_G, and DNA_T where the primer strand was 5′-^{32}P labeled. Reactions were quenched and quantified as above.

Table 2: Effects of Modifying the 2′-Substituents on the Ability of a Compound To Inhibit Primase Activity

compound[a]	2′-substitution	K_i vs ATP (μM)	K_i vs GTP (μM)
ITP[b]	α-OH, β-H	*	*
dITP	α,β-H	ND	80*
araITP	α-H, β-OH	10*	1.5*
araATP	α-H, β-OH	1.5*	20*
2′-methyl-ITP	α-OH, β-CH_3	22	20
2′-methyl-araITP	α-CH_3, β-OH	29	23

[a] Assays were performed as described under Experimental Procedures. Poly(dT) was used as the template to measure inhibition versus ATP, and $(dC)_{40}$ was used as the template to measure inhibition versus GTP. An asterisk denotes reactions where primase incorporated the tested analogue. [b] Inclusion of ITP in assays containing either ATP or GTP resulted in increased product formation consistent with ITP as an alternate substrate.

RESULTS

Interaction of Primase with 2′-Modified Inosine Nucleotides. Previous studies have shown that araNTPs potently inhibit primase (*23*), and in fact, primase prefers to polymerize araNTPs rather than NTPs, the enzyme's physiological substrate (*24*). To provide further insights into the interactions of primase with the 2′-position of the ribose ring, particularly the effect of a methyl group at the 2′-position, we examined a series of ITP analogues for their ability to interact with primase.

Including ITP in primase assays stimulates both GTP and ATP incorporation at low concentrations, as measured on the templates $(dC)_{40}$ and poly(dT), respectively (Table 2). In the assays using $(dC)_{40}$ as the template, primase generated products of altered mobility when assays also contained ITP, indicating that primase will polymerize the ITP (data not shown). Inclusion of ITP in assays containing poly(dT) results in increased initiation events without altering product distribution at low ATP concentration, suggesting that ITP is efficiently incorporated and extended while not altering electrophoretic mobility. Thus, the stimulation of GTP and ATP incorporation likely results from primase using ITP as an alternative substrate, consistent with the notion that inosine can serve as a universal base in some assays (*34*, *35*).

Similar to previous studies (*24*), we found that removing the 2′-hydroxyl resulted in a compound that inhibited primase relatively weakly (K_i = 80 μM, competitive with respect to GTP). Primase incorporated only trace amounts of dITP into products, as evidenced by the appearance of only small amounts of products with altered mobility when assays contained dITP (Table 2 and data not shown). Adding back a hydroxyl to the 2′-C in the ara configuration now results in a very potent inhibitor. In assays containing $(dC)_{40}$ and [α-^{32}P]GTP, primase synthesized copious quantities of products with altered electrophoretic mobility when reactions contained even low araITP concentrations (Figure 1C). Increasing the araITP concentration results in a dramatic reduction in the average length of the primer products, consistent with incorporation of the araITP resulting in chain termination. Interestingly, inclusion of 50 μM araITP in

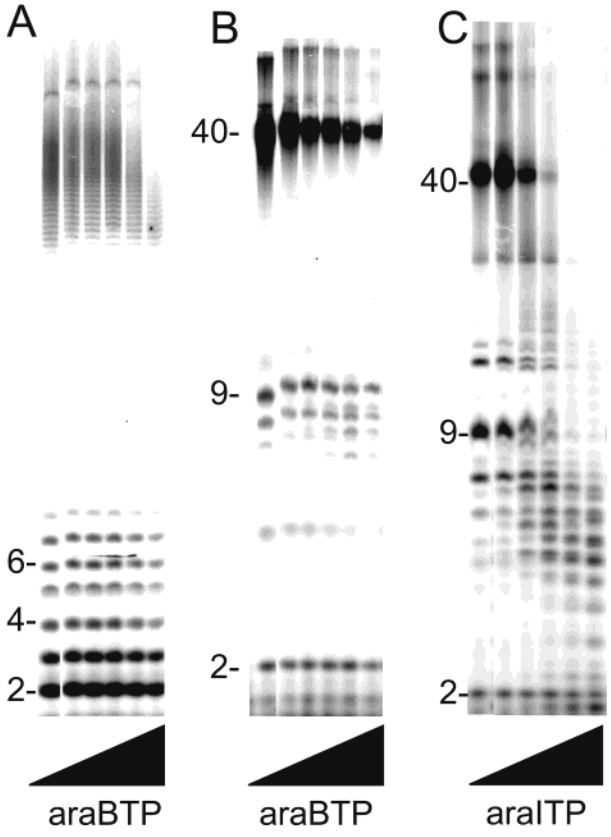

FIGURE 1: Effect of aranucleotides on primase-generated primers. Assays were performed with 60 μM template and 200 μM [α-^{32}P]-NTP. Primer lengths are noted to the left of each panel. (A) ATP polymerization on the template poly(dT) in the presence of 0, 0.5, 0.75, 1, 5, and 10 μM araBTP. (B) GTP polymerization on the template $(dC)_{40}$ in the presence of 0, 25, 50, 100, 150, and 200 μM araBTP. Incorporation of araBTP into primers is evidenced by the presence of new bands with increasing inhibitor concentration. (C) GTP polymerization on the template $(dC)_{40}$ in the presence of 0, 0.75, 5, 15, 30, and 50 μM araITP.

assays almost completely abolished the synthesis of unit length primers (i.e., those that pol α can readily elongate) while increasing the total moles of primers synthesized by 10-fold.[4] Since these products are only two to three nucleotides long, however, pol α cannot readily elongate them.

Since the presence of a 2′-hydroxyl in either the ara or ribo configuration appears essential for primase to efficiently polymerize a nucleotide, we attempted to mimic the effects of having substituents in both the ara and ribo configuration at the 2′-C by synthesizing 2′-methyl-ITP and 2′-methyl-araITP. However, these compounds were much less potent inhibitors than araITP (Table 2) and were not detectably incorporated into products (data not shown). The latter conclusion derives from the absence of any products with altered electrophoretic mobility when either 2′-methyl-ITP or 2′-methyl-araITP was included in assays. While it is possible that incorporation of these analogues into products would not induce any changes in electrophoretic mobility, this seems unlikely since both here and in previous work, we have found that even relatively small changes in sugar structure affect the electrophoretic mobility of the resulting products. Thus, introduction of the hydrophobic methyl group did not prevent enzyme binding, only polymerization. Interestingly, concentrations resulting in significant reduction of unit length primer production had little effect on total product synthesis, suggesting that these compounds had less effect on primase initiation than on elongation.

Table 3: Inhibition of Primer Synthesis by Nucleoside Triphosphates Incapable of Forming Normal Watson−Crick Hydrogen Bonds[a]

compound	K_i vs ATP (μM)	K_i vs GTP (μM)
DCBTP	24	45
5(6)-NBTP	22	52
BTP	20	50
ATP		49*
araBTP	5	24*
araATP	1.5*	20*
araCTP	45	85

[a] Assays were performed as described under Experimental Procedures. Poly(dT) was used as the template to measure inhibition versus ATP, and $(dC)_{40}$ was used as the template to measure inhibition versus GTP. An asterisk denotes reactions where primase incorporated the tested analogue.

Synthesis and Evaluation of Non-Hydrogen-Bonding Nucleotides. We tested the hypothesis that nucleoside analogues incapable of forming the Watson−Crick H-bonds with the template base would potently inhibit primase. On the basis of the low fidelity of primase, we reasoned that primase might not require correct Watson−Crick hydrogen bonding between the incoming NTP and the template base being copied. To test this hypothesis, three benzimidazole-based nucleotides that lack, or can only weakly form, Watson−Crick hydrogen bonds were tested for their ability to inhibit and/or serve as alternate substrates for primase.

Three "non-hydrogen-bonding" nucleotides were tested as primase inhibitors, benzimidazole ribofuranosyl triphosphate (BTP), dichlorobenzimidazole ribofuranosyl triphosphate (DCBTP), and a 50:50 mixture of 5- and 6-nitrobenzimidazole ribofuranosyl triphosphate [5(6)-NBTP].[5] All three compounds inhibited primase activity competitively with respect to ATP and GTP on the templates poly(dT) and $(dC)_{40}$, respectively (Table 3 and Figure 2). In each case, ATP incorporation was inhibited to a greater degree than GTP incorporation. Surprisingly, the K_i values for the three benzimidazole compounds were extremely similar with respect to both ATP and GTP polymerization, even though the structure of the bases near the hydrogen-bonding face varied substantially. We also measured the ability of a noncognate natural nucleotide, ATP, to inhibit GTP polymerization and found that it inhibited primase activity with a similar potency to the three benzimidazole-derived nucleo-

[4] Primase products refer to actual molar amounts of primer synthesized and are calculated by dividing each length primer by the number of radiolabeled nucleotides present in that length primer. Since more [α-^{32}P]NTPs are present in each longer product (e.g., unit length primers) than in a short product, the relative intensity of long versus short products is much greater than the actual molar ratio of long to short products.

[5] Synthesis of 5-nitrobenzimidazole resulted in an equimolar mixture of 5- and 6-nitrobenzimidazole nucleosides that were not readily resolved (*4*). The two regioisomers were confirmed by 1-D NOE experiments. Because the two were not well resolved, the 50:50 mixture (as determined by NMR integration of equivalent protons) was used for initial studies. Unlike with the benzimidazole nucleoside, no diglycosylated cation was detected, suggesting that the addition of the electron-withdrawing nitro group destabilized the formation of the cation sufficiently to preclude its formation.

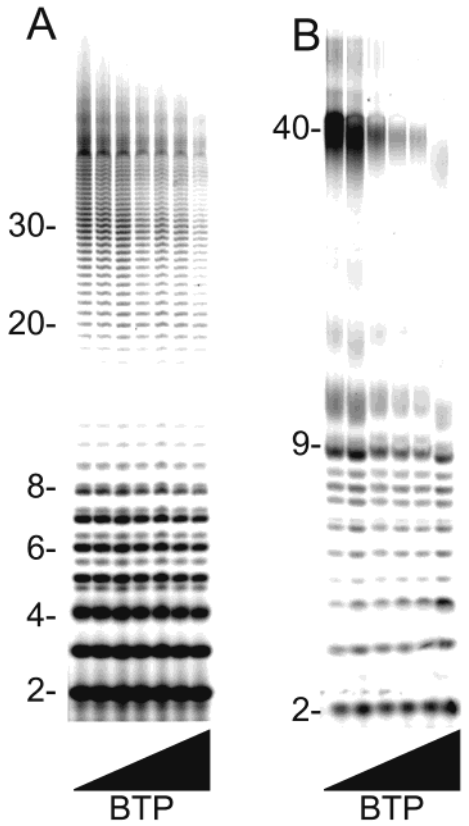

FIGURE 2: Effect of BTP on primase- generated primers. Assays were performed with 60 μM template and 200 μM [α-^{32}P]NTP. Primer lengths are noted to the left of each panel. (A) ATP polymerization on the template poly(dT) in the presence of 0, 10, 20, 30, 40, 50, and 60 μM BTP. (B) GTP polymerization on the template $(dC)_{40}$ in the presence of 0, 50, 100, 150, 200, and 250 μM BTP.

tides. As previously observed with the 2′-*C*-methyl compounds, concentrations of inhibitor that only slightly reduced (20%) the total moles of primer synthesized resulted in severe inhibition (80%) of unit length primer synthesis. The lack of enhanced inhibition by 5(6)-NBTP was particularly surprising given the structural similarity between nitrobenzimidazole and the reported universal base 5-nitroindole (*36*). Inspection of the sequencing gels suggested that primase did not polymerize the analogues, as evidenced by a lack of any new bands of altered electrophoretic mobility (data not shown). Together, these data indicate that primase will bind nucleotides that contain bases incapable of forming normal Watson−Crick hydrogen bonds; however, polymerization is significantly impaired.

Synthesis and Evaluation of Arabenzimidazole Triphosphate (AraBTP). As noted earlier, inversion of the 2′-hydroxyl of natural nucleotides results in compounds that are both very good primase substrates and potent inhibitors. Thus, to enhance the potency of the non-hydrogen-bonding analogues, we attached the benzimidazole base to arabinose and tested its effect on primase. We limited these studies to benzimidazole, since ring substitution did not provide enhanced binding in our ribose-based inhibitors.

As predicted, araBTP proved to be a more potent inhibitor of unit length primer formation than the ribo counterpart. K_i values versus ATP and GTP were 5 and 24 μM, respectively, values 2−3-fold lower than the corresponding ribo compound (Table 3 and Figure 1A,B). Inhibition by a "natural" araNTP, araATP, was likewise measured and found to be only slightly more potent than araBTP. AraATP inhibited primase with a K_i of 1.5 μM versus ATP and 20 μM versus GTP. Similarly, araCTP inhibited both ATP and GTP polymerization, albeit with a higher K_i than either araBTP or araATP.

Similar to the effects of araITP described earlier, including araATP in assays on poly(dT) resulted in a dramatic increase in the total moles of primers synthesized while potently inhibiting the synthesis of longer products. In contrast, araBTP did not appear to greatly increase total initiation events even though it still inhibited primase almost as potently as araATP. The implications of these differences will be addressed in the Discussion section.

If incorporated into the growing primer, aranucleotides act as extremely potent chain terminators, and the resulting products exhibit substantially altered electrophoretic mobility. To determine if primase polymerized araBTP, we first looked for products of altered electrophoretic mobility. In reactions containing poly(dT) and 200 μM ATP, inclusion of up to 100 μM araBTP, conditions that resulted in 33% total inhibition of primase activity and 60% inhibition of unit length primers, did not result in any products of altered electrophoretic mobility (data not shown). In contrast, primase incorporated very small amounts of araBTP in reactions containing $(dC)_{40}$ and up to 200 μM GTP (Figure 1B). Interestingly, incorporation of araBTP was not observed in products shorter than five nucleotides, in contrast to the results with either araITP or araATP where products as short as a dinucleotide contained araIMP or araAMP (contrast Figure 1C with Figure 1B). These data suggest that primase only polymerizes araBTP into longer, more stable primers.

To rule out the possibility that primase readily polymerized araBTP but the resulting products had electrophoretic mobility identical to that of products consisting entirely of ribonucleotides, we examined the effects of adding araBTP to primase assays containing the template $d(TC)_{30}$ and only [α-^{32}P]ATP. Under these conditions, the enzyme has the choice of no primer synthesis, misincorporation of ATP across from a template deoxycytidylate to generate the pppApA dinucleotide, or dinucleotide synthesis using araBTP as one of the two nucleotides. Consistent with previous studies showing that primase has relatively high fidelity during the synthesis of the dinucleotide, primase did not generate any pppApA dinucleotide in assays containing only [α-^{32}P]ATP. Adding up to 120 μM araBTP did not result in dinucleotide formation, suggesting that araBTP does not act as a specific GTP analogue (Figure 3A). Likewise, primase does not readily initiate primer synthesis when given only GTP, although some misincorporation occurs as evidenced by formation of di- and trinucleotides. Again, however, inclusion of araBTP did not result in increased formation of short products. Rather, synthesis of the pppGpG and pppGpGpG di- and trinucleotides was potently inhibited (Figure 3B). Thus, primase does not readily incorporate araBTP into short products as either an ATP or a GTP analogue, although it does readily bind it. By way of comparison, inclusion of araATP and [α-^{32}P]GTP results in the synthesis of massive amounts of dinucleotide, presumably pppGp(araA), and a 5-fold stimulation of total primer products (Figure 3C). These data demonstrate that primase readily utilizes the cognate aranucleotide during initiation;

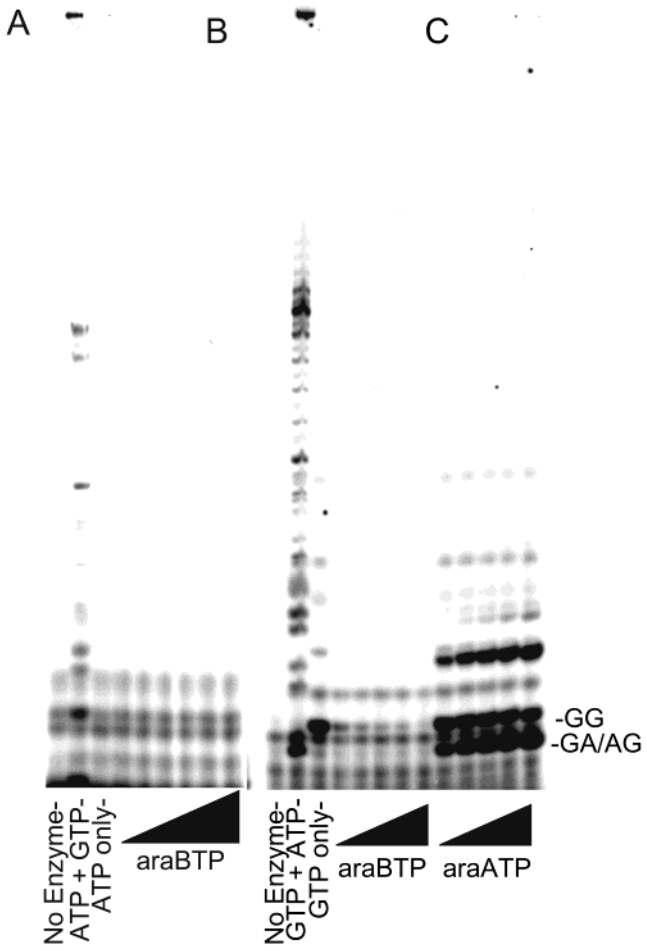

FIGURE 3: Primase does not readily polymerize araBTP as either an ATP or GTP analogue. Assays contained 60 μM d(TC)$_{30}$. (A) Test of araBTP as a specific GTP analogue. Assays were performed using 200 μM [α-^{32}P]ATP. Lane 2 shows full primer extension in the presence of GTP. Lanes 3−9 contain no GTP and increasing araBTP concentrations of 0, 20, 40, 60, 80, 100, and 120 μM. (B) Test of araBTP as an ATP analogue. Assays were performed as in (A) except 200 μM [α-^{32}P]GTP was used instead. Lane 2 shows normal primer synthesis when 200 μM ATP is included in the assay. Lane 3 lacks ATP and shows significant misincorporation of GTP across from template T. GG dinucleotide is noted to the left of the panel. Lanes 4−8 have increasing araBTP concentrations of 50, 100, 150, 200, and 250 μM and no ATP. (C) Effect of increasing araATP concentrations on primer synthesis (10, 20, 30, 40, and 50 μM). Assays contained 200 μM [α-^{32}P]GTP.

however, this results in potent chain termination along with a dramatic activation of primer initiation.

Even though the bicyclic ring system of benzimidazole more closely resembles a purine than a pyrimidine, araBTP can inhibit pyrimidine polymerization. Under normal experimental conditions, primase greatly prefers to polymerize purine nucleotides and, in fact, requires the 5′-terminal nucleotide of the primer to be a purine (*37*). Thus, even for templates that require primase to polymerize pyrimidines, measuring total product formation cannot distinguish between effects of the inhibitor on purine polymerization and pyrimidine polymerization. To overcome this problem, we used an indirect method to examine the ability of araBTP to compete with a pyrimidine nucleotide (*38*). On the template d(ATC)$_{20}$, primase initiates primer synthesis by generating the pppApG dinucleotide and then incorporates UTP to generate the pppApGpU trinucleotide (as well as longer

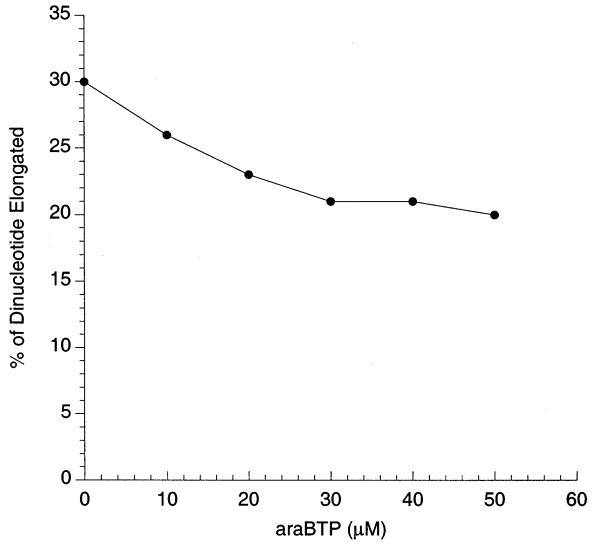

FIGURE 4: Effect of araBTP on pyrimidine incorporation. Assays were performed with 60 μM d(ATC)$_{20}$, 200 μM [γ-^{32}P]ATP, and 200 μM GTP. Data were quantified by calculating products greater than dinucleotide/total products synthesized.

products). To determine if araBTP inhibits UTP polymerization independent of any effects on either ATP or GTP polymerization, we measured the effect of increasing araBTP concentrations on the conversion of the pppApG dinucleotide into pppApGpU trinucleotide and longer products. If araBTP can inhibit UTP polymerization, then the fraction of the dinucleotide converted into the trinucleotide should decrease. Figure 4 shows that increasing the concentration of araBTP decreases the elongation of the dinucleotide, indicating that araBTP can inhibit pyrimidine polymerization by primase.

AraBTP Is a Very Weak Inhibitor of Pol α. Since araBTP cannot form normal Watson−Crick hydrogen bonds with the template base, we anticipated that it should be at most a very weak inhibitor of DNA polymerase activity. To test this prediction, pol α activity was measured in assays containing poly(dT)−oligo(rA) and [α-^{32}P]dATP. AraBTP inhibited pol α extremely weakly (K_i = 600 μM, competitive with dATP). In contrast, araATP potently inhibited pol α activity on this substrate (K_i = 1 μM, competitive with dATP). Thus, ablating the Watson−Crick hydrogen-bonding face of the incoming nucleotide dramatically reduces its ability to interact with the polymerase. Because pol α will incorporate aranucleotides (*24*), we explicitly tested for incorporation of araBTP by pol α. Exogenous primers were annealed to templates where the next correct base for primer extension was either A, G, C, or T. The products were analyzed for percentage of +1 extension in assays containing 1 μM primer template and either 5 μM correct dNTP or 500−1000 μM araBTP (Table 4). These data demonstrate that pol α can incorporate araBTP across from all four natural bases; however, the reaction is extremely inefficient and requires very high concentrations of araBTP.

DISCUSSION

In contrast to DNA polymerases that typically replicate DNA with high fidelity, DNA primase exhibits an extraordinarily low level of fidelity (*18*). Here, we have taken advantage of this difference to develop a potent primase inhibitor, araBTP, that exhibits minimal effects on pol α.

Table 4: Pol α Does Not Readily Polymerize AraBTP

	% of template elongated with		
primer-template[a]	5 μM dNTP	500 μM araBTP	1 mM araBTP
DNA_A	3.3	1.1	1.3
DNA_G	4.9	0.5	0.8
DNA_T	7.6	1.3	1.6
DNA_C	12.9	2.9	3.4

[a] Assays were performed as described under Experimental Procedures in the presence of either DNA_A, DNA_G, DNA_T, or DNA_C substrates where the next template base to be replicated is either A, G, T, or C, respectively. Assays contained either the cognate dNTP for replication of the next correct template base (dTTP, dCTP, dATP, or dGTP, respectively) or araBTP.

This compound combines primase's "preferred" sugar, arabinose, thereby providing the compound with its relatively high potency, with a base that is incapable of forming Watson−Crick hydrogen bonds with the normal bases, thereby preventing DNA polymerases from strongly interacting with it. We also found that primase will accept multiple synthetic base analogues, indicating that it may be possible to use this approach and develop a large number of selective and potentially more potent primase inhibitors.

The key feature of primase that makes it possible to obtain inhibitors that target primase without targeting most DNA polymerases is its relatively low fidelity relative to DNA polymerases. Indeed, three different benzimidazole derivatives of quite different size and chemical properties inhibited primase with similar potency. Surprisingly, however, primase did not readily incorporate either the ribofuranosyl or arabinofuranosyl form of these analogues into the growing primer. These results contrast with primase's promiscuous misincorporation of the natural NTPs (*19*). The lack of incorporation of the benzimidazole derivatives may be a template-dependent effect such that, with other templates, primase might incorporate higher levels of these compounds. Alternatively, these results could indicate that additional specific interactions between the base and enzyme are required for polymerization. For example, Kool and coworkers found that a nitrogen at the 3-position of purine analogues greatly enhances the rate at which some DNA polymerases incorporate purine analogues (*39*). Experiments to test these ideas with primase are in progress.

While the araBTP specifically inhibits primase relative to pol α, in vitro, the question remains as to how specific it will be relative to other nucleotide polymerizing enzymes. DNA replication in vivo involves large multiprotein complexes, which could alter the specificity of an enzyme toward our inhibitors (*40*). Pol δ and ε, the other two DNA polymerases involved in DNA replication, replicate DNA with high fidelity. While we anticipate that compounds such as araBTP will have minimal effects on these enzymes as well as other high-fidelity DNA polymerases, it remains critical to test these other polymerases. In a few cases, DNA polymerases have been shown to polymerize hydrophobic, non-hydrogen-bonding deoxynucleotide analogues across from the natural bases (*47*, *48*). Likewise, RNA polymerases involved in transcription synthesize RNA with high fidelity; hence araBTP should not affect these enzymes. Some DNA polymerases, however, exhibit very low fidelity when synthesizing DNA. For example, pol ι misincorporates nucleotides at frequencies near 0.01 (*1*), close to the frequency with which primase misincorporates nucleotides. Thus, pol ι may well be affected by compounds such as araBTP that cannot form normal Watson−Crick base pairs. However, the arabinose sugar may affect the extent of inhibition since some polymerases discriminate against arabinose (*41*, *42*), and it is unclear how this enzyme interacts with araNTPs.

Primase incorporated araBTP only poorly and even then only into longer products when the primer-template duplex will be most stable. Interestingly, primase misincorporates the natural NTPs most efficiently under conditions where the primer-template is relatively stable (*19*). The enzyme is most likely to misincorporate NTPs onto primers that are rich in G·C base pairs and least likely to misincorporate an NTP during dinucleotide synthesis (i.e., a primer length of 1). These data suggest that polymerization of the araBTP is a slow reaction, and the (in)stability of the primer-template duplex limits the extent of incorporation. After formation of the primase−primer-template−araBTP quaternary complex, the enzyme can either polymerize the araBTP, allow the araBTP to dissociate, or allow the primer (or primer-template) to dissociate. Increasing either the length or the G·C content of the primer would enhance primer-template stability, thereby increasing the likelihood of araBTP polymerization.

Curiously, even though the araBTP is not readily polymerized by primase, araBTP inhibits primase almost as potently as araATP, a compound that primase readily polymerizes and results in chain termination. Normally with nucleotide polymerizing enzymes, one expects compounds that the enzymes incorporate and then cause chain termination to give more potent inhibition than compounds that just bind to the enzyme. The similar potency of inhibition likely results from a unique feature of primase's mechanism: when primase begins to synthesize a primer and the primer dissociates from the enzyme before it reaches at least seven nucleotides in length, primase very rapidly reinitiates primer synthesis rather than dissociating from the template (*6*, *16*). Importantly, this reinitiation process is much faster than the normal initiation process that occurs after primase binds single-stranded DNA. AraBTP appears to primarily inhibit primase by binding to the enzyme to form a primase−template-primer−araBTP quaternary complex, thereby slowing the rate of polymerization, and secondarily allow the dissociation of short primers from the primase−primer-template−araBTP quaternary complex. In contrast, adding araATP to primase assays results in shorter products due to facile araATP incorporation and concomitant chain termination. When this process results in a very short primers, primase will very rapidly reinitiate primer synthesis. Experimentally, this has two consequences: (1) as described earlier, a large increase in the number of initiation events as compared to assays lacking araATP and (2) mitigation of the inhibitory potency because reinitiation is much faster than the normal initiation process.

Incorporation of an aranucleotide into a unit length primer may also tend to mitigate the apparent severity of inhibition due to the absence of a 2′-hydroxyl in the ribo configuration. Normally, when primase synthesizes a primer at least seven nucleotides long, further primase activity is negatively regulated until pol α elongates the primer (*16*). While the actual mechanism of reactivation remains obscure, the minimal requirement for reactivation is dNTP polymeriza-

tion. If araNTP polymerization by primase mimics the effects of dNTP polymerization by pol α, then incorporation of araNTPs would eliminate the negative regulation of primase that normally occurs after unit length primer synthesis. Interestingly, primase interacts very similarly with primers containing either a dNMP or an araNMP at the 3′ terminus; it will not further elongate either primer.

Both these and previous studies have demonstrated the importance of the 2′-hydroxyl of the nucleotide sugar (*23*, *24*, *43*). A 2′-hydroxyl dramatically enhances the ability of primase to polymerize a nucleotide although, surprisingly, the actual stereochemistry of the hydroxyl is not critical. Indeed, primase actually prefers NTPs with the 2′-hydroxyl in the unnatural ara configuration. Removing the 2′-hydroxyl results in a compound that primase does not readily polymerize and is a very weak primase inhibitor. Curiously, adding a methyl group at the 2′-position, regardless of the stereochemistry of the 2′-hydroxyl, resulted in compounds that were not detectably polymerized by primase, although they still bound reasonably well. These data suggest that the region of primase around the 2′-position is hydrophilic, and a hydroxyl in either configuration can interact with these hydrophilic groups. In contrast, a methyl group in either configuration or the presence of just two H's at the 2′-position generates energetically unfavorable interactions with these hydrophilic groups and, consequently, interferes with NTP binding and/or polymerization. Alternatively, accommodating the methyl group may result in the placement of a 2′-hydroxyl in a position where it cannot make critical interactions with hydrophilic groups on the enzyme. Since the 2′-methyl-NTPs still bound primase quite well, these interactions are important for polymerization but not for binding.

In addition to providing the RNA primers needed to synthesize all new strands of DNA, primase plays a critical, but not understood, role in coupling DNA replication with the DNA checkpoint (*44*−*46*). In response to DNA damaging agents, yeast normally delay entry into S phase. However, mutations in the primase p49 subunit can eliminate this delay (*44*). Furthermore, these mutations are lethal in combination with mutations in two repair-checkpoint genes (RAD53 and MEC1). Studies by Michael et al. using *Xenopus* extracts indicate that the critical species for checkpoint activation is the stable, pol α−primase−primer-template species that is formed after primase activity (*45*). Having available highly specific primase inhibitors should facilitate further studies delineating the role of primase in the DNA checkpoint.

In these studies, we have focused solely on purine analogues due to primase's preference for pyrimidine-rich templates under normal experimental conditions. With the exception of the nucleotide that will become the primer 5′ terminus, however, primase readily incorporates pyrimidines into the growing primer chain (*37*). In combination with the data showing that araBTP can inhibit UTP polymerization, this observation indicates that pyrimidine analogues may also potently inhibit primase. Nucleotides bearing benzimidazole, dichlorobenzimidazole, and nitrobenzimidazole bases all bound primase quite well and to a similar extent, suggesting that primase will accept a wide variety of purine analogues. Thus, it may be possible to attach either chemically reactive or fluorescent groups onto the nucleotide to provide novel probes for studying catalysis by the pol α−primase complex. Studies to test these hypotheses are in progress.

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BI026468R