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Polymerization of the triphosphates of AraC, 2′,2′-difluorodeoxycytidine (dFdC) and OSI-7836 (T-araC) by human DNA polymerase α and DNA primase

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Abstract

OSI-7836 (4′-thio-araC, T-araC) is a nucleoside analogue that shows efficacy against solid tumor xenograft models. We examined how the triphosphates of OSI-7836 (T-araCTP), cytarabine (araCTP), and gemcitabine (dFdCTP) affected the initiation of new DNA strands by the pol α primase complex. Whereas dFdCTP very weakly inhibited primase, both T-araCTP and araCTP potently inhibited this enzyme. Primase polymerized T-araCTP and araCTP more readily than its natural substrate, CTP, and incorporation resulted in strong chain termination. dFdCTP, araCTP, and T-araCTP inhibited pol α competitively with respect to dCTP. When exogenously added primen-templates were used, pol α incorporated all three analogues into DNA, and incorporation caused either weak chain termination (dFdCTP), strong termination (araCTP), or extremely strong termination (T-araC). Furthermore, pol α polymerized T-araCTP only nine-fold less well than dCTP, whereas it polymerized araCTP and dFdCTP 24- and 83-fold less well, respectively. The presence of these three analogues in the template strand resulted in significant pausing by pol α, although the site and severity of pausing varied between the analogues. During the elongation of primase-synthesized primers, a reaction that is thought to mimic the normal sequence of events during the initiation of new DNA strands, pol α polymerized all three compounds. However, incorporation of araCTP and dFdCTP resulted in minimal chain termination, while incorporation of T-araCTP still caused extremely strong termination. The implications of these results with respect to how these compounds affect cells are discussed.

Keywords: Gemzar®; (gemcitabine; dFdC; 2′2′-difluorodeoxycytidine); 1-beta-D-arabinofuranosylcytosine (cytosine arabinoside, araC); OSI-7836 (T-araC, 4′-thio-araC, 4′-thio-beta-D-arabinofuranosylcytosine); polymerase alpha; human; DNA primase

Nucleoside analogues are an important class of cancer chemotherapeutics that are used for the treatment of various malignancies. These compounds can target DNA replication both directly and indirectly. For example, 5-fluorouracil indirectly targets replication by altering dNTP levels, while cytarabine triphosphate (araCTP) directly affects DNA synthesis by interacting with DNA polymerases to slow the rate of polymerization. Of particular interest to our group was the nucleoside analog OSI-7836 (4′-thio-araC, T-araC), a structural analog of araC (Fig. 1) currently in Phase I development for the treatment of solid tumors [1-3]. T-araC is more efficacious in the treatment of solid tumor xenografts than either gemcitabine or araC, a compound that exhibits little activity ([4-7], and unpublished data). Activity of T-araC may be dependent on

Abbreviations: araC, cytosine arabinoside,1-β-D-arabinofuranosylcytosine; dFdC, gemcitabine,2′,2′-difluorodeoxycytidine; OSI-7836, 4-thio-araC,T-araC,4′-thio-beta-D-arabinofuranosylcytosine; BSA, bovine serum albumin; TBE, Tris-borate-EDTA; Tris, tris[hydroxymethyl]aminomethane; EDTA, ethylenediaminetetraacetic acid; pol α, human DNA polymerase alpha; pol α; primase, human polymerase alpha primase complex

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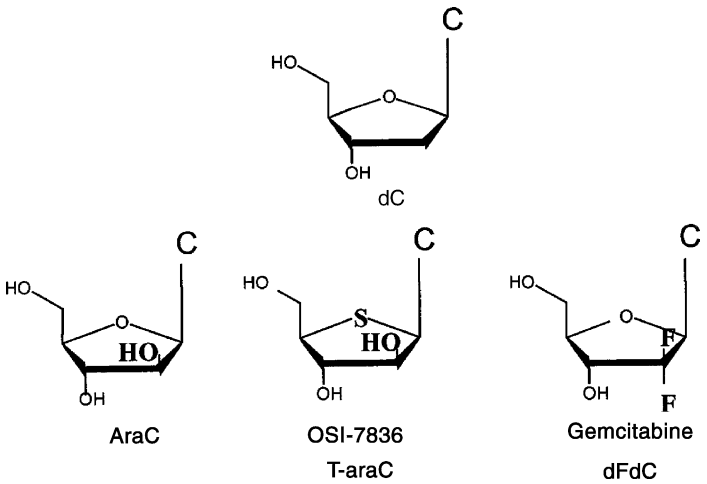

Fig. 1. Structures of the normal dC and the modified nucleosides, araC, OSI-7836, and dFdC. The differences as compared to dC of each modified nucleoside are in bold and larger font.

conversion to the triphosphate form inside the cell, which has been shown to occur over a relatively long period of time as compared to dFdC or araC [5]. Therefore, its efficacy in solid tumor xenografts may be due to this slow conversion to the triphosphate form and subsequent longer availability of this tripohosphate for use in DNA replication [5]. Similar to other nucleoside analogues, T-araC is incorporated into DNA, suggesting that its antitumor activity most likely involves its effects on DNA replication and/or its incorporation into DNA. Eukaryotic DNA replication involves a host of different proteins that include at least four different nucleotide polymerizing enzymes, pol α, pol δ, pol ε, and DNA primase [8,9]. DNA pol α and primase form a tightly bound complex that is responsible for initiating the synthesis of all new strands of DNA. On single-stranded DNA, primase synthesizes a short RNA primer, approximately 8–10 nucleotides long, and then moves intramolecularly to the pol α active site [10]. Pol α then elongates this RNA primer via dNTP polymerization and polymerizes 25 or so dNTPs before dissociating. This product is further elongated by pol δ and/or pol ε, which perform the bulk of both leading and lagging strand synthesis [9].

Currently, there are no data on the effects of T-araC on pol α catalyzed elongation of DNA primer:templates; on how araCTP, dFdCTP, or T-araC affect primase or the pol α-primase coupled reactions; or on the effects of an incorporated (T-araC) on translesion synthesis. Therefore, to better understand the potential mechanisms of T-araC antitumor activity, we examined the effects of these compounds on some of the individual components of DNA replication primarily responsible for the initiation of DNA synthesis. Specifically, we compared: (a) the utilization of araCTP, dFdCTP and T-araCTP by polymerase α as substitutes for normal nucleotides in DNA synthesis using defined primentemplates; (b) the effects of these analogues on the pol α-primase coupled reaction and; (c) the extent of translesion synthesis by pol α when one of these lesions is in the template strand.

1. Materials and methods

1.1. Materials

Cytosine arabinoside triphosphate (araCTP) was obtained from Sigma Chemical Company. dATP, dGTP, dTTP, and dCTP were obtained from United States Biochemicals. T-araCTP was generously provided by Southern Research Institute (SRI). AraC phosphoramidite was purchased from Glen Research. Synthetic oligonucleotides of defined sequence that contained only the four natural deoxynucleotides were obtained from either Oligos, etc. or Operon Technologies, Inc. The 35 bp araC template and 35 bp normal template were synthesized by Qiagen/Operon Technologies. The 35 bp templates containing dFdC or T-araC and the 20 bp primer were synthesized at OSI Pharmaceuticals using an Applied Biosystem Incorporated synthesizer. Acetylated BS A was obtained from Promega. Polymerase α was expressed and purified using a baculovirus expression system [11]. The cloned human DNA pol α-primase (four-subunit complex) and the two subunit primase complex were expressed and purified as previously described [12,13].

1.2. Synthesis of T-araC phosphoramidite (Fig. 2)

The OSI-7836 CE-phosphoramidite (Fig. 2) monomer was synthesized by analogy to the preparation of araC methyl-phosphoramidite by Beardsley, et al. [14].

1.3. Synthesis of dFdCphosphoramidite

dFdC phosphoramidite was synthesized from gemcitabine following previously published methods [15].

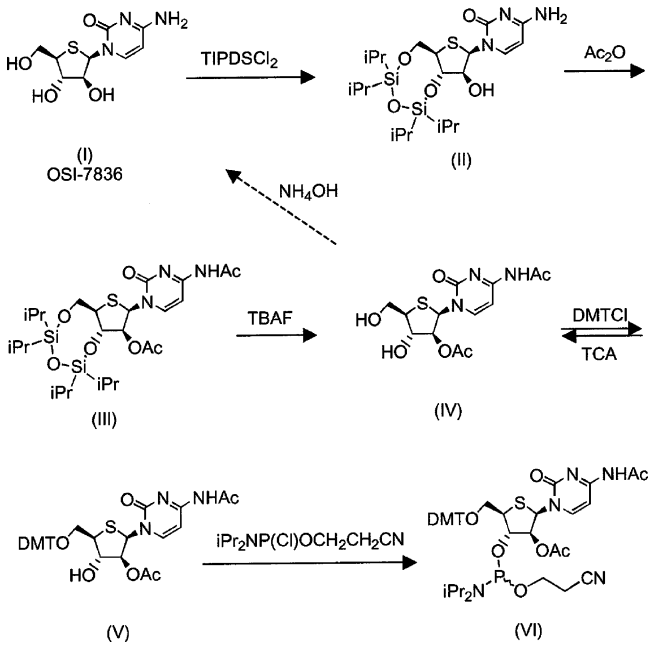

Fig. 2. Synthesis of OSI-7836 phosphoramidite. $TIPDSCl_2$: 1,3-dichloro-1,1,3,3-tetraisopropyldisilane; Ac_2O: acetic anhydride; NH_4OH: ammonium hydroxide; TBAF: tetrabutyl ammonium fluoride; DMTCI: 4.4′-dimethoxytrityl chloride; TCA: trichloroacetic acid; $IPr_2NP(Cl)OCH_2CH_2CN$: 2-cyanoethyl diisopropylchlorophosphoramidite.

1.4. Primers and templates

The primer and templates used for the pol α assays are shown in Table 1. All reactions used to determine the K_M and V_{max} for the enzymes were running start reactions, with the modified nucleotide being the third nucleotide incorporated from the end of the primer. For the DNA #1, two dAMPs are inserted before the insertion of the modified nucleotide. Those primers and templates used in experiments to determine the ability of pol α to polymerize over an incorporated, modified nucleoside (translesion synthesis, DNA #2) are also shown in Table 1.

1.5. Synthesis and digestion of oligonucleotides

Synthesis of all oligonucleotides was performed on an ABI Synthesizer using standard reagents and coupling times with exceptions for phosphoramidites of araC, dFdC and T-araC. For these modified nucleotide phosphoramidites, the coupling time was elongated to 6 min. One exception was the synthesis of the araC-containing 35 mer (Table 1), which was synthesized by Qiagen-Operon Technologies, using their conditions. Oligonucleotides containing the normal dC or any of the modified nucleosides were digested to nucleosides using DNAse I, snake venom phosphodiesterase, and bacterial alkaline phosphatase and standard reaction conditions [16]. The nucleosides were then separated by HPLC using a Supelco C-18-S reverse phase column, an Hewlett Packard HPLC with a diode array detector, and a isocratic 50 mM KPO_4 (pH 6.5), 8% MeOH buffer.

1.6. Labeling and annealing

The primer (1.0 nmols) was end-labeled using 120 μCi of [γ-^{32}P]dATP (3000 Ci/mmol, NEN Life Sciences) and 50 U of T_4 polynucleotide kinase (10 U/μl, Boehringer Mannheim) in a final volume of 100 μl of 1× kinase buffer (Boehringer Mannheim). After 30 min at 37 °C the unincorporated nucleotides were removed using Centri Sep spin columns (Princeton Separations).

Labeled primer and template (1:1.5) were annealed in H_2O at a primer concentration of 5–10 μM.

For K_M and V_{max} determinations, the samples were incubated at 90 °C for 5 min in a heat block. The heat block was then turned off and the samples were allowed to cool slowly to room temperature.

For translesion polymerization experiments, the samples were incubated at 70 °C for 5 min in a heat block, placed on ice for 5 min and then placed at room temperature overnight. The annealed samples were stored at −20 °C until use, but not past 3 weeks post end-labeling.

1.7. Incorporation reaction conditions

To determine the K_M and V_{max} for the various modified nucleosides, the annealed DNA #1 (Table 1) was incubated with human polymerase α under the following conditions: Five pmol of a specific primer/template in a total reaction volume of 5.0 μl were incubated at 37 °C for 8 min in 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithio-

Table 1
Sequences of primers and templates

Primer/template	
DNA #1	5′-GCCGCATAATTCCAACAAAG 3′-CGGCGTATTAAGGTTGTTTCTT **G** CCAAGCTCGTAC
DNA #2	5′-CCCAAACGATATTCACAAAG 3′-GGGTTTGCTATAAGTGTTTCAA **X** GGAAGCTCGTAC
$d(T_3G)_{15}$	TTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTGTTTG
$d(C_2G)_{20}$	CCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCG
$d(C_3G)_{15}$	CCCGCCCGCCCGCCCGCCCGCCCGCCCGCCCGCCCGCCCGCCCGCCCGCCCG

G indicates site of incorporation of dCTP, araCTP dFdCTP or OSI-7836TP (T-araCTP) across from this base to determine K_M and V_{max}. **X** indicates the presence of dC, araC, dFdC, or T-araC in the DNA strand. Assays were performed as described under Section 1.

threitol, and 1 μg/mL acetylated BSA. Each reaction also contained: 0.1 units pol α, 80 μM of dATP as the running start nucleotide, and various concentrations of the normal or modified nucleotide.

Methods developed by Boosalis, 1987 [17] to measure the K_M and V_{max} for each of these nucleotides using pol α were utilized in these studies.

1.8. Elongation reactions

Elongation reactions were performed as for the incorporation reactions, but included appropriate dNTPs to allow elongation past the site of araC, dFdC, or T-araC incorporation.

1.9. Translesion synthesis conditions

To determine translesion synthesis, DNA #2 (Table 1) was annealed as above. HPLC analysis of these oligos indicated the presence of the normal or modified nucleoside was present in the correct molar ratios (data not shown). The reaction conditions for translesion synthesis using pol α were the same as those for incorporation reactions, except that each reaction mixture contained a concentration range of 0.1–400 μM of a mixture of all four deoxynucleotides. All reactions were quenched by addition of a 2× dye stock solution consisting of 98% formamide, 20 mM EDTA, 0.025% SDS, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol, and 0.05% (w/v) orange G. Products were separated by denaturing polyacrylamide gel electrophoresis (16% acrylamide, 8 M urea in 1× TBE.) The amount of radiolabeled products was visualized using an Amersham Storm 840 Imager and quantified using the ImageQuant software program.

1.10. Primase assays

Reactions (10 μl) were performed as previously described and typically contained 50 mM Tris–HC1, pH 7.9, 5 mM $MgCl_2$, 60 μM DNA template (total nucleotide), 0.05 mg/mL BSA, 1 mM dithiothreitol, and 100–800 μM [α-^{32}P]NTPs [18]. 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). The oligonucleotides used in these assays are shown in Table 1.

The relative V_{max}/K_M for polymerization of CTP versus T-araCTP was determined as described previously [19]. Assays contained the template $d(C_2G)_{20}$, 200 μM NTPs [α-^{32}P]GTP], and increasing concentrations of either araCTP or T-araCTP. Since incorporation of these compounds result in chain termination, the V_{max}/K_M for polymerization of CTP versus T-araCTP could be determined using the methods described in [19].

1.11. Primase coupled pol α assays

Reactions (10 μL) were performed as previously described and typically contained pol α-primase, 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 60 μM template DNA (total nucleotide), 0.1 mg/mL BSA, 0.2 mM NTPs, and 5–10 μM [α-^{32}P]dNTPs [13]. Reactions were initiated by adding enzyme and incubated at 37 °C for 1 h. After quenching the reactions with gel loading buffer, products were separated and analyzed as described above for the primase assays. The oligonucleotides used in these assays are shown in Table 1.

2. Results

2.1. DNA primase

In order to measure the effects of cytosine-based nucleotide analogs on primase, we designed the templates

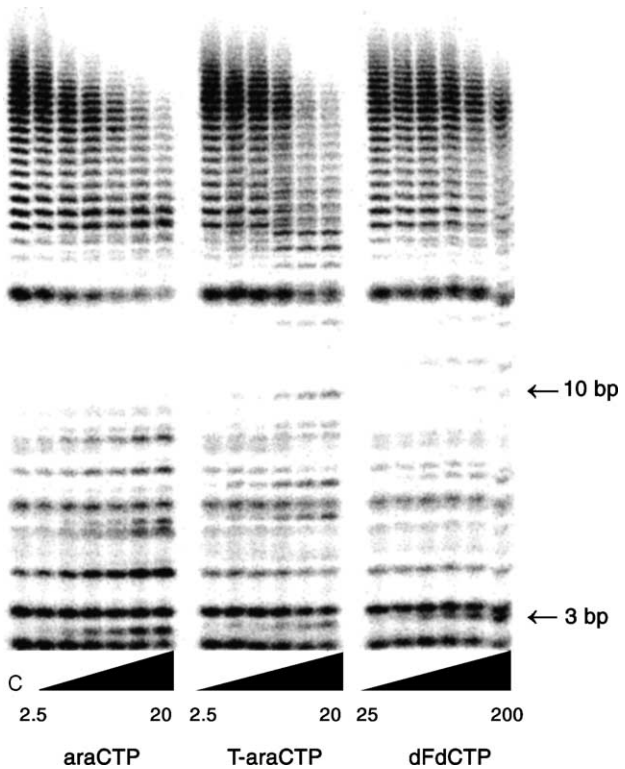

Fig. 3. Inhibition of primase activity by araCTP, T-araCTP and dFdCTP on the template $d(C_3G)_{15}$. Assays were performed as described under Section 1. Briefly, the 10 μl reactions were incubated for 1 h at 37 °C and contained 50 mM Tris–HCl, pH 7.9, 5 mM $MgCl_2$, 60 μM $d(C_3G)_{15}$ template (total nucleotide), 0.05 mg/mL BSA, 1 mM dithiothreitol, and 200 μM [α-^{32}P]ATP [18] and contained 200 μM NTPs and; no further addition (C); 2.5, 5, 7.5, 10, 15, or 20 μM araCTP or T-araCTP, or; 25, 50, 75, 100, 150, or 200 μM dFdCTP. The lane marked C contained no analogue. The length of products is noted to the right of the image.

$d(T_3G)_{15}$, $d(C_2G)_{20}$, and $d(C_3G)_{15}$ (Table 1). Each of these templates contain either 2 or 3 pyrimidines in order to accommodate primase's strong preference to initiate primer synthesis opposite pyrimidines, followed by a deoxyguanylate to measure the effects of the analogues under conditions where it could form a correct base-pair. Fig. 3 shows that $d(C_3G)$is supports high levels of primase activity in the presence of the cognate NTPs (lane C). Similar levels of primase activity were observed on $d(T_3G)$is and $d(C_2G)_{20}$ (data not shown).

AraCTP, T-araCTP, and dFdCTP inhibited primase activity on the templates $d(C_3G)_{15}$ and $d(T_3G)_{15}$, but with vastly different potency (Fig. 3 and data not shown). Whereas low concentrations of T-araCTP potently inhibited primase, much higher concentrations of dFdCTP were required to obtain similar effects. A detailed kinetic analysis of inhibition by these three analogues revealed that they inhibited primase activity competitively with respect to NTPs and T-araCTP was the most potent inhibitor (Table 2).

The data in Fig. 3 also indicate that primase readily polymerizes araCTP, T-araCTP, and dFdCTP. Including either compound in assays results in products of altered mobility as compared to assays containing only the natural NTPs. Significant incorporation of araCTP and T-araCTP occur even when the concentration of analogue is 10-fold lower than the NTP concentration, indicating that both compounds are very good substrates for primase. In contrast, primase only polymerizes dFdCTP at relatively high concentrations.

Primase incorporates araCTP and T-araCTP opposite template deoxyguanylates, and incorporation results in strong chain termination. In assays containing $d(T_3G)_{15}$ and only ATP, primase synthesized products 2 and 3 nucleotides long (Fig. 4), indicating that the enzyme encountered a template dG and then dissociated. Upon adding either araCTP or T-araCTP, a new product that exhibited altered electrophoretic mobility appeared, indicating that primase polymerized the analogues opposite the deoxyguanylate. Since ATP is present in all reactions, these data also indicate that polymerization of araCTP and T-araCTP results in strong chain termination. If incorporation of araCTP and T-araCTP did not result in strong chain termination, large amounts of longer products should have been synthesized since the assays contained the next correct NTP (ATP). Since only trace amounts of longer products are synthesized, it is concluded that incorporation of araCTP and T-araCTP results in strong chain termination.

Table 2
Inhibition of primase by NTP analogues

Template	K_i (μM)		
	dFdCTP	AraCTP	T-araCTP
$d(C_3G)_{15}$	300 ± 60	50 ± 25	25 ± 10
$d(T_3G)_{15}$	240 ± 40	60 ± 30	20 ±10

Assays were performed as described under Section 1. Briefly, 10 μl reactions containing 50 mM Tris–HCl, pH 7.9, 5 mM $MgCl_2$, 60 μM DNA template, 0.05 mg/mL BSA, 1 mM dithiothreitol, 200 μM [α-^{32}P]GTP], pol α primase and increasing concentrations of either dFdCTP, araCTP or T-araCTP were incubated at 37 °C for 1 h. The reactions were analyzed by PAGE and phosphoimagery.

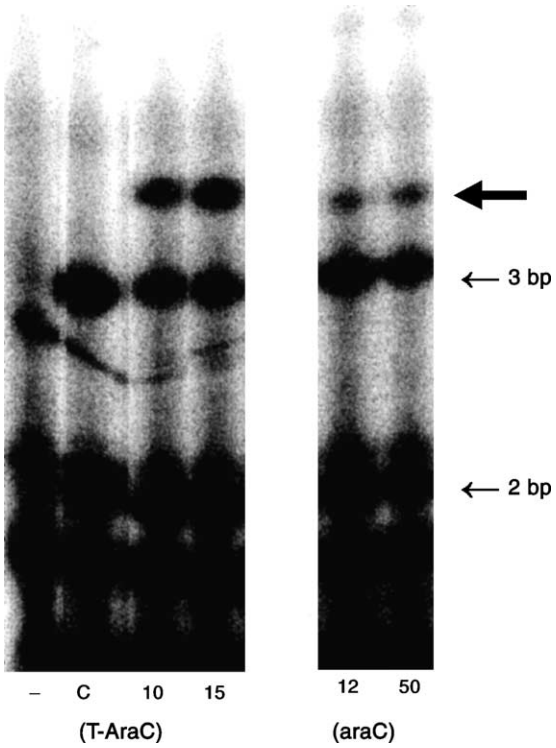

Fig. 4. Incorporation of T-araCTP and AraCTP by primase. Assays were performed as described under, Section 1. Briefly, the 10 μl reactions were incubated for 1 h at 37 °C and contained 50 mM Tris–HCl, pH 7.9, 5 mM 60 μM $d(T_3G)_{15}$ template (total nucleotide), 0.05 mg/mL BSA, 1 mM dithiothreitol, and 200 μM [α-^{32}P] ATP, and the indicated concentrations of either T-araCTP or araCTP. The lane marked (–) lacked enzyme and the lane marked (C) contained no analogue. The mobility of the dinucleotide and trinucleotide are noted on the side of the gel. The large arrow notes the product due to incorporation of the analogue.

The fact that incorporation of araCTP and T-araCTP resulted in chain termination allowed us to measure how efficiently primase polymerizes T-araCTP as compared to CTP for the template $d(C_2G)_{20}$. In the presence of CTP and GTP, primase readily synthesizes long primers, and almost no trinucleotide is formed. When either T-araCTP or araCTP is also present in the reaction, primase now has a choice after generating the pppGpG dinucleotide. It can either incorporate CTP, which will lead to the synthesis of even longer products via continued NTP polymerization, or it can incorporate the analogue, which will result in chain termination and consequent accumulation of a trinucleotide. Thus, by measuring the amount of analogue-terminated trinucleotide synthesized at various ratios of CTP/analogue, the relative V_{max}/K_M for CTP versus analogue polymerization can be obtained [18]. Primase preferred to polymerize T-araCTP rather than CTP by a factor of 1.7, and preferred to polymerized araCTP rather than CTP by a factor of 1.4.

2.2. DNA polymerase α

Initial studies focused on determining the effects of araCTP, dFdCTP, or T-araCTP on polymerization by pol α. In each case, polymerization was measured using the "running start" assay developed by Boosalis et al. [17]. In accordance with previous studies [20,21], araC insertion resulted in very strong chain termination; incorporation of dFdC caused a major pause at the site just after the incorporation of dFdC; but elongation could occur in the presence of elevated levels of dGTP and dTTP (Fig. 5). Like araC, incorporation of T-araC caused very strong chain termination at the site of incorporation (Fig. 5).

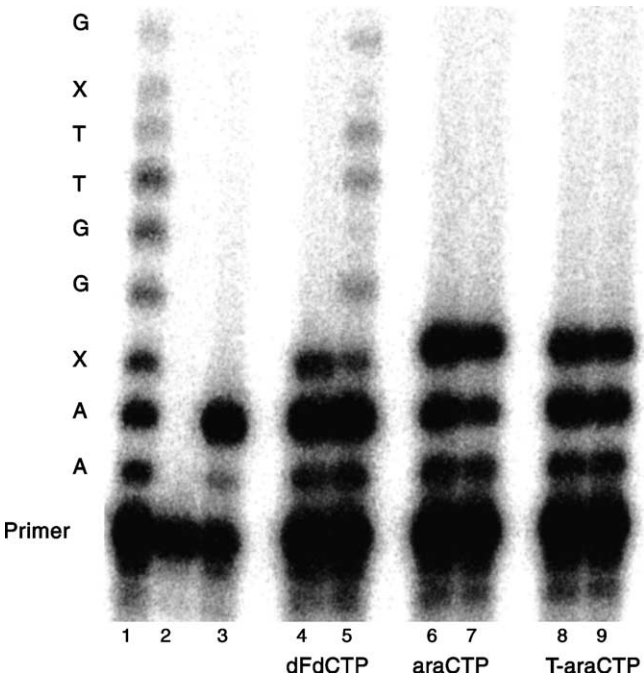

Fig. 5. Ability of pol α to continue synthesis following incorporation of dC, dFdC, T-araC, or AraC reactions containing DNA #1 (Table 1) for insertion of dCTP, dFdCTP, OSI-7836TP or araCTP across from the third base (G) following the end of the primer. Reaction conditions are described in Section 1. Briefly, 5 pmol of 5′-end labeled primer/template were incubated at 37 °C for 8 min in 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 1 μg/mL acetylated BSA. Each reaction also contained: 0.1 units pol α, 25 μM of dATP as the running start nucleotide, 50 μM of the normal dCTP or modified nucleotise and 100 μM of dGTP and 100 μM of TTP for elongation past the site of incorporation of the modified nucleoside. The sequence of the synthesized strand is shown on the left-hand side of the figure. The "X" represents position of incorporation of a modified nucleotide across from a G in the template strand. Lane 1: control synthesis using insertion of the normal Watson–Crick basepairing nucleotide (dC) across from the G in the template strand. Lane 2: complete reaction mixture without the pol α. Lane 3: reaction mixture containing only 25 μM of dATP, the running start nucleotide. Lanes 4, 6, and 8: reaction mixture containing only 25 μM dATP and 50 μM of dFdCTP, T-araCTP, or araCTP; showing the ability of pol α to incorporate the lesion into the DNA. Lanes 5, 7, and 9: reaction mixture containing 25 μM dATP, 100 μM each of dGTP and dTTP and either 50 μM of dFdCTP, T-araCTP or araCTP with Lane 5 showing the ability of pol α to incorporate dFdC and then continue synthesis, whereas, Lanes 7 and 9 indicate the chain terminating effect of incorporation of T-araC (Lane 7) or araC (Lane 9).

Table 3
K_M, V_{max}, and mutation frequency for pol α on DNA #1 (Table 1)

Compound	K_M	V_{max}	V_{max}/K_M	Misinsertion frequency
dCTP	0.64 ± 0.2	1.5 ± 0.5	2.34	–
araCTP	2.03 ± 0.4	0.2 ± 0.04	0.098	0.042
dFdCTP	3.4 ± 0.6	0.1 ± 0.05	0.029	0.012
OSI-7836TP	1.72 ± 0.15	0.47 ± 0.36	0.27	0.12

Methods developed by Boosalis, 1987 [17] were used to determine the K_M and V_{max} for each nucleoside as described in Section 1. Briefly, the annealed DNA #1 (Table 1) was incubated in a total volume of 5 μl at 37 °C for 8 min. Using 0.1 units pol α, 80 μM of dATP as the running start nucleotide, and various concentrations of the normal or modified nucleotide. Misinsertion frequency = V_{max}/K_M (modified)/V_{max}/K_M (normal).

Table 3 summarizes the steady-state kinetic parameters and misinsertion frequencies (MF) for all nucleotides tested. All three compounds exhibit increased K_M and decreased V_{max} as compared to dCTP. When compared to control dCTP values, T-araCTP (T-araCTP) had the least effect on either the K_M (2.7-fold increase) or the V_{max} (3.2-fold decrease). For araC, there was a 3.2-fold increase in the K_M and a 7.5-fold decrease in the V_{max}. For dFdC there was a 5.3-fold increase in the K_M and a 15-fold decrease in the V_{max}. The calculated misinsertion frequencies for polymerase α indicated that the order of misinsertion is T-araC > araC > dFdC.

2.3. Primase-coupled polymerase α activity

We examined the effects of araCTP, T-araCTP and dFdCTP on the pol α-catalyzed elongation of primase-synthesized primers (i.e. primase-coupled pol α activity). This reaction is of major interest because it recapitulates the primary function of pol α-primase in vivo, the initiation of new DNA strands. Initially, the ability of each compound to inhibit the synthesis of elongated primers was measured in the presence of the template $d(T_3G)_{15}$, ATP, CTP, dATP and dCTP.

All three compounds inhibited product synthesis, but with very different potency (Fig. 6). In assays containing 500 μM NTPs and 10 μM dNTPs, the IC_{50}s were 2.1 μM (araCTP), 0.65 μM (T-araCTP), and 140 μM (dFdCTP). In addition, each compound had very different effects on the size distribution of products. As compared to products generated in control reactions, T-araCTP resulted in significantly shorter products, araCTP had only very slight effects on product length while dFdCTP had no detectable effect (data not shown).

In order to further explore polymerization of each analogue, we examined the effects of the analogues on primase-coupled pol α activity on the template $d(T_3G)_{15}$ in the absence of dCTP. Under these conditions, primase synthesizes a primer and pol α can polymerize [α-^{32}P]dATP opposite the template thymidylates. Upon encountering a template deoxyguanylate, for which pol α lacks the required dCTP, the enzyme complex will

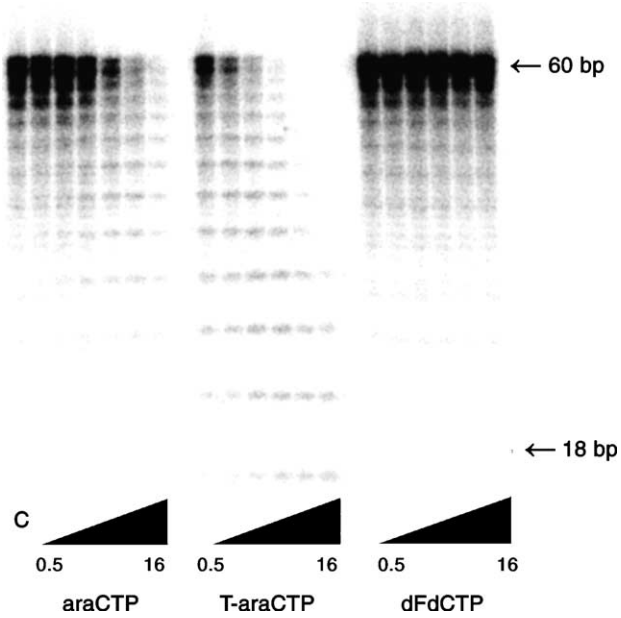

Fig. 6. Inhibition of primase-coupled pol α activity by araCTP, T-araCTP, and dFdCTP on the template $d(T_3G)_{15}$ Assays were performed as described under Section 1. Briefly, 10 μl reactions were incubated for 1 h at 37 °C and contained pol α-primase, 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 60 μM $d(T_3G)_{15}$ template (total nucleotide), 0.1 mg/mL BSA, 5–10 μM [α-^{32}P]dATP, 0.2 mM NTPs [13] and either no inhibitor (C), or 0.5, 1,2,4, 8 or 16 μM of the indicated compound. The lane marked C contained no analogue. The lengths of products are noted to the right of the image.

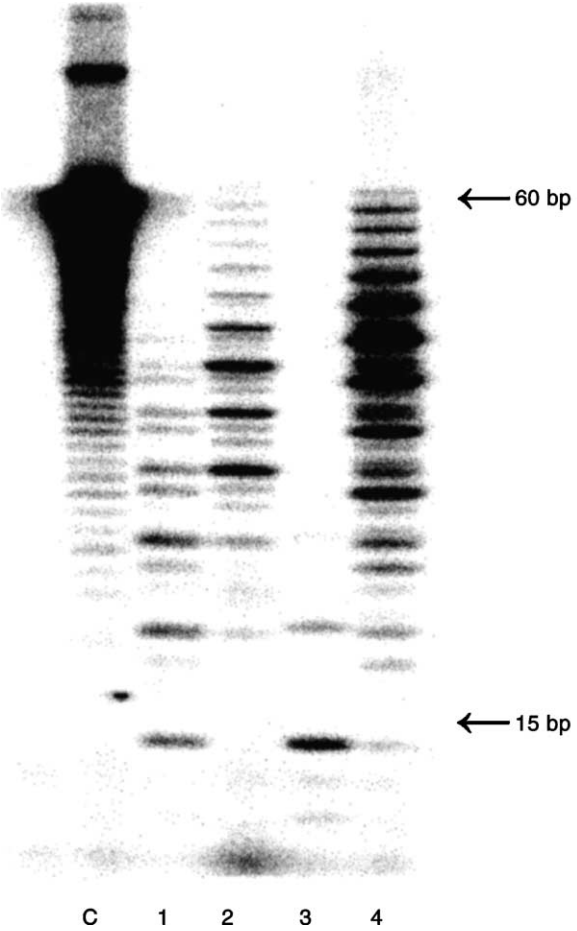

Fig. 7. Incorporation of dFdCTP, araCTP and T-araCTP during primase-coupled pol α activity on the template $d(T_3G)_{15}$. Assays were performed as described under Section 1. Briefly, 10 μl reactions were incubated for 1 h at 37 °C and contained pol α-primase, 50 mM Tris–HCl, pH 7.5, 5 mM MgCb, 60 μM $d(T_3G)_{15}$ template (total nucleotide), 0.1 mg/mL BSA, 5–10 μM [α-^{32}P]dATP, 0.2 mM NTPs [13]. Assays contained ATP, CTP, dATP and either C (10 μM dCTP): (1) no further addition; (2) 10 μM araCTP; (3) 10 μM T-araCTP; or (4) 10 μM dFdCTP.

polymerize CTP [13,22]. Fig. 7 shows that in the presence of both dATP and dCTP, products approximately 60 nucleotides long are synthesized. Omitting dCTP results in products up to approximately 30 nucleotides long, 8–10 of which comprise the primer. Adding 10 μM araCTP to the assays results in products of greater length and of altered electrophoretic mobility, indicating that araCTP was polymerized opposite the template deoxyguanylates and that chain termination did not occur. Including dFdCTP markedly increased product length to near that of those synthesized in the assays containing dCTP, indicating that pol α incorporated dFdCTP and that incorporation did not inhibit subsequent polymerization events. In contrast, adding T-araCTP resulted in significantly shorter products of altered electrophoretic mobility, indicating that pol α-primase incorporated the analogue and chain termination ensued. Similar results were obtained on the template $d(C_3G)_{15}$ upon the omission of dCTP (data not shown).

2.4. Translesion synthesis by DNA polymerase α

In an effort to elucidate the effects of internal incorporation of araC, dFdC, or T-araC, the ability of pol α to polymerize dNTPs opposite templates containing either araC, T-araC or dFdC at the third base following the end of a DNA primer was determined (Table 1). There was slight pausing of synthesis across from the dFdC lesion compared to control at 10–400 μM dNTP (4–8% versus 3–4% pausing for control; Fig. 8) and, synthesis past the dFdC lesion was similar to the control from 50 to 400 μM dNTP (2.5–5% pausing). There was pronounced pausing of translesion synthesis at the base just before the araC lesion at dNTP concentrations of 2 μM and above (20–30% versus 12–20% pausing for control; Fig. 8), and elongation was concentration dependent.

For the T-araC template, pausing of synthesis occurred across from T-araC and was concentration dependent from 10 to 400 μM dNTP (5–14% versus 3–4% pausing for control; Fig. 8). The amount of elongation past the T-araC lesion was reduced as compared to all other templates (2% as compared to 4–5% elongation; Fig. 8).

3. Discussion

We have examined the effects of dFdCTP, araCTP and T-araCTP on human DNA primase and pol α. T-araCTP was

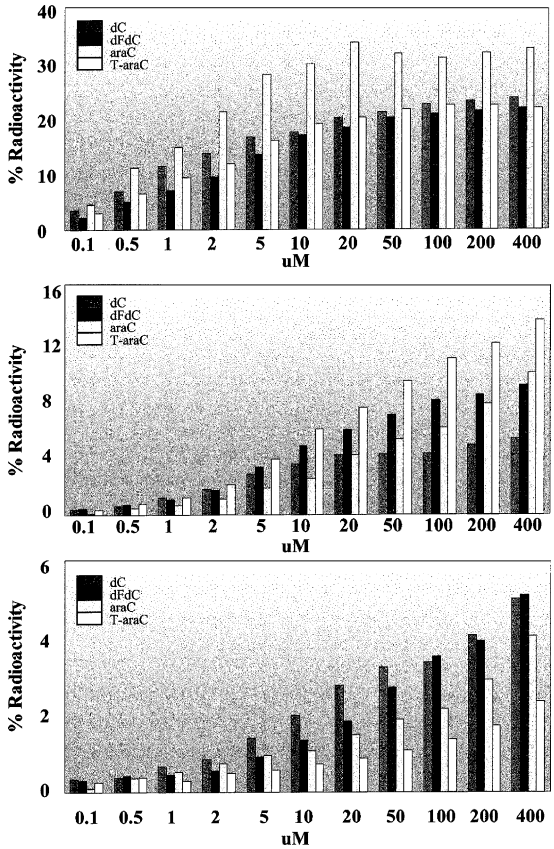

Fig. 8. Translesion synthesis by pol α for control and lesion-containing DNA translesion synthesis as described in Section 1 using DNA #2 from Table 1. Briefly, five pmol of 5′-end labeled primer/template were incubated at 37 °C for 8 min in 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 1 μg/mL acetylated BSA. Each reaction also contained: 0.1 units pol α, and 0.1–400 μM of a mixture of all four deoxynucleotides. The percent of radioactivity incorporated is depicted as a function of the concentration of an equimolar mixture of dGTP, dATP, dCTP, dTTP: (A) incorporation of the first two bases prior to the position of the control (blue), dFdC (red), araC (It. blue), T-araC (yellow) in the template; (B) Incorporation across from the control (blue), dFdC (red), araC (It. blue), T-araC (yellow) in the template (3rd bp); and (C) Incorporation past the site of control (blue), dFdC (red), araC (It. blue), T-araC (yellow) in the template.

the most potent inhibitor of primase, and incorporation of T-araCTP resulted in chain termination. Similarly, T-araCTP was the most potent inhibitor of pol α. Whereas polymerization of dFdCTP and araCTP resulted in moderate and strong chain termination, respectively, polymerization of T-araCTP resulted in extremely strong chain termination. This was true both when pol α elongated exogenously added primentemplates as well as primase-generated primentemplates. When present in the template, all three nucleotide analogues caused pausing by pol α. However, the strength and position of the pause varied for each analogue.

T-araCTP was the strongest inhibitor of primase, while dFdCTP was the weakest inhibitor. Compared to araCTP, primase polymerized T-araCTP approximately 20% more readily than araCTP, but incorporation resulted in stronger chain termination. Presumably, the more efficient polymerization and chain termination reflects the altered chemical properties of sulfur as compared to oxygen at the 4′-position. It has previously been demonstrated that although primase polymerizes both araATP and 2-fluoro-araATP more efficiently than ATP, incorporation results in very strong chain termination [19,23,24]. The similarity of the effects of three different araNTPs indicates that NTPs containing a 2′-hydroxyl in the ara configuration should generally be both good substrates and potent chain terminators for primase.

dFdCTP interacted with primase very similarly to how dCTP interacts with primase [19], even though the chemical properties of the 2′-position are extremely different between these two compounds. Both compounds are relatively weak inhibitors and poor substrates of primase. The weak polymerization of these two compounds emphasizes the importance of the 2′-hydroxy, and suggests that primase makes a specific contact with the 2′-hydroxyl.

Studies with pol α have demonstrated that araATP, araCTP, and dFdCTP potently inhibit the elongation of exogenously added DNA primer:templates and inhibition is competitive with respect to the natural substrate [19,21,25]. In addition to binding the analogues, pol α readily polymerizes them with incorporation of either araCTP or araATP resulting in immediate, strong chain termination [21,25-28]. Interestingly, after polymerization of dFdCTP, pol α polymerizes one additional dNTP, at which point further synthesis is strongly inhibited [21]. In our studies, similar results for araC and dFdC were observed. In comparison, T-araC, like araC, caused immediate and strong chain termination, suggesting that the 2′-ara-hydroxyl plays an important role in the chain termination aspect of these compounds.

In contrast to results using pol α alone during primase-coupled pol α activity, both araCTP and dFdCTP were readily incorporated into the growing RNA–DNA strands when primase-coupled pol α complex was used. Importantly, pol α continued polymerizing additional dNTPs after incorporation of these two analogues. Why pol α should exhibit such divergent properties depending upon the source of the primer, remains a mystery. However, these data are consistent with previous studies showing that calf thymus pol α-primase readily incorporates araATP into internucleotide linkages during the elongation of primase synthesized primers but not when elongating exogenously added primer templates [22]. Importantly, pol α continued polymerizing additional dNTPs after incorporation of these two analogues. The rapid incorporation of these analogues into internucleotide linkages during primase coupled pol α activity would account for the observation in whole cells, that the majority of these analogues are both incorporated into DNA and are found at internucleotide linkages [29,30]. In contrast, incorporation of these analogues results in either moderate

(dFdCTP) or strong (araCTP) chain termination when pol α elongates exogenously added primentemplates. These data are consistent with previous studies showing that calf thymus pol α-primase readily incorporates araATP into internucleotide linkages during the elongation of primase synthesized primers but not when elongating exogenously added primer:templates.

In comparison, T-araC still appeared to cause a significant reduction in the elongation by the primase-coupled pol α complex. Despite this reduction in elongation, studies in cells and tumors demonstrate that the vast majority of T-araC present in DNA following treatment is also located in intranucleotide linkages [31].

A key question is how large amounts of T-araCTP becomes incorporated into internucleotide linkages in whole cells with only small amounts found at the 3′-terminus of DNA. In contrast to the results with araCTP, T-araCTP remained a very strong chain terminator even when using a primase-synthesized primer. Potentially, pol δ and/or pol ε, two additional DNA polymerases involved in replication, might incorporate T-araCTP into internucleotide linkages, thereby accounting for the results observed in whole cells. Alternatively, accessory proteins with which pol α likely interacts in vivo might greatly alter the properties of pol α, such that it can continue polymerizing dNTPs after incorporating a T-araCTP. Work with DNA synthesomes has suggested that these complexes of DNA replication machinery may more accurately reflect the manner in which cells will handle DNA analogs and have demonstrated elongation with araC and dFdC [32]. It would be interesting to see how T-araC would behave in such a system. Alternately or in addition, the relative absence of T-araC at the 3′-terminus of DNA could result from rapid excision of these residues via cellular exonuclease activity. For example, if pol α incorporated T-araC into DNA with consequent chain termination, pol α might dissociate and a cellular exonuclease could then excise the T-araC. Whatever the mechanism, these data demonstrate that while the use of isolated systems are essential to understanding the effects of the analogs on DNA polymerases and DNA synthesis, they are also limited in their ability to approximate what occurs in whole cells and tissues.

The results from the translesion syntheses studies demonstrated that the presence of dFdC, araC, or T-araC in internucleotide linkages alters pol α synthesis. Since the concentration range of the normal nucleotide triphosphates used in these studies encompassed the physiological range and these modified nucleosides are present in internucleotide linkages in vivo following therapeutic doses of the compound, the effects on polyermase a and the pol α-primase complex may occur in cells and tissues. This pausing of translesion synthesis in turn may be enough to trigger pathways in the cell that would lead to apoptosis observed in xenograft models.

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