

DNA polymerase θ specializes in incorporating synthetic expanded-size (xDNA) nucleotides

Tatiana Kent^{1,†}, Timur D. Rusanov^{1,†}, Trung M. Hoang¹, Willem A. Velema², Andrew T. Krueger², William C. Copeland³, Eric T. Kool² and Richard T. Pomerantz^{1,*}

¹Fels Institute for Cancer Research, Department of Medical Genetics and Molecular Biochemistry, Temple University Lewis Katz School of Medicine, Philadelphia, PA 19140, USA, ²Department of Chemistry, Stanford University, Stanford, CA 94305, USA and ³Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709, USA

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ABSTRACT

DNA polymerase θ (Pol θ) is a unique A-family polymerase that is essential for alternative end-joining (alt-EJ) of double-strand breaks (DSBs) and performs translesion synthesis. Because Pol θ is highly expressed in cancer cells, confers resistance to ionizing radiation and chemotherapy agents, and promotes the survival of homologous recombination (HR) deficient cells, it represents a promising new cancer drug target. As a result, identifying substrates that are selective for this enzyme is a priority. Here, we demonstrate that Pol θ efficiently and selectively incorporates into DNA large benzo-expanded nucleotide analogs (dxAMP, dxGMP, dxTMP, dxAMP) which exhibit canonical base-pairing and enhanced base stacking. In contrast, functionally related Y-family translesion polymerases exhibit a severely reduced ability to incorporate dxNMPs, and all other human polymerases tested from the X, B and A families fail to incorporate them under the same conditions as Pol θ . We further find that Pol θ is inhibited after multiple dxGMP incorporation events, and that Pol θ efficiency for dxGMP incorporation approaches that of native dGMP. These data demonstrate a unique function for Pol θ in incorporating synthetic large-sized nucleotides and suggest the future possibility of the use of dxG nucleoside or related prodrug analogs as selective inhibitors of Pol θ activity.

INTRODUCTION

Synthetic nucleotide analogs possessing non-canonical structures and properties are widely used for medicinal purposes, biomedical research, high-throughput sequenc-

ing and show promise for synthetic biology applications (1–6). With regard to therapeutic applications, nucleoside and nucleotide analogs have been developed widely as prodrugs to treat cancer and viral infections (1). For example, the anti-hepatitis C virus (HCV) nucleotide prodrug sofosbuvir is now widely used to treat HCV patients due to its ability to act as a chain terminator of HCV NS5A polymerase after it is converted into triphosphate form in cells (1,7). Several nucleoside and nucleotide analogs are also used as chemotherapy agents for both hematological malignancies and solid tumors. For example, gemcitabine, a prodrug deoxycytidine analog that inhibits DNA synthesis, is used to treat various carcinomas including pancreatic cancer, non-small cell lung cancer, breast cancer, bladder cancer, and is currently being tested in blood cancers (1). Sapacitabine is a newly developed nucleoside analog that exhibits a unique mechanism of action by catalyzing a single-strand break in DNA after it is incorporated by the replication machinery (1). The single-strand break is subsequently converted into a double-strand break (DSB) during the next round of replication. Since persistent DSBs are lethal to cells deficient in homologous recombination (HR)—the primary DSB repair pathway during S and G2 cell-cycle phases—sapacitabine causes selective killing of cells deficient in HR, which is a proven mechanism of personalized medicine for cancers mutated in *BRCA1* or *BRCA2* (*BRCA*) (8,9). For example, since *BRCA1* and *BRCA2* tumor suppressor proteins are essential for proper HR function and thus maintaining genome integrity, cells defective in these factors are highly susceptible to DNA breaks occurring in S and G2 cell-cycle phases and exhibit genome instability phenotypes. Many more anti-cancer and anti-viral nucleoside and nucleotide pro-drugs are currently in development to increase bioavailability and reduce toxic side-effects and drug resistance (1).

Synthetic nucleotide analogs also show great promise for synthetic biology applications. For example, unnatural nucleotides containing hydrophobic nucleobases or

*To whom correspondence should be addressed. Tel: +1 215 707 7623; Email: richard.pomerantz@temple.edu

†These authors contributed equally to the paper as first authors.

alternatively-H-bonded nucleobases have recently been developed for the purpose of expanding the genetic code (6,10–12). Such alternative base pairs can adopt a Watson–Crick-compatible pair geometry within a polymerase active site, and at least one example can be incorporated during multiple rounds of DNA replication in bacteria (6,13). Prior to this work, the Kool lab developed synthetic size-expanded deoxyribonucleoside monophosphates (dxNMPs) and triphosphates (dxNTPs) which include a benzene ring within the base moiety of each canonical nucleoside (Figure 1A) (5). Although dxNMPs significantly expand the width of the double-helix (14), they retain canonical base pairing interactions and exhibit stronger base stacking interactions, increasing the thermostability of double-strand xDNA compared to canonical DNA (5,15). Despite the increased size, xDNA was also shown to be utilized as genetic information in bacteria, successfully encoding amino acids of green fluorescent protein (4). In that study, data suggested that error-prone Y-family bacterial polymerases aided in the synthesis and bypass of the large-sized base pairs. In separate studies, the Y-family DNA polymerase, Dpo4, was shown to perform relatively efficient nucleotide incorporation opposite template dxNMPs *in vitro*, in comparison to A family Pol I (Klenow fragment) which exhibits higher fidelity DNA synthesis (16). Together, these studies demonstrate that certain sterically flexible DNA polymerases can accommodate size-expanded dxNMPs in the template DNA strand.

Although some DNA polymerases can perform DNA synthesis opposite xDNA bases in the template (4,17–18), to date the incorporation of dxNMPs into a primer strand by a template-dependent DNA polymerase has not been tested. For most polymerases, unfavorable steric interactions would be expected to disfavor dxNTPs as an incoming substrate. The large nucleobase is likely to induce steric hindrance within the active site of DNA polymerases, which may prevent proper positioning of the nucleotide near the 3'-hydroxyl at the primer terminus for the phosphodiester transfer reaction. Interestingly, previous studies have shown efficient utilization of dxNMPs by terminal deoxynucleotidyl transferase, which unlike common DNA polymerases does not require a template for the nucleotidyl transfer, thus potentially explaining its ability to accommodate large unnatural nucleotides (19).

Importantly, error-prone translesion DNA polymerases have been selected throughout evolution to perform replication opposite damaged DNA bases, which often contain bulky adducts (20,21). Furthermore, certain translesion polymerases are also capable of incorporating nucleotides containing structurally altered or enlarged bases that arise from oxidative damage (22–24). Thus, the possibility exists that certain translesion polymerases may effectively utilize size-expanded nucleotides as substrates for DNA synthesis. In particular, the polymerase domain encoded by human POLQ—referred to herein as DNA polymerase θ (Pol θ)—has been characterized as a highly promiscuous enzyme that exhibits translesion synthesis activity and the unique ability to synthesize DNA across a DSB during a process called microhomology-mediated end-joining (MMEJ) or alternative end-joining (alt-EJ) (25–30). Although Pol θ is among the A-family of polymerases, it ex-

hibits low-fidelity DNA synthesis and translesion synthesis activities akin to Y-family polymerases. The translesion synthesis activity of Pol θ has been attributed to a unique insertion motif which has also been shown to facilitate MMEJ (25,26). In the latter activity, Pol θ extends minimally paired ssDNA overhangs generated by 5'-3' exonucleases at DSBs which is necessary for end-joining of the broken DNA ends (25). Overall, Pol θ appears to be the most versatile and promiscuous of the error-prone translesion polymerases and therefore might conceivably incorporate size-expanded nucleotides, whereas many other cellular polymerases may be hindered in accepting them as substrates.

Intriguingly, several studies strongly indicate Pol θ as an ideal cancer drug target. For example, Pol θ is highly upregulated in multiple cancer types, and high levels of the polymerase have been shown to correspond to a poor survival rate for breast cancer patients regardless of their specific breast cancer type (31–33). Pol θ has also been shown to confer resistance to ionizing radiation and other chemotherapy agents, such as cisplatin and the Poly (ADP ribose) polymerase I (PARP1) inhibitor Rucaparib which preferentially kill cancer cells harboring mutations in integral HR factors BRCA1 or BRCA2 (BRCA) (34–36). Recent studies also show that suppression of *POLQ* expression causes synthetic lethality in HR deficient cells including breast and ovarian cancer cell lines (35,37). In contrast, loss of Pol θ activity has no major effects in BRCA proficient cells or mice (35,37). Thus, inhibition of Pol θ or PARP1 may have similar synthetic lethal effects in HR deficient cells. Since the polymerase domain of *POLQ* was shown to play a major role in the survival of BRCA deficient cells (37), identification of nucleotides that are exclusively incorporated by this polymerase will inform strategies to develop prodrug nucleotide analogs that specifically inhibit Pol θ for personalized medicine in patients with HR-deficient cancers. Here, we tested the ability of Pol θ to incorporate dxNMPs to investigate their potential for future development as selective Pol θ inhibitors.

MATERIALS AND METHODS

Primer extension

Primer extension was performed by incubating 90 nM of the indicated polymerase with 100 nM of indicated radio-labeled or cy3-labeled primer-template in the presence of the indicated nucleotides at 37°C in the following buffer: 25 mM Tris–HCl pH 8.8, 10 mM MgCl₂, 0.1 mg/ml bovine serum albumin (BSA), 0.01% NP-40, 5 mM dithiothreitol (DTT), 10% glycerol. Reactions were performed for 30 min unless noted otherwise. Reactions were terminated by the addition of 2× stop buffer (50 mM ethylenediaminetetraacetic acid (EDTA), 90% formamide). DNA products were resolved in denaturing urea polyacrylamide gels and visualized by autoradiography for radio-labeled DNA or fluorescence scanning for cy3-labeled DNA. Percent extension was determined by dividing the intensity of the bands representing the extension products by the sum of the intensity of the bands representing initial and extended products. Concentrations of nucleotides used were as follows. Figures 1 and 2: 5 μ M dTTP, dxTTP, dATP, dxATP; 10 μ M dGTP, dxGTP, dCTP, dxCTP. Figure 3: 5 μ M dTTP, dxTTP, dATP, dx-

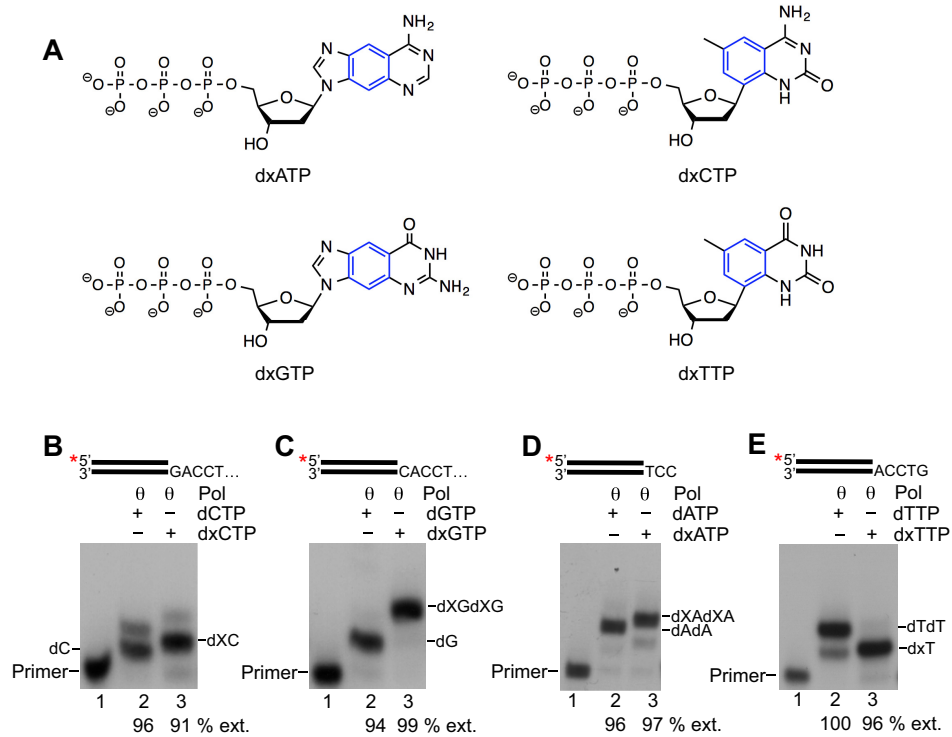


Figure 1. Polθ effectively incorporates dxNMPs. (A) Structures of dxNTPs. Blue denotes benzene rings. (B–E) Denaturing gels showing Polθ primer extension in the presence of the indicated nucleotide and primer-template sequence. *, 5' radio-label.

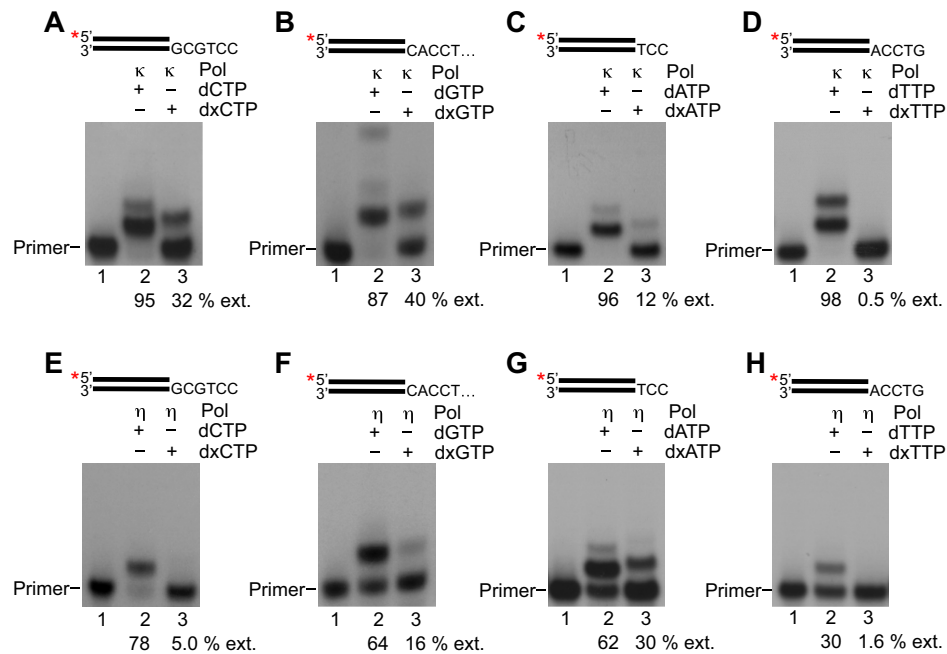


Figure 2. Y-family polymerases exhibit a limited ability to incorporate dxNMPs. (A–D) Denaturing gels showing primer extension by Polκ in the presence of the indicated nucleotide and primer-template sequence. (E–H). Denaturing gels showing primer extension by Polη in the presence of the indicated nucleotide and primer-template sequence. *, 5' radio-label.

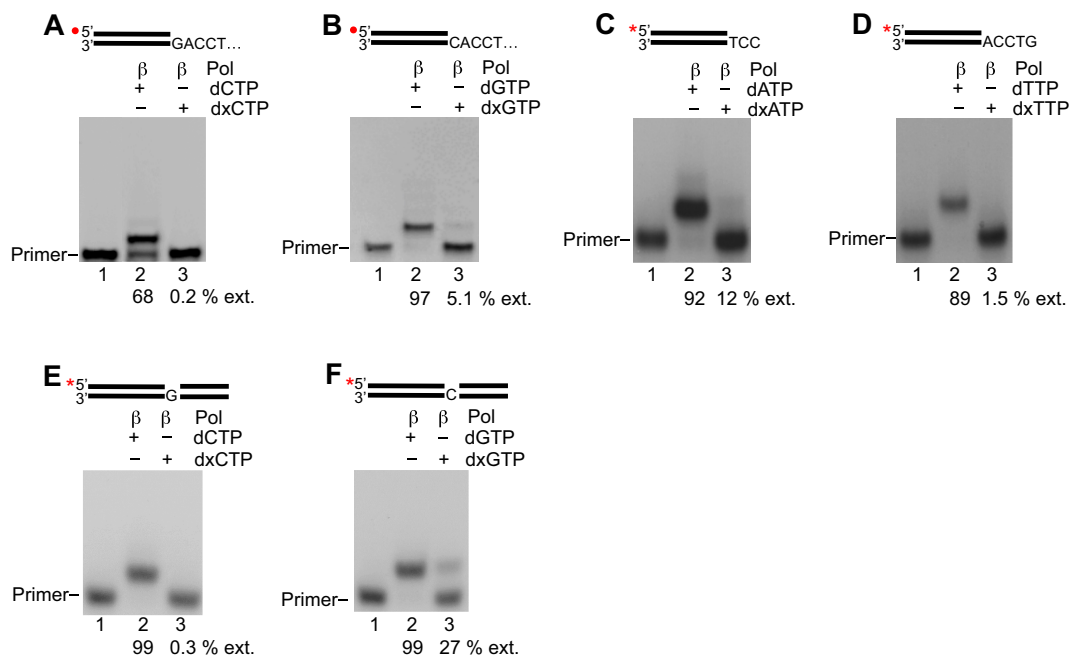


Figure 3. X-family polymerase β fails to incorporate dxNMPs. (A–F) Denaturing gels showing primer extension by Pol β in the presence of the indicated nucleotide and primer-template. *, 5' radio-label. ●, 5' Cy3-conjugate.

ATP; 50 μ M dGTP, dxGTP, dCTP, dxCTP. Figure 4: all nucleotides were at 50 μ M. Figure 5: all nucleotides were at 5 μ M except as indicated otherwise. Figure 6, panels A–C: all nucleotides were at 5 μ M. Supplementary Figure S1: all nucleotides were at 50 μ M. Rescue primer extension assays in Figure 4 were performed by two steps of nucleotide addition as indicated in Figure schematic. Each nucleotide addition step was followed by a 5 min time interval. Reactions without a rescue step were performed for a single 5 min time interval with the indicated nucleotides. All concentrations are listed as final in the reactions.

Determination of relative V_{\max} and K_m for nucleotide incorporation under steady-state conditions

Steady-state kinetics for single nucleotide incorporation for the determination of relative V_{\max} and K_m was performed similar to a previous study (38). Conditions for <20% primer extension were first identified to ensure initial rates of extension and that DNA concentrations were not limiting. 100 nM 5' radio-labeled primer-template (RP443/RP444 or RP443/RP445) and 1 nM Pol θ were mixed in 1 \times buffer (25 mM Tris-HCl pH 8.8, 10 mM MgCl₂, 0.1 mg/ml BSA, 0.01% NP-40, 5 mM DTT, 10% glycerol) at room temp. Reactions were initiated by the addition of dGTP or dxGTP at indicated concentrations and terminated after 2 min by the addition of 2 \times stop buffer (50 mM EDTA, 90% formamide). Reaction products were resolved in denaturing urea polyacrylamide gels and visualized by autoradiography. Multiple films were generated under variable exposure times to ensure bands were not overexposed. Percent extension was determined by dividing the intensity of the extended products by the sum of the intensities of the unextended and extended products. ImageJ

was used to quantify the intensities of radio-labeled DNA. Reactions were performed in triplicate and average velocities for each nucleotide concentration were determined. The substrate (nucleotide) concentrations were regarded as constant throughout the reaction. The data were fit to Hanes-Woolf plots which were used to determine the relative V_{\max} and K_m for each nucleotide.

Proteins

Human Pol θ , Pol η and Pol δ were purified as described (25). Exonuclease deficient human Pol γ was purified as described (39). Human Pols β and μ were purchased from Enzymax. Human Pol κ and Pol ϵ were kind gifts from Dr. Aggarwal (Mt Sinai School of Medicine).

DNA

Primer-templates were annealed by mixing together a 2:1 ratio of template strand to primer strand followed by heating to 95–100°C then slowly cooling to room temp. Primer strand was radio-labeled by using bacteriophage T4 polynucleotide kinase (New England Biolabs) in the presence of ³²P- γ -ATP (Perkin Elmer). In some instances a Cy3 5' labeled RP25 primer strand was used as indicated. The following primer-template pairs (primer/template) were used in each figure. Figures 1 and 3: RP25/RP409, RP25/RP408, RP25/RP166, RP25/RP424. Figure 2: RP25/RP409, RP166/RP167, RP25/RP166, RP25/RP424. Figures 4 and 5: RP25/RP409, RP25/RP408. Figure 5F: RP25/RP16. Figure 6: RP25/RP409, RP25/RP408, RP25/RP166, RP25/RP424, RP443/RP444, RP443/RP445.

Oligos (Integrated DNA technologies (5'-3')): RP25, CA CAGATTCTGGCAGGCTGCAGATCGC; RP25Cy3, Cy3-CACAGATTCTGGCAGGCTGCAGATCGC;

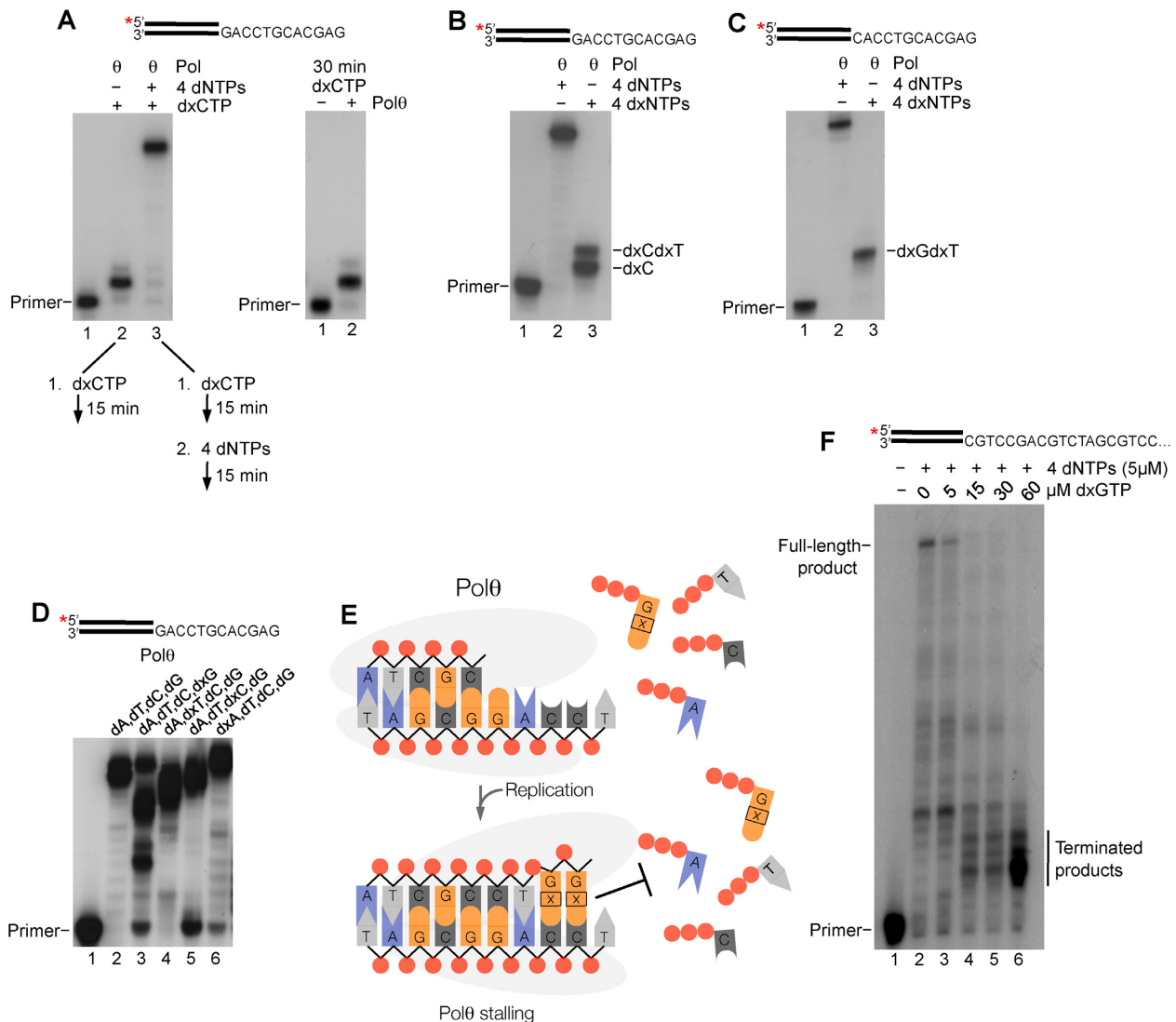


Figure 5. Polθ is inhibited after multiple dxNMP incorporation events. (A) Denaturing gels showing Polθ primer-template extension in the presence of the indicated nucleotides and primer-template sequence. Explanation of nucleotide order of addition (bottom). (B–D) Denaturing gels showing Polθ primer-template extension in the presence of the indicated nucleotides and primer-template sequence. (E) Model of Polθ activity observed in panel D, lane 3. Polθ becomes arrested after incorporating two consecutive dxGMPs probably due to steric hindrance within its active site. (F) Denaturing gel showing Polθ primer extension in the presence of the indicated nucleotides. *, 5' radio-label.

RESULTS

Polθ efficiently incorporates expanded-size dxNMPs

To determine whether Polθ is capable of incorporating size-expanded dxNMPs into DNA, we first examined the ability of the purified polymerase domain to perform primer extension in the presence of a single complementary dxNTP *in vitro*. Size-expanded dxNTPs, which contain a benzene ring within the base moiety of each nucleoside (Figure 1A), were synthesized as described (19). These expanded nucleosides form canonical base pairs and increase the width of double-stranded DNA by 2.4 Å (14). The bases of dxNMPs also exhibit stronger base stacking interactions (5). Polθ was incubated with the indicated radio-labeled primer-templates which respectively encode for one of the four different bases immediately downstream from the 3' primer terminus (Fig-

ure 1B–E). Primer-template extension was then initiated by the addition of either the complementary canonical dNTP or the respective large-sized dxNTP at equimolar concentration. Reactions were terminated after 30 min and radio-labeled DNA products were resolved in denaturing urea polyacrylamide gels and visualized by autoradiography.

Surprisingly, Polθ demonstrated efficient use of all four dxNTPs as substrates for primer-template extension under the given experimental conditions (Figure 1B–E). The slowed mobility of the extended primer in the presence of the dxCTP compared to dCTP is consistent with the slightly higher molecular weight of the size-expanded nucleoside (dxCMP) (Figure 1B). Polθ further extends a fraction of the primers due to misincorporation of dxCMP opposite the next template base, adenine (A) (Figure 1B). This result is consistent with previous studies showing that Polθ exhibits

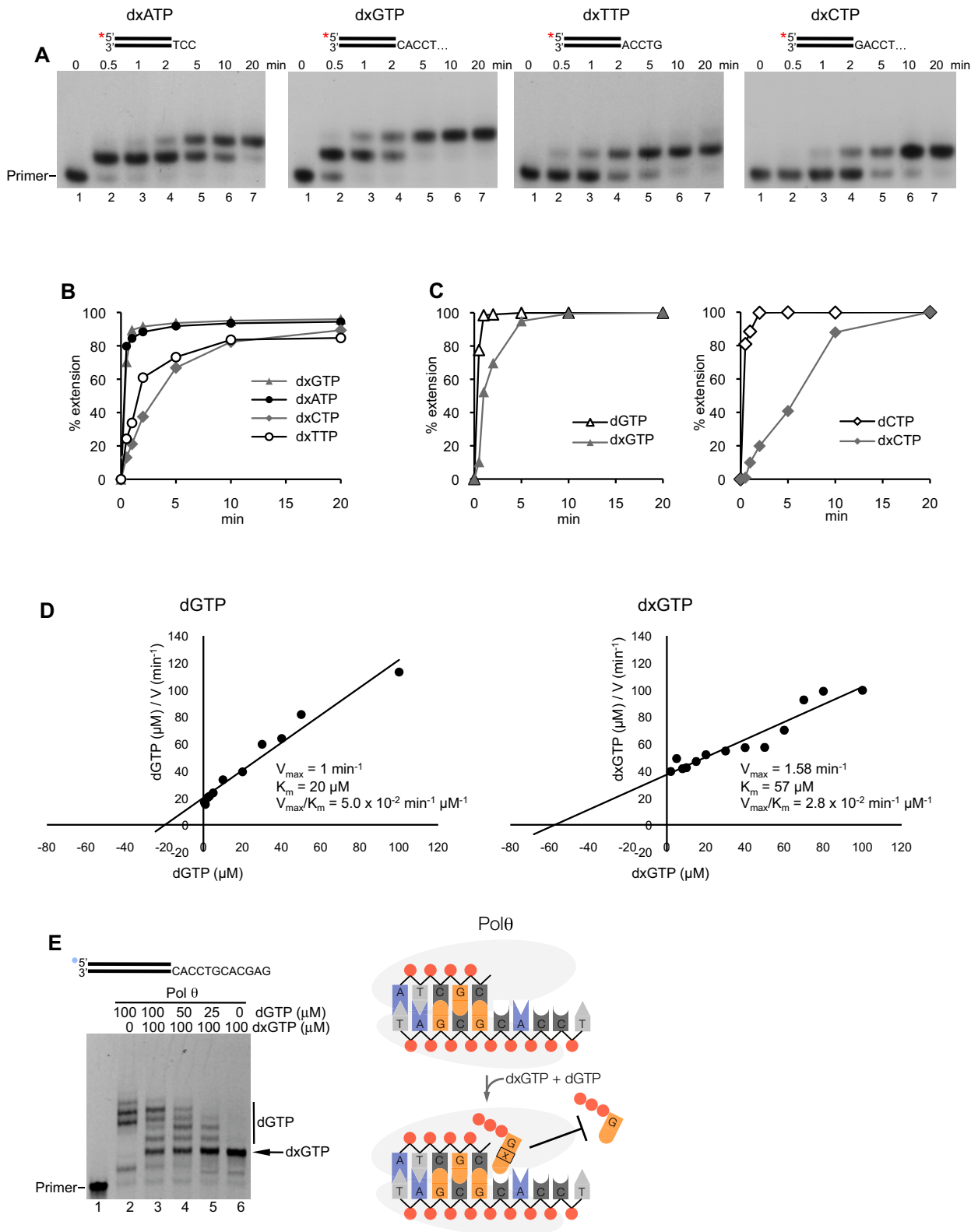


Figure 6. Polθ exhibits a relatively high efficiency of dxGMP incorporation. (A) Denaturing gels showing a time course of Polθ primer extension in the presence of the indicated expanded-size dxNTP. (B) Plot showing relative velocities of Polθ incorporation of dxNMPs. (C) Plots showing relative velocity of Polθ incorporation of the indicated nucleotide. (D) Hanes-Woolf plots of Polθ steady-state incorporation of the indicated nucleotide. Each data point represents an average from three separate experiments. Relative K_m , V_{max} and V_{max}/K_m are indicated. (E) Denaturing gel showing Polθ primer extension in the presence of the indicated nucleotides (left). Schematic illustrating the ability of dxGTP to compete with dGTP during Polθ primer extension (right). *, 5' radio-label. ●, 5' Cy3-conjugate.

a high rate of misincorporation (10^{-2} to 10^{-3}) (28). Interestingly, in the case of dxGTP, Pol θ incorporated two consecutive dxGMPs, but only incorporated a single dGMP on the same template under identical conditions (Figure 1C). This suggests that the presence of the incorporated large-sized nucleoside (dxGMP) in the enzyme's active site facilitates the subsequent misincorporation event, possibly due to increased stacking interactions between dxNMPs or to better alignment of the second incipient large pair with the first (Figure 1C). In the case of dxATP, two consecutive incorporation events are also observed (Figure 1D, lane 3). On this template the canonical nucleotide (dAMP) is also incorporated twice due to the error-prone nature of Pol θ (Figure 1D, lane 2). Interestingly, in the presence of dxTTP and dTTP, a different pattern emerged. Here, the canonical nucleotide (dTTP) was incorporated twice, whereas the large-sized nucleotide (dxTTP) was incorporated only once (Figure 1E). This suggests that Pol θ may exhibit a reduced efficiency of dxTTP incorporation compared to other dxNMPs, which could reflect the somewhat weaker stacking ability of the xT base compared with the other expanded bases (40). Overall, the results demonstrate that Pol θ effectively incorporates all four size-expanded dxNMPs and document the first case of polymerase dependent synthesis of xDNA by incorporation of synthetic size-expanded nucleotides.

Y-family translesion polymerases exhibit a limited ability to incorporate dxNMPs

To test whether the expanded nucleotides are selectively processed by Pol θ , we next examined the ability of other error-prone translesion polymerases to incorporate dxNMPs. The experiments in Figure 1 were repeated using identical conditions; however, Pol θ was substituted with Y-family translesion polymerases κ or η . Y-family polymerases perform low-fidelity DNA synthesis (i.e. error rate 10^{-2} to 10^{-3}) on undamaged DNA and accommodate bulky or non-canonical damaged DNA bases within their active sites during translesion synthesis (20,41). In contrast to the results observed with Pol θ in Figure 1B, Pol κ exhibited a relatively low efficiency of dxCMP incorporation (Figure 2A). For example, Pol θ and Pol κ respectively exhibited 91% and 32% primer extension in the presence of dxCTP, whereas both enzymes nearly fully incorporated the canonical dCMP under identical conditions (compare Figures 1B and 2A). Pol κ also exhibited a severely reduced ability to incorporate dxAMP compared to Pol θ (compare Figures 2C and 1D). Unexpectedly, Pol κ failed to perform primer extension in the presence of dxTTP, again using identical conditions as Pol θ in Figure 1 (Figure 2D). Although Pol κ exhibited the highest efficiency of incorporation (40%) in the presence of dxGMP, this was still significantly lower than Pol θ which extended 99% of the primers with dxGMP under identical conditions (compare Figures 1C and 2B). The ability of Y-family Pol η to incorporate dxNMPs was examined using the same conditions. The results show that Pol η exhibits a similar low efficiency of dxNMP incorporation as Pol κ (compare panels A–D to E–H in Figure 2). Specifically, Pol η fails to incorporate size-expanded pyrimidines (dxCMP, dxTTP) (Figure 2E and H) and exhibits a

severely limited ability to incorporate size-expanded purines (dxGMP, dxAMP) (Figure 2F and G). Taken together, these results demonstrate that Y-family polymerases exhibit a severely limited ability to incorporate dxNMPs compared to Pol θ .

X, B and A family polymerases strongly select against incorporating dxNMPs

We next examined the ability of X-family Pol β to incorporate dxNMPs. X-family polymerases perform gap filling during non-homologous end-joining (NHEJ) and base excision repair (BER). Overall, X-family polymerases exhibit a relatively high error rate (10^{-2} to 10^{-4}) compared to B-family replicative polymerases ($<10^{-5}$) which also exhibit exonuclease activity for proofreading misincorporation errors (41,42). X-family polymerases, however, are more accurate than Y-family polymerases which exhibit relatively high error rates (10^{-2} to 10^{-3}) on undamaged DNA (41,42). Consistent with its higher fidelity than Y-family polymerases, Pol β exhibited a substantially lower efficiency of dxNMP incorporation (0.2–12% extension) compared to Pols κ and η (0.5 – 40% extension) (compare Figures 2 and 3A–D). For example, Pol β failed to incorporate dxCMP and dxTTP and showed only a very slight ability to incorporate dxGMP and dxAMP during the 30 minute time course (Figure 3A–D). Because Pol β performs gap filling during base excision repair, we also examined its ability to incorporate purine and pyrimidine based dxNMPs on a primer-template substrate containing a small gap with a 5' phosphate on the oligonucleotide downstream from the primer. On this substrate Pol β failed to incorporate dxCMP and showed only a small increase in dxGMP incorporation (Figure 3E and F). X-family Pol μ also failed to effectively use dxNTPs as substrates like Pol β (Supplementary Figure S1). Thus, these data demonstrate that X-family polymerases strongly select against size-expanded nucleotides.

Next, we decided to investigate the ability of B-family replicative polymerases δ and ϵ to incorporate dxNMPs. These enzymes are responsible for replicating the genome in eukaryotes and therefore exhibit relatively high fidelities of nucleotide incorporation. For example, Pol δ and Pole were shown to exhibit error rates less than 10^{-5} (41). Pol δ and Pole possess proofreading activity which contributes to these low error rates. In order to examine the incorporation of dxNMPs by Pol δ and Pole it was necessary to devise a slightly different experimental method due to their robust exonuclease activities which are stimulated when one or more nucleotides are omitted from the reaction. We therefore developed the following primer-template extension assay which limits the exonuclease activities of Pol δ and Pole while detecting their ability to incorporate dxNMPs. Here, all four nucleotides were added to the reaction, however, dGTP was replaced with dxGTP. Thus, in the event that these high-fidelity enzymes are capable of efficient incorporation of dxNMPs, full primer extension and little or no exonuclease activity should be observed. However, if these enzymes are unable to efficiently incorporate dxNMPs, their respective exonuclease activities should be activated at the cytosine template base located immediately downstream from the 3' terminus of the primer. Indeed,

we found that the exonuclease activity of Pole was strongly stimulated when dxGTP was added along with dTTP, dATP and dCTP, indicating that Pole is unable to efficiently incorporate dxGMP (Figure 4A, lane 2). We next repeated the reaction with dxGTP, dTTP, dATP and dCTP, however, after 5 min an equimolar concentration of dGTP was added for a further 5 min. Subsequent addition of dGTP rescued the polymerase activity of Pole as indicated by full extension of the primer (Figure 4A, lane 3); this activity is modeled in Figure 4C. Since the exonuclease function is activated following a misincorporation event or when the correct incoming nucleotide is lacking, these data reflect one of the following scenarios. In the first scenario, Pole efficiently incorporates the complementary size-expanded nucleotide which rapidly triggers its exonuclease activity, resulting in immediate excision of the unnatural nucleotide. In the second more likely scenario, the polymerase fails to efficiently incorporate dxGMP which would also trigger its exonuclease activity since the correct canonical nucleotide (dGTP) is initially withheld from the reaction (Figure 4C). In the case of the first scenario where Pole exhibits dxGMP incorporation activity prior to exonuclease activity, the polymerase is likely to enter into a repetitive cycle of dxGMP incorporation and excision opposite the cytosine template base immediately downstream from the primer. Evidence of this incorporation-excision cycle would be indicated by some detection of dxGMP incorporation. However, a band representative of dxGMP incorporation is not observed. Thus, the data indicate that Pole exhibits little or no ability to incorporate dxGMP under the conditions tested.

We next repeated the assay with Pol δ . In contrast to the results with Pole, Pol δ showed slight incorporation of dxGMP (Figure 4A, lane 5). Minor exonuclease digestion of the primer was also observed in this reaction. Thus, although Pol δ shows some ability to incorporate dxGMP, its proofreading function is stimulated and probably acts to excise the large-sized nucleotide from the primer. To determine if the polymerase activity of Pol δ can be rescued, dGTP was added after 5 min and the reaction was allowed to proceed for an additional 5 min. Similar to the results obtained with Pole: addition of the canonical nucleotide rescued the polymerase activity of Pol δ (Figure 4A, lane 6) (Figure 4C). Hence, these data show that Pol δ and Pole are capable of continued DNA synthesis even when dxGTP and dGTP are present at equimolar concentrations, which demonstrates their ability to either select against dxGMP incorporation or effectively proofread a misincorporated dxGMP (Figure 4C). The rescue assay was then repeated with Pol δ and Pole, but dxCMP was added as the size-expanded nucleotide instead of dxGMP (Figure 4B). Similar to the results in panel A, the addition of the large-sized nucleotide (dxCTP) activated the respective proofreading activities of Pole and Pol δ , and the subsequent addition of the canonical nucleotide (dCTP) rescued primer extension by both polymerases. Taken together, the results presented in Figure 4 demonstrate that B-family replicative polymerases either strongly select against dxNTPs or efficiently excise these large-sized nucleotides after they are incorporated. Next, we examined whether B-family Pol α which functions as a replicative primase selected against incorporating size-expanded nucleotides. In contrast to Pols

δ and ϵ , Pol α lacks exonuclease activity and therefore may incorporate dxNMPs during a prolonged incubation time of 30 minutes. The results show that Pol α fails to incorporate purine and pyrimidine based dxNMPs like Pols δ and ϵ . These data therefore demonstrate that all three B-family replicative polymerases strongly select against incorporating size-expanded nucleotides.

Because Pol θ is an A-family polymerase, the mitochondrial replicative Poly, which is also an A-family member, may similarly use size-expanded nucleotides as substrates. This could conceivably cause toxicity in patients treated with prodrug chain terminator versions of size-expanded nucleotide or nucleoside analogs. We tested the ability of an exonuclease deficient mutant version of Poly to incorporate purine and pyrimidine versions of dxNMPs in Figure 4E. Remarkably, in contrast to Pol θ , the related Poly fails to use purine and pyrimidine based dxNTPs as substrates under identical conditions (Figure 4E). Hence, the data presented insofar demonstrate that Pol θ exhibits a unique ability to utilize dxNTPs as substrates and suggest that chain terminator versions of size-expanded nucleotides could be developed as specific inhibitors of Pol θ .

Pol θ is inhibited after multiple dxNMP incorporation events

Previous studies have demonstrated that Pol θ exhibits a relatively high efficiency of mismatch extension (27). This suggests that Pol θ may also efficiently extend from a dxNMP located at the 3' primer terminus. To examine this, we first incubated Pol θ with the primer-template in the presence of dxCTP to allow for nearly full incorporation of the nucleotide (Figure 5A, left panel, lane 2). To assess whether Pol θ is capable of efficiently extending from the incorporated dxCMP, the reaction was repeated, however, all 4 canonical dNTPs were added after the initial 15 min incubation with dxCTP. The result shows that Pol θ fully extends the primer after the subsequent addition of canonical nucleotides, demonstrating that the polymerase efficiently extends from the expanded-size nucleotide (dxCMP) (Figure 5A, left panel, lane 3). As a control, we show that Pol θ fails to extend from dxCMP in the absence of canonical dNTPs even after 30 min (Figure 5A, right panel).

Following these results, we chose to examine whether Pol θ can extend from multiple consecutively incorporated dxNMPs. To test this, we simply repeated the primer extension reaction with all 4 dxNMPs. In contrast to the results observed in panel A, Pol θ stalled after a single dxCMP incorporation event and showed a minimal ability to incorporate a second consecutive dxNMP (dxTTP) (Figure 5B). In a different sequence context, Pol θ stalled after two consecutive dxNMP incorporation events (Figure 5C). These data demonstrate that two consecutive dxNMP incorporation events strongly inhibit Pol θ , presumably due to distortion of the polymerase's active site which may prevent proper positioning of the next incoming nucleotide or suppress forward translocation of the enzyme. Further analysis shows that two consecutive dxNMP incorporation events suppress Pol θ activity even in the presence of all four nucleotides (Figure 5D). For example, in Figure 5D Pol θ primer extension was analyzed in the presence of a single size-expanded nucleotide mixed with equimolar concentrations of the re-

maining three canonical nucleotides. Since the template sequence contains two consecutive cytosine bases, a significant population of the polymerase becomes arrested at the second cytosine position when dxGTP is present in the reaction (Figure 5D, lane 3; Figure 5E). Furthermore, the majority of enzymes fail to reach the end of the template during this particular reaction which is likely due to additional dxGMP incorporation events downstream (Figure 5D, lane 3).

To further examine the ability of multiple dxGMP incorporation events to inhibit Pol θ , we performed primer extension with increasing amounts (0–60 μ M) of dxGTP in the presence of all four canonical dNTPs at a constant concentration of 5 μ M (Figure 5F). Remarkably, the results show that dxGTP begins to inhibit Pol θ DNA synthesis activity when added at an equimolar concentration (i.e. 5 μ M) as canonical nucleotides (Figure 5F, lane 3). At higher concentrations, dxGTP prevents Pol θ from fully extending the primer (Figure 5F, lanes 4–6). This is likely due to the inability of Pol θ to extend the primer after multiple dxGMP incorporation events (Figure 5E). Taken together, these data indicate that chain terminator versions of dxGTP may act as potent inhibitors of Pol θ at relatively low concentrations (i.e. <15 μ M).

Pol θ preferentially incorporates purine based dxNMPs

We proceeded to examine the relative velocities of dxNMP incorporation by Pol θ under steady-state conditions. The results show that Pol θ exhibits a substantially higher rate of incorporating size-expanded nucleotides derived from purine bases (dxGMP, dxAMP) (Figure 6A,B). For example, primer extension is nearly completed within 30 s in the presence of dxGTP or dxATP. In contrast, full primer extension in the presence of dxCTP or dxTTP under identical conditions requires 5–10 min. We speculate that increased base stacking interactions by dxGMP and dxAMP contribute to their higher rate of incorporation. We next compared relative velocities of incorporation of large-sized and canonical purine and pyrimidine nucleotides, again under steady-state conditions. Unexpectedly, Pol θ showed only a slightly lower velocity for dxGMP incorporation compared to dGMP (Figure 6C, left). In contrast, dxCMP, a pyrimidine analog, was incorporated at a substantially slower rate relative to canonical dCMP (Figure 6C, right). Since Pol θ exhibited roughly similar rates of incorporation between dxGMP and dGMP (Figure 6C), we chose to further examine the steady-state kinetics of these nucleotides. Consistent with the data presented in Figure 6C, we found that Pol θ exhibits similar steady-state kinetics for dxGMP ($V_{\max}/K_m = 2.8 \times 10^{-2} \text{ min}^{-1} \mu\text{M}^{-1}$) and dGMP ($V_{\max}/K_m = 5.0 \times 10^{-2} \text{ min}^{-1} \mu\text{M}^{-1}$) (Figure 6D). Thus, these results demonstrate that Pol θ incorporates dxGMP with remarkable efficiency despite its large size, and suggest that dxGTP can readily compete with canonical dGTP during Pol θ DNA synthesis activity.

To test directly whether dxGTP can compete with canonical dGTP during Pol θ replication, we analyzed primer extension in the presence of either dxGTP or dGTP as controls, and a combination of dxGTP and dGTP at various concentrations. The control reaction in the presence of

dxGTP reveals a clearly identifiable pattern of Pol θ inhibition after two dxGMP incorporation events (Figure 6E, lane 6). The control reaction in the presence of dGTP, however, results in a different pattern. Here, several consecutive dGMPs are incorporated due to multiple misincorporation and mismatch extension events (Figure 6E, lane 2). The differential patterns of dGMP versus dxGMP incorporation allows for the determination of which nucleotide is preferentially incorporated in reactions containing both nucleotides. Next, the reactions were repeated with 100 μ M dxGTP and increasing amounts of canonical dGTP. As expected from the similar steady-state kinetics of dxGMP and dGMP incorporation, similar amounts of dxGMP and dGMP incorporation events were observed when equimolar concentrations of these nucleotides were added to the reaction (Figure 6E, lane 3). Hence, these data along with those presented in Figure 5F demonstrate the ability of dxGTP to compete with dGTP at equimolar concentrations and suggest the dxGMP structure as a lead for development of cell-permeable prodrug competitive inhibitors of Pol θ .

DISCUSSION

Recent studies indicate Pol θ as a promising drug target for the development of precision medicine in HR deficient cancers, such as breast and ovarian cancers possessing *BRCA* mutations (35,37). However, there are currently no reported selective inhibitors of this polymerase, which appears to promote the survival of HR deficient cells through its role in the repair of DSBs via the alt-EJ pathway (37). Since Pol θ is highly error-prone and probably the most versatile of all human polymerases due to its limited template requirements and dual cellular functions in translesion synthesis and alt-EJ, we considered the possibility that this unusual polymerase might be able to efficiently incorporate size-expanded nucleotides (dxNMPs) which contain benzene rings within their respective base moieties. Indeed, we find that Pol θ not only effectively incorporates dxNMPs, but uniquely exhibits this novel function compared to all other human polymerases tested from the X, B, A and Y families.

Although Pol θ is among the A-family of polymerases, it includes three insertion motifs which alter the activity of the enzyme relative to other A-family DNA polymerase members. For example, insertion loop 2 which lies between the thumb and palm subdomains confers both translesion synthesis and end-joining activities onto the polymerase (25,26). Although crystal structures of Pol θ have recently been solved (43), it remains unclear how loop 2 or other insertion motifs affect the polymerase's fidelity or ability to utilize non-canonical templates such as DNA end-joining intermediates and ssDNA. The structural studies, however, do identify conserved positively charged residues that contribute to translesion synthesis activity by binding to phosphates at the 3' terminus of the primer (43). We found that these conserved residues contribute to the processivity of the enzyme which may increase the polymerase's ability to accommodate non-canonical templates and nucleotides by allowing it to remain tightly bound to DNA during catalysis (30). Regardless of the exact mechanisms by which Pol θ synthesizes DNA with substantially reduced fidelity and tem-

plate requirements compared to other polymerases, the enzyme clearly exhibits unique characteristics that may be exploited for developing selective nucleotide inhibitors.

Here, we find that Pol θ exhibits a unique ability to incorporate expanded-size dxNMPs, and that the polymerase incorporates dxGMP and dGMP with surprisingly similar steady-state kinetics. Functionally similar translesion polymerases from the Y-family (Pol κ , Pol η), which are also highly error-prone, show a severely reduced ability to incorporate dxNMPs compared to Pol θ , and in some cases are unable to incorporate particular dxNMPs even after long time intervals. We further find that X- and B- family polymerases fail to effectively incorporate dxNMPs. Since B-family replicative polymerases δ and ϵ exhibit proofreading activity, these enzymes can conceivably incorporate a dxNMP then rapidly excise the unnatural nucleotide due to its large base moiety. For example, we observed some evidence for Pol δ dxNMP incorporation, however, we did not detect any dxNMP incorporation by Pol ϵ . Interestingly, although the respective proofreading functions of Pol δ and Pol ϵ were activated by the presence of dxNTPs, both enzymes effectively switched to their polymerase activity when equimolar amounts of the respective canonical dNTP was subsequently added to the reaction. These data therefore demonstrate that replicative Pols δ and ϵ can efficiently perform DNA synthesis in the presence of equimolar amounts of dxNTPs without being inhibited. This observation suggests that prodrug inhibitors derived from dxNMPs would have little or no effect on chromosomal replication in non-cancerous proliferating cells which is important for minimizing toxicity. Consistent with this idea, we found that Pol α , the replicative primase, also failed to incorporate dxNMPs. Poly which replicates mitochondrial DNA similarly failed to use these size-expanded nucleotides as substrates. Taken together, these data suggest that prodrug chain terminator versions of dxNMPs would not induce toxicity in normal cells which do not rely on Pol θ activity for their proliferation.

In contrast to replicative polymerases, we found that Pol θ incorporates dxGMP even when an equimolar amount of dGTP is present in the reaction, which is consistent with its ability to incorporate dGMP and dxGMP with similar kinetics. We further find that Pol θ becomes arrested after multiple dxGMP incorporation events. This suggests that two closely spaced dxNMPs in the primer strand induce a severe distortion in the polymerase's active site which suppresses further DNA synthesis either due to preventing proper positioning of the next incoming nucleotide or disabling forward translocation of the polymerase.

Since Pol θ exhibits similar steady-state kinetics of dxGMP and dxGTP incorporation, the possibility exists that analogs of dxGMP might be developed as selective inhibitors of Pol θ . Other human polymerases from the A, B, X and Y families either fail to incorporate dxGMP or exhibit a markedly reduced ability to incorporate this nucleotide compared to Pol θ . Future chain terminator analogs of dxGTP might show enhanced inhibition of Pol θ since they would not require multiple incorporation events. In addition, phosphate prodrug variants of dxG might enable cellular activity against the polymerase which has been shown to promote the survival of HR deficient breast cancer

cells (37). Future studies are needed to determine whether Pol θ can be targeted by prodrug size-expanded nucleotide analogs for potential applications in HR deficient cells.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. R.T.P. and E.T.K. filed a provisional patent for the use of size-expanded (xDNA) nucleotide and nucleoside analogs as Pol θ inhibitors and cancer therapeutics.

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