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Replicative Bypass of O^2 -alkylthymidines Lesions *In Vitro*

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Abstract

DNA alkylation represents a major type of DNA damage and is generally unavoidable due to ubiquitous exposure to various exogenous and endogenous sources of alkylating agents. Among the alkylated DNA lesions, O^2 -alkylthymidines (O^2 -alkylTd) are known to be persistent and poorly repaired in mammalian systems, and have been shown to accumulate in esophagus, lung and liver tissue of rats treated with tobacco-specific *N*-nitrosamines, i.e. 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N*'-nitrosonornicotine (NNN). In this study, we assessed the replicative bypass of a comprehensive set of O^2 -alkylTd lesions, with the alkyl group being a Me, Et, *n*Pr, *i*Pr, *n*Bu, *i*Bu or *s*Bu, in template DNA by conducting primer extension assays with the use of major translesion synthesis DNA polymerases. The results showed that human Pol η and, to a lesser degree, human Pol κ , but not human polymerase ι or yeast polymerase ζ , were capable of bypassing all O^2 -alkylTd lesions and extending the primer to generate full-length replication products. Data from steady-state kinetic measurements showed that human Pol η exhibited high frequencies of misincorporation of dCMP opposite those O^2 -alkylTd lesions bearing a longer straight-chain alkyl group. However, the nucleotide misincorporation opposite branched-chain lesions was not selective, with dCMP, dGMP, and dTMP being inserted at similar efficiencies, though the total frequencies of nucleotide misincorporation opposite the branched-chain lesions differed and followed the order of O^2 -*i*PrdT > O^2 -*i*BudT > O^2 -*s*BudT. Together, the results from the present study provided important knowledge about the effects of the length and structure of the alkyl group in the O^2 -alkylTd lesions on the fidelity and efficiency of DNA replication mediated by human Pol η .

Graphical Abstract

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Supporting Information Available: Standing-start primer extension, steady-state kinetic assay results and quantification of full-length extension product from primer extension assays. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

DNA alkylation; human Pol η ; translesion synthesis; O^2 -alkyl dT

Introduction

According to the Center for Disease Control and Prevention, smoking is considered the leading cause of preventable diseases, and it accounts for more than 480,000 premature deaths per year in the United States alone.¹ Cigarette smoke contains more than 5,000 chemical species, 73 of which are considered carcinogenic to laboratory animals and/or humans.² Among these compounds are various polycyclic aromatic hydrocarbons and tobacco-specific *N*-nitrosamines, which, after metabolic activation, are known to alkylate DNA.^{2, 3} Depending on the chemical nature of the alkylating agent involved, the size of the alkyl functionality adducted to DNA ranges from simple methyl and ethyl groups, e.g. those arising from ethylating agents present in tobacco,³ to more complex alkyl groups from the tobacco-specific *N*-nitrosamines. In the latter respect, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N'*-nitrosonornicotine (NNN) can be metabolized by cytochrome P450 enzymes and ultimately result in the addition of a pyridyloxobutyl (POB) or pyridylhydroxybutyl (PHB) group to thymine, cytosine and guanine in DNA.⁴

Among the various alkylated DNA lesions, O^2 -alkylthymidines (O^2 -alkyl dT) are known to be resistant to repair and thus persist in mammalian systems.^{5–7} For instance, O^2 -POB dT and O^2 -PHB dT could accumulate in the esophagus, lung and liver tissue of rats treated with NNK and NNN, and were detectable in various human tissues.^{2, 3, 8–10} Additionally, the levels of O^2 -ethylthymine in human leukocyte DNA were found to be significantly higher in smokers than nonsmokers.³ If remained unrepaired, these alkylated DNA adducts may perturb genomic integrity by impeding DNA replication and transcription, and inducing mutations in these processes.²

Unrepaired DNA adducts may stall the replication fork, which results in cell cycle arrest and allows time for the cell to repair the damaged DNA.^{11–15} Nevertheless, some DNA lesions are more difficult to repair and may result in prolonged stalling of the replication fork, thereby leading to apoptosis. In order to avoid apoptosis, cells are equipped with translesion synthesis (TLS) DNA polymerases which possess more spacious and flexible active sites

than replicative DNA polymerases to facilitate lesion bypass.^{16, 17} The TLS polymerases include polymerases η , ι , κ and Rev1 in the Y-family, and polymerase ζ in the B-family. These polymerases function in the bypass of various DNA lesions, and some are known to bypass specific DNA lesions with similar or better fidelity and efficiency than the corresponding unmodified nucleosides.^{16–21} In this vein, polymerase η (Pol η) has been shown to be highly efficient and accurate when bypassing the UV-induced thymine-thymine cyclobutane pyrimidine dimers, which is recognized as the major role of Pol η .^{16, 19, 20, 22, 23} The importance of this polymerase is manifested in patients suffering from the variant form of xeroderma pigmentosum (XPV), and these individuals display elevated UV-induced mutagenesis and susceptibility toward developing skin cancer because they carry inactivating mutations in *POLH* gene which encodes for Pol η .^{22–24} Although this is recognized as the main functional role of Pol η , the polymerase is capable of bypassing many other DNA lesions with different efficiency and fidelity.^{25, 26}

Previous studies showed that O^2 -EtdT is partially blocking to DNA synthesis mediated by T7 DNA polymerase *in vitro*, and both dAMP and dTMP can be incorporated opposite the lesion.²⁷ Recently, our laboratory showed that polymerase V (an ortholog of human polymerase η) plays a major role in the bypass of various O^2 -alkylthymidine lesions in *Escherichia coli*, and the polymerase was indispensable for the lesion-induced T $\rightarrow$ A and T $\rightarrow$ G mutations in *E. coli* cells.²⁸ In addition, Basu and coworkers²⁹ showed that Pol η promotes the replication across the O^2 -MedT and O^2 -POBdT lesions in human cells. Thus, in the present study, we chose to focus on human Pol η by characterizing biochemically how the efficiency and fidelity of nucleotide insertion opposite the O^2 -alkyl dT lesions are modulated by the length and structure of the alkyl group.

Experimental Procedures

Materials

Human Pol η , κ , and yeast Pol ζ were purchased from Enzymax (Lexington, KY), and the recombinant full-length human Pol ι was kindly provided by Prof. Linlin Zhao (Central Michigan University). All other enzymes were obtained from New England BioLabs (Ipswich, MA) and unmodified oligodeoxyribonucleotides (ODNs) were acquired from Integrated DNA Technologies (Coralville, IA). [γ -³²P]-ATP was obtained from Perkin-Elmer (Boston, MA), and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

Substrate preparation

20-mer lesion-containing ODNs were generated by ligating 12-mer O^2 -alkyl dT-containing ODNs d(ATGGCGXGCTAT), where 'X' designates the O^2 -alkyl dT with the alkyl group being a Me, Et, *n*Pr, *n*Bu, *i*Pr, *i*Bu or *s*Bu (Figure 1)²⁸ to an 8-mer ODN d(GATCCTAG) in the presence of a 27-mer scaffold d(GTAGCTAGGATCATAGCACGCCATTAG), as previously described.²⁵ All ligation products were then purified by polyacrylamide gel electrophoresis (PAGE) and annealed to a 13-mer ³²P-labeled primer (10 nM) to yield the primer-template complex for *in vitro* primer extension and steady-state kinetic measurements (Figure 1).

Primer extension assays

Primer extension assays were performed under standing-start conditions by incubating the aforementioned primer-template complex (at a final concentration of 10 nM) at 37°C for 1 hr with various concentrations of human Pol η (Figure 2), κ (Figure S1) and ι (Figure S2), or for 5 hr with yeast Pol ζ (Figure S3), all four dNTPs (250 μ M each), MgCl₂ (5 mM) and a reaction buffer. The reaction buffer contained 25 mM potassium phosphate (pH 7.0), 5 mM MgCl₂, 5 mM DTT, 100 μ g/ml BSA and 10% glycerol.³⁰ The reaction was then terminated by adding an equal volume of formamide gel-loading buffer [80% formamide, 10 mM EDTA (pH 8.0), 1 mg/ml xylene cyanol and 1 mg/ml bromophenol blue]. The reaction mixtures were subsequently resolved on a 20% (19:1) denaturing polyacrylamide gel and the gel band intensities analyzed using a Typhoon 9410 Variable Mode Imager (Amersham Biosciences Co.).

Steady-state kinetic assay

Standing-start steady-state kinetic assays were performed following previously published procedures.³¹ The control or lesion-containing primer-template complexes (at a final concentration of 10 nM) were incubated with the above-mentioned reaction buffer, 5 nM human Pol η and various concentrations of individual dNTPs at 37°C for 10 min (Figure 3, S4 and S5). In this vein, the dNTP concentrations were optimized to allow for less than 20% nucleotide incorporation opposite the lesion or the corresponding unmodified nucleoside site. The reaction was again terminated by adding an equal volume of formamide gel-loading buffer. Reaction mixtures were then resolved on a 20% (19:1) denaturing PAGE, and gel-band intensities quantified by phosphorimaging analysis, as described above.

From the gel images, we first determined the observed rate for nucleotide incorporation, V_{obs} , by dividing the quantified amount of product formed by the incubation time (i.e. 10 min).^{30, 32, 33} The steady-state kinetic parameters (i.e. V_{max} and K_{m}) for nucleotide incorporation were then determined by plotting V_{obs} versus dNTP concentration and fit to the Michaelis-Menten equation using Origin 6.0 (Origin-Lab, Northampton, MA):³¹

$$V_{\text{obs}} = \frac{V_{\text{max}}[\text{dNTP}]}{K_{\text{m}} + [\text{dNTP}]}$$

The k_{cat} values were calculated by dividing V_{max} with the concentration of human Pol η employed. The efficiency of nucleotide incorporation was determined by the ratio of $k_{\text{cat}}/K_{\text{m}}$, and the frequency of incorrect nucleotide insertion (f_{inc}) was calculated from the ratio of $k_{\text{cat}}/K_{\text{m}}$ obtained for the insertion of incorrect nucleotide over that for the correct nucleotide incorporation:³¹

$$f_{\text{inc}} = \frac{(k_{\text{cat}}/K_{\text{m}})_{\text{incorrect}}}{(k_{\text{cat}}/K_{\text{m}})_{\text{correct}}}$$

It is of note that the 3' flanking nucleobase of the lesion in the template is a guanine; as a result, we observed, apart from the product with a single dCMP insertion, the product arising

from the incorporation of two dCMPs. We included both products for the determination of the steady-state kinetic parameters for dCMP incorporation.

Results

In this study, we aimed to investigate how the structures of the alkyl group in O^2 -alkyldT lesions influence the replicative bypass mediated by human Pol η , κ , ι and yeast Pol ζ .

Primer extension assay

Primer extension assays were first conducted to assess human Pol η , κ , ι and yeast Pol ζ 's ability to extend a 13-mer primer in the presence of a 20-mer template containing an unmodified dT or site-specifically inserted O^2 -alkyldT. The results showed that, when all four dNTPs are present, human Pol η was capable of successfully bypassing all seven O^2 -alkyldT lesions and generating full-length extension products (Figure 2). Quantification of the full-length extension product revealed that human Pol η -mediated primer extension was impeded to a lesser degree by those lesions carrying a branched-chain alkyl group than those with the corresponding straight-chain alkyl group, i.e. O^2 -iPrdT > O^2 -nPrdT (53% and 29%, respectively) and O^2 -iBudT, O^2 -sBudT > O^2 -nBudT (22%, 16% and 6.4% respectively) (Table S1). The values represent the percentage of full-length product observed for the lesion-containing substrate relative to that found for the control substrate. Pol κ was also able to bypass all the O^2 -alkyldT lesions and extend the primer to the end of the DNA template, but the main products formed were 18mer and 19mer products (Figure S1 and Table S1). Pol ι was able to generate shorter 18mer extension products for the templates harboring an O^2 -MedT or O^2 -EtdT; this polymerase, however, was incapable of extending further the primer after inserting one nucleotide opposite O^2 -nPrdT, O^2 -iPrdT, O^2 -nBudT, O^2 -iBudT or O^2 -sBudT (Figure S2 and Table S1). Yeast Pol ζ , on the other hand, was capable of producing full-length product when bypassing O^2 -MedT and O^2 -EtdT (at 50% and 7.4%, respectively), but was blocked by the other O^2 -alkyldT lesions (Figure S3 and Table S1). Thus, Pol η is the most efficient TLS polymerase involved in bypassing the O^2 -alkyldT lesions *in vitro* and was thus chosen for steady-state kinetic analysis.

Steady-state kinetic analysis

We next performed steady-state kinetic assays to assess the efficiency and fidelity of human Pol η in inserting nucleotides opposite the O^2 -alkyldT lesions (Table 1 and Figures 3, S4, and S5). The results showed that, among the four natural nucleotides, dAMP was inserted opposite the O^2 -MedT and O^2 -EtdT at the highest efficiency, though the presence of the two lesions reduces markedly the efficiency of dAMP incorporation, i.e. at 2.09% and 2.15%, respectively, relative to the corresponding insertion for the unmodified control substrate. Additionally, our results showed that human Pol η was more efficient in incorporating the correct nucleotide opposite the O^2 -alkyldT lesions with a branched-chain alkyl group (i.e. O^2 -iPrdT, O^2 -iBudT and O^2 -sBudT, at 0.42%, 0.34% and 0.18%, respectively, Figure 4) than the corresponding lesions with a straight-chain alkyl group (i.e. O^2 -nPrdT and O^2 -nBudT, at 0.08% and 0.07%, respectively, Figure 4).

We next analyzed the differences in human Pol η -mediated incorporation of incorrect nucleotides opposite the various O^2 -alkyldT lesions. Human Pol η displayed high fidelity when bypassing O^2 -MedT and O^2 -EtdT. However, human Pol η incorporates the incorrect dCMP opposite O^2 -*n*PrdT and O^2 -*n*BudT at relatively high frequencies (76% and 100%, respectively, relative to the incorporation of the correct dAMP). In contrast, all the O^2 -alkyldT lesions with a branched-chain alkyl functionality directed primarily promiscuous nucleotide misincorporation, where no marked preference was found for the misincorporation of dCMP, dGMP or dTMP (Table 1 and Figure 5).

Discussion

In the present study, we investigated the replicative bypass of O^2 -MedT, O^2 -EtdT, O^2 -*n*PrdT, O^2 -*i*PrdT, O^2 -*n*BudT, O^2 -*i*BudT and O^2 -*s*BudT by major TLS polymerases, including human Pol η , κ , ι and yeast Pol ζ . We uncovered the different capabilities of the TLS polymerases in bypassing these lesions and generating full-length extension products. We also demonstrated that the structure of the alkyl group attached to the O^2 position of thymidine influences the efficiency and fidelity of human Pol η -catalyzed nucleotide incorporation *in vitro*.

We found that, in the mutual presence of all four dNTPs, human Pol η could bypass all O^2 -alkyldT lesions and generate full-length replication products (Figure 2). In addition, human Pol η was found to generate more full-length replication product when bypassing those O^2 -alkyldT lesions bearing a branched-chain alkyl group relative to their straight-chain counterparts, i.e. O^2 -*i*PrdT > O^2 -*n*PrdT and O^2 -*i*BudT, O^2 -*s*BudT > O^2 -*n*BudT (Table S1). These results suggest that the O^2 -alkyldT lesions with a branched-chain alkyl group are more readily bypassed by human Pol η than the corresponding straight-chain lesions.

Human Pol κ mainly generated shorter 18mer and 19mer products, though small amounts of full-length extension product could also be detected (Figure S1 and Table S1). Our results about the difficulty of Pol κ in bypassing the O^2 -alkyldT lesions is in line with previous findings. Pol κ distinguishes itself from the other Y-family polymerases by its capability in bypassing efficiently and accurately dG adducts with various sizes of alkyl groups being attached to the minor-groove N^2 position^{34–36} as well as an N^2 - N^2 -guanine interstrand cross-link.³⁷ These abilities are due to the unique structure of Pol κ , whose active site can readily accommodate minor-groove modifications in DNA.^{34, 38} At first glance, this may suggest that Pol κ is capable of bypassing O^2 -alkyldT lesions. However, a number of recent studies showed that O^2 -MedT, O^2 -EtdT and O^2 -POBdT are not good substrates for Pol κ or its ortholog in *E. coli*, i.e. Pol IV.^{28, 29, 36, 39–41} This may be attributed to the fact that, unlike the N^2 -modified dG derivatives which still pair favorably with dCTP, the O^2 -alkyldT lesions do not have strong tendency to form Watson-Crick hydrogen bonding with any of the four canonical nucleotides.^{28, 29, 36, 39–41}

Pol ι , however, was unable to generate full-length replication products, as reflected by the lack of further extension of the primer after insertion of a single nucleotide opposite all the lesions except O^2 -MedT and O^2 -EtdT, where Pol ι could generate a shorter 18mer product for templates containing the latter two lesions (Figure S2). Yeast Pol ζ , on the other hand,

was capable of yielding full-length replication products as it bypasses O^2 -MedT and O^2 -EtdT, with more full-length extension product generated for the former substrate, but was completely blocked by all other O^2 -alkyldT lesions (Table S1 and Figure S3).

Our steady-state kinetic assay results revealed that the efficiency and fidelity of human Pol η -mediated nucleotide insertion opposite the O^2 -alkyldT lesions are influenced by the structure of the alkyl group. In particular, we found that human Pol η inserts dAMP opposite O^2 -MedT and O^2 -EtdT (relative to dT) more efficiently than the other O^2 -alkyldT lesions. Interestingly, human Pol η exhibited higher efficiencies in incorporating the correct nucleotide opposite the O^2 -alkyldT lesions possessing a branched-chain alkyl group, i.e. O^2 -*n*PrdT, O^2 -*i*BudT and O^2 -*s*BudT when compared to their straight-chain counterparts. We also determined the efficiency of human Pol η in incorporating the incorrect nucleotides opposite these minor-groove lesions. We observed that the incorrect nucleotide, dCMP, was incorporated at high frequency opposite the longer straight-chain lesions, i.e. O^2 -*n*PrdT and O^2 -*n*BudT. In contrast, human Pol η exhibited no strong preference in the incorporation of the incorrect nucleotides opposite the lesions with branched alkyl groups.

These results, in conjunction with previous findings about the major role of Pol V in bypassing the same O^2 -alkyldT lesions in *E. coli*, and Pol η 's function in bypassing the O^2 -MedT and O^2 -POB-dT in HEK293T human embryonic kidney cells, indicate that human Pol η may be the main TLS polymerase involved in the replicative bypass of the O^2 -alkyldT lesions.^{28, 29} Human Pol η is known to accurately bypass thymine-thymine cyclobutane pyrimidine dimers (CPD) with a similar accuracy and efficiency as bypassing the corresponding unmodified nucleosides.^{16–21} Pol η is unique among the Y-family polymerases due to its spacious active site which has the ability to accommodate the two cross-linked thymine bases in the CPD lesion.³⁶ Additionally, human Pol η is capable of promoting replication through many other DNA lesions including O^6 -alkyldG, 8-oxodG, O^4 -alkyldT, O^2 -MedT and O^2 -POBdT.^{30, 36, 41, 42} This may explain why human Pol η is able to incorporate a nucleotide opposite all seven O^2 -alkyldT lesions and extend past them. Moreover, the high frequency of misincorporation of dCMP opposite the longer straight-chain lesions, i.e. O^2 -*n*PrdT and O^2 -*n*BudT, may be attributed to Pol η 's recognition of these lesions as cytosine instead of thymine. Nevertheless, the addition of a branched-chain alkyl group to the O^2 position of thymine may render it difficult for human Pol η to recognize the hydrogen bonding property of alkylated nucleobase and result in a lack of selectivity in nucleotide misincorporation.

Our laboratory recently investigated the roles of the TLS polymerases in the bypass of the major-groove O^4 -alkyldT lesions *in vitro* and in human cells. In keeping with the findings made for the O^2 -alkyldT lesions in the current study, human Pol η was the only TLS polymerase capable of bypassing all the O^4 -alkyldT lesions and generating full-length extension product *in vitro*.³⁰ Additionally, human Pol η and ζ were found to promote the bypass of the O^4 -alkyldT lesions in HEK293T cells.⁴² On the other hand, the nature of nucleotide misincorporation mediated by the polymerase is vastly different when bypassing the dT lesions with the same alkyl groups being attached to the O^2 and O^4 positions. Unlike our current findings where dCMP was the main misincorporation event identified for the minor-groove O^2 -alkyldT lesions, dGMP was found to be the major nucleotide

misincorporated opposite the major-groove O^4 -alkyldT lesions both *in vitro* and in cells.^{30, 42} This study combined with our previous findings demonstrated that the efficiency and fidelity of human Pol η -mediated replicative bypass of the alkylated thymidine lesions are modulated not only by the size and shape of the alkyl group, but also by the position of the alkyl group in duplex DNA.

Together, the results from this study provided important insights into the roles of human Pol η , κ , ι and yeast Pol ζ in bypassing the minor-groove O^2 -alkyldT lesions, as well as the impact these lesions have on the efficiency and fidelity of human Pol η . Further understanding the impact the structure of the alkyl group on the efficiency and fidelity of human Pol η calls for structural studies in the future. In addition, it will be important to determine the roles of the TLS polymerases in bypassing the O^2 -alkyldT lesions in human cells as well as the impacts these lesions have on the efficiency and fidelity of cellular DNA replication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NOCs	<i>N</i> -nitroso compounds
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NNN	<i>N'</i> -nitrosonornicotine
POB	pyridyloxobutyl
PHB	pyridylhydroxybutyl
O^2 -alkyldT	O^2 -alkylthymidine
TLS	translesion synthesis
Pol	polymerase
ODN	oligodeoxyribonucleotide
PAGE	polyacrylamide gel electrophoresis
XPV	xeroderma pigmentosum variant

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a **R****O²-AlkyldT**

– CH ₃	O ² -MedT
– CH ₂ CH ₃	O ² -EtdT
– CH ₂ CH ₂ CH ₃	O ² - <i>n</i> PrdT
– CH(CH ₃) ₂	O ² - <i>i</i> PrdT
– CH ₂ CH ₂ CH ₂ CH ₃	O ² - <i>n</i> BudT
– CH ₂ CH(CH ₃) ₂	O ² - <i>i</i> BudT
– CH(CH ₃)CH ₂ CH ₃	O ² - <i>s</i> BudT

b5'–³²P–CTAGGATCATAGC–3'3'–³²P–GATCCTAGTATCGXGCGGTA–5'**Figure 1.**

(a) The structures of the O²-alkyldT lesions examined in this study and (b) the primer-template complex used for the *in vitro* primer extension and steady-state kinetic assays, X= dT, O²-MedT, O²-EtdT, O²-*n*PrdT, O²-*i*PrdT, O²-*n*BudT, O²-*i*BudT or O²-*s*BudT.

Figure 2.

Representative gel images from the primer extension assays under standing-start conditions for primer-template complexes containing an unmodified dT, O^2 -MedT, O^2 -EtdT, O^2 -nPrdT, O^2 -iPrdT, O^2 -nBudT, O^2 -iBudT or O^2 -sBudT with human Pol η . The final concentration of the primer-template complex was 10 nM, and the final concentrations of human Pol η are indicated.

Figure 3.

Representative gel images for steady-state kinetic assays in measuring the individual nucleotide incorporation opposite unmodified dT (a), O²-nPrdT (b) and O²-nBudT (50 nM) (c) with human Pol η. The final concentration of the primer-template complex was 10 nM, and the final concentration of human Pol η was 5 nM. The highest concentrations of individual dNTPs used are indicated in the figure, and the concentration ratio between neighboring lanes was 0.50.

Figure 4.

The efficiencies for the human Pol η -catalyzed insertion of the correct nucleotide, dAMP, opposite unmodified dT, O^2 -MedT, O^2 -EtdT, O^2 -nPrdT, O^2 -iPrdT, O^2 -nBudT, O^2 -iBudT or O^2 -sBudT (relative to dT). The results represent the mean $\pm$ standard deviation of results from at least three independent measurements.

Figure 5. Relative efficiencies for the Pol η-mediated nucleotide incorporation opposite dT, O^2 -MedT, O^2 -EtdT, O^2 -nPrdT, O^2 -iPrdT, O^2 -nBudT, O^2 -iBudT and O^2 -sBudT.

Table 1

Steady-state kinetic parameters for human Pol α -mediated incorporation of individual dNTPs opposite the O^2 -MedT, O^2 -EtdT, O^2 -nPrdT, O^2 -iPrdT, O^2 -BudT, O^2 -sBudT and O^2 -unmodified dT substrates. The results are shown as the mean $\pm$ standard deviation of results from at least three independent measurements.

dNTP	k_{cat} (min^{-1})	K_m (μM)	k_{cat}/K_m ($\mu\text{M}^{-1}\text{min}^{-1}$)	f_{inc}^a
Undamaged dT Substrate
dTTP	$1.2 \times 10^{-2} \pm 2 \times 10^{-3}$	12 ± 2	$9.6 \times 10^{-4} \pm 7 \times 10^{-5}$	2.0×10^{-3}
dGTP	$5.3 \times 10^{-2} \pm 7 \times 10^{-3}$	9.2 ± 1	$5.9 \times 10^{-3} \pm 1 \times 10^{-3}$	1.2×10^{-2}
dCTP	$2.5 \times 10^{-2} \pm 4 \times 10^{-3}$	23 ± 1	$1.1 \times 10^{-3} \pm 2 \times 10^{-4}$	2.1×10^{-3}
dATP	$1.5 \times 10^{-2} \pm 2 \times 10^{-3}$	$3.0 \times 10^{-2} \pm 4 \times 10^{-4}$	$5.1 \times 10^{-1} \pm 8 \times 10^{-2}$	1.0
O^2-MedT
dTTP	$5.6 \times 10^{-2} \pm 1 \times 10^{-2}$	$4.8 \times 10^{-2} \pm 8 \times 10^{-1}$	$1.2 \times 10^{-4} \pm 3 \times 10^{-5}$	1.1×10^{-2}
dGTP	$3.5 \times 10^{-2} \pm 3 \times 10^{-3}$	89 ± 7	$3.9 \times 10^{-4} \pm 1 \times 10^{-5}$	3.7×10^{-2}
dCTP	$3.7 \times 10^{-2} \pm 3 \times 10^{-3}$	$1.4 \times 10^{-2} \pm 7$	$2.6 \times 10^{-4} \pm 9 \times 10^{-6}$	2.5×10^{-2}
dATP	$4.5 \times 10^{-2} \pm 6 \times 10^{-4}$	$4.3 \times 10^{-1} \pm 4 \times 10^{-2}$	$1.1 \times 10^{-2} \pm 1 \times 10^{-3}$	1.0
O^2-EtdT
dTTP	$3.0 \times 10^{-3} \pm 3 \times 10^{-4}$	22 ± 5	$1.4 \times 10^{-4} \pm 2 \times 10^{-5}$	1.3×10^{-2}
dGTP	$2.3 \times 10^{-2} \pm 3 \times 10^{-3}$	23 ± 4	$1.0 \times 10^{-3} \pm 1 \times 10^{-4}$	9.2×10^{-2}
dCTP	$1.5 \times 10^{-2} \pm 2 \times 10^{-3}$	48 ± 3	$3.1 \times 10^{-4} \pm 2 \times 10^{-5}$	2.9×10^{-2}
dATP	$8.1 \times 10^{-3} \pm 4 \times 10^{-4}$	0.77 ± 0.1	$1.1 \times 10^{-2} \pm 1 \times 10^{-3}$	1.0
O^2-nPrdT
dTTP	$2.7 \times 10^{-2} \pm 3 \times 10^{-3}$	$4.4 \times 10^{-2} \pm 6 \times 10^{-1}$	$6.1 \times 10^{-5} \pm 4 \times 10^{-6}$	1.5×10^{-1}
dGTP	$1.6 \times 10^{-2} \pm 3 \times 10^{-3}$	$1.8 \times 10^{-2} \pm 4$	$8.9 \times 10^{-5} \pm 2 \times 10^{-5}$	2.2×10^{-1}
dCTP	$2.2 \times 10^{-2} \pm 3 \times 10^{-3}$	$70 \pm 1 \times 10^1$	$3.1 \times 10^{-4} \pm 2 \times 10^{-5}$	7.6×10^{-1}
dATP	$3.6 \times 10^{-2} \pm 3 \times 10^{-3}$	$89 \pm 1 \times 10^1$	$4.1 \times 10^{-4} \pm 4 \times 10^{-5}$	1.0
O^2-iPrdT				

	dNTP	k_{cat} (min^{-1})	K_{m} (μM)	$k_{\text{cat}}/K_{\text{m}}$ ($\mu\text{M}^{-1}\text{min}^{-1}$)	f_{inc}^a
dTTP	$4.9 \times 10^{-3} \pm 4 \times 10^{-4}$	15 ± 2	$3.4 \times 10^{-4} \pm 2.1 \times 10^{-5}$	1.6×10^{-1}
	dGTP	$2.3 \times 10^{-2} \pm 3 \times 10^{-2}$	60 ± 7	$3.9 \times 10^{-4} \pm 2.9 \times 10^{-5}$	1.9×10^{-1}
	dCTP	$3.4 \times 10^{-2} \pm 1 \times 10^{-3}$	52 ± 8	$6.5 \times 10^{-4} \pm 7.9 \times 10^{-5}$	3.1×10^{-1}
	dATP	$2.4 \times 10^{-2} \pm 3 \times 10^{-3}$	11 ± 1	$2.1 \times 10^{-3} \pm 2.2 \times 10^{-4}$	1.0
dTTP	$1.7 \times 10^{-2} \pm 2 \times 10^{-3}$	$3.5 \times 10^{-2} \pm 4 \times 10^{-1}$	$4.8 \times 10^{-5} \pm 8 \times 10^{-6}$	1.4×10^{-1}
	dGTP	$2.5 \times 10^{-2} \pm 3 \times 10^{-3}$	$4.4 \times 10^{-2} \pm 6 \times 10^{-1}$	$5.6 \times 10^{-5} \pm 2 \times 10^{-6}$	1.7×10^{-1}
	dCTP	$1.3 \times 10^{-2} \pm 2 \times 10^{-3}$	38 ± 6	$3.4 \times 10^{-4} \pm 4 \times 10^{-5}$	1.0
	dATP	$2.0 \times 10^{-2} \pm 5 \times 10^{-3}$	88 ± 7	$3.4 \times 10^{-4} \pm 4 \times 10^{-5}$	1.0
dTTP	$3.9 \times 10^{-3} \pm 6 \times 10^{-4}$	16 ± 3	$2.4 \times 10^{-4} \pm 1 \times 10^{-5}$	1.4×10^{-1}
	dGTP	$2.3 \times 10^{-2} \pm 3 \times 10^{-3}$	69 ± 7	$3.3 \times 10^{-4} \pm 4 \times 10^{-5}$	1.9×10^{-1}
	dCTP	$3.1 \times 10^{-2} \pm 5 \times 10^{-3}$	73 ± 15	$4.2 \times 10^{-4} \pm 4 \times 10^{-5}$	2.5×10^{-1}
	dATP	$8.7 \times 10^{-3} \pm 2 \times 10^{-3}$	5.1 ± 1	$1.7 \times 10^{-3} \pm 2 \times 10^{-4}$	1.0
dTTP	$2.1 \times 10^{-2} \pm 3 \times 10^{-3}$	$2.2 \times 10^{-2} \pm 2 \times 10^{-1}$	$9.3 \times 10^{-5} \pm 2 \times 10^{-5}$	1.0×10^{-1}
	dGTP	$2.5 \times 10^{-2} \pm 2 \times 10^{-2}$	$3.1 \times 10^{-3} \pm 6 \times 10^{-1}$	$8.1 \times 10^{-5} \pm 1 \times 10^{-5}$	8.8×10^{-2}
	dCTP	$3.1 \times 10^{-2} \pm 2 \times 10^{-3}$	90 ± 9	$1.3 \times 10^{-4} \pm 3 \times 10^{-5}$	1.4×10^{-1}
	dATP	$8.7 \times 10^{-2} \pm 5 \times 10^{-3}$	32 ± 3	$9.2 \times 10^{-4} \pm 1 \times 10^{-4}$	1.0

^aFrequency of nucleotide misincorporation = $[k_{\text{cat}}/K_{\text{m}} (\text{incorrect nucleotide})]/[k_{\text{cat}}/K_{\text{m}} (\text{correct nucleotide})]$