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***In Vitro* Lesion Bypass Studies of O^4 -Alkylthymidines with Human DNA Polymerase η**

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Abstract

Environmental exposure and endogenous metabolism can give rise to DNA alkylation. Among alkylated nucleosides, O^4 -alkylthymidine (O^4 -alkylTd) lesions are poorly repaired in mammalian systems and may compromise the efficiency and fidelity of cellular DNA replication. To cope with replication-stalling DNA lesions, cells are equipped with translesion synthesis DNA polymerases that are capable of bypassing various DNA lesions. In this study, we assessed human DNA polymerase η (Pol η)-mediated bypass of various O^4 -alkylTd lesions, with the alkyl group being Me, Et, *n*Pr, *i*Pr, *n*Bu, *i*Bu, (*R*)-*s*Bu, or (*S*)-*s*Bu, in template DNA by conducting primer extension and steady-state kinetic assays. Our primer extension assay results revealed that human Pol η , but not human polymerases κ and ι or yeast polymerase ζ , was capable of bypassing all O^4 -alkylTd lesions and extending the primer to generate full-length replication products. Data from steady-state kinetic measurements showed that Pol η preferentially misincorporated dGMP opposite O^4 -alkylTd lesions with a straight-chain alkyl group. The nucleotide misincorporation opposite most lesions with a branched-chain alkyl group was, however, not selective, where dCMP, dGMP, and dTMP were inserted at similar efficiencies opposite O^4 -*i*PrdT, O^4 -*i*BudT, and O^4 -(*R*)-*s*BudT. These results provide important knowledge about the effects of the length and structure of the alkyl group in O^4 -alkylTd lesions on the fidelity and efficiency of DNA replication mediated by human Pol η .

Graphical abstract

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.chemrestox.5b00509.

Standing- and running-start primer extension and steady-state kinetic assay results (PDF)

The authors declare no competing financial interest.

INTRODUCTION

Genomic integrity is constantly challenged by a plethora of endogenous and exogenous DNA damaging agents, which can lead to the formation of 10^4 – 10^5 DNA lesions per cell per day.^{1,2} These DNA damaging agents include alkylating agents, reactive oxygen species, ultraviolet (UV) irradiation, ionizing radiation, and various environmental chemicals.^{2–5} DNA alkylation represents a major type of DNA damage and is generally unavoidable due to ubiquitous exposure to exogenous (e.g., chemotherapeutic drugs, tobacco, and tobacco combustion products) and endogenous (e.g., cellular methyl donors and byproducts of lipid peroxidation) sources of alkylating agents.^{6,7} These agents are capable of transferring alkyl groups to various positions within DNA, including the ring nitrogen, exocyclic nitrogen, and oxygen atoms of nucleobases as well as the phosphate backbone.^{6–11} Depending on the chemical nature of the alkylating agents involved, the size of the alkyl functionality adducted to DNA ranges from simple methyl and ethyl groups, e.g., those emanating from monofunctional methylating agents used in cancer chemotherapy⁶ and ethylating agent(s) present in tobacco and its smoke,¹² to more complex alkyl groups, including the pyridyloxobutyl and pyridylhydroxybutyl functionalities arising from metabolites of some tobacco-derived N-nitrosamines.¹³ Among various alkylated DNA lesions, O^4 -alkylthymidine (O^4 -alkylTd) lesions are known to be poorly repaired and persist in mammalian tissues.^{14,15}

The induction of DNA damage in dividing cells may result in cell cycle arrest, which allows time for the DNA repair machinery to resolve the damage, thereby maintaining genomic stability.^{2,4,5,16,17} Some DNA lesions can be more difficult to repair, which may result in sustained stalling of the DNA replication fork. To avoid apoptosis emanating from such replication blockage, cells are equipped with translesion synthesis (TLS) DNA polymerases that are capable of bypassing various DNA lesions to support continued DNA replication. TLS polymerases, including polymerases η , ι , κ , and Rev1 in the Y-family and polymerase ζ in the B-family, possess more spacious active sites than replicative DNA polymerases.^{18,19} Some TLS polymerases are known to bypass certain DNA lesions with an accuracy and efficiency that are similar to or even better than that of bypassing the corresponding unmodified nucleosides.^{18–23} In this vein, polymerase η (Pol η) has been shown to

preferentially insert the correct nucleotide, dAMP, opposite UV-induced thymine–thymine cyclobutane pyrimidine dimers with high efficiency.^{18,21,22,24,25} This has been established as the major functional role of Pol η , the importance of which is manifested in patients suffering from the variant form of xeroderma pigmentosum (XPV). XPV patients are deficient in Pol η and exhibit elevated mutagenesis and susceptibility to developing skin cancer.^{24–26} Although this is considered to be Pol η 's main role, this polymerase is capable of bypassing many other DNA lesions with varying degrees of fidelity and efficiency.^{27,28}

Klenow fragment (Kf) of *Escherichia coli* DNA polymerase I and *Drosophila melanogaster* Pol α were previously shown to preferentially incorporate dGMP over dAMP opposite O^4 -MedT by more than 10-fold and to successfully extend past the lesion.²⁹ Interestingly, Kf and Pol α exhibited similar frequencies of misincorporation, suggesting that the exonuclease activity of Kf may not recognize O^4 -MedT:G as a mismatch.²⁹ A recent replication study with the use of single-stranded M13 plasmid showed that SOS-induced DNA polymerases assume somewhat redundant roles in bypassing the eight O^4 -alkylidT lesions carrying various structures of the alkyl functionality (Figure 1) in *E. coli* cells, with the exception of the two diastereomers of O^4 -sBudT lesions whose bypass requires Pol V.³⁰ Furthermore, O^4 -alkylidT lesions induce only T $\rightarrow$ C mutations in *E. coli* cells, which are not perturbed by depletion of any SOS-induced DNA polymerases, suggesting that the miscoding potential of these lesions is independent of the TLS DNA polymerases involved.³⁰ Nevertheless, the bypass efficiencies for all O^4 -alkylidT lesions except O^4 -nPrdT were substantially reduced when all three SOS-induced DNA polymerases were simultaneously deleted,³⁰ which revealed the involvement of TLS DNA polymerases in bypassing these lesions in *E. coli* cells. Thus, in this study, we chose to employ human Pol η as a model TLS DNA polymerase to biochemically characterize how the efficiency and fidelity of nucleotide insertion opposite O^4 -alkylidT lesions are modulated by the length (from methyl to butyl), branching (*i*Pr, *i*Bu, and *s*Bu vs their straight-chain counterparts), and stereochemistry (the *R* vs *S* diastereomers of *s*Bu) of the alkyl group (Figure 1).

EXPERIMENTAL PROCEDURES

Materials

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

Substrate Preparation

The 12-mer O^4 -alkylidT-containing ODNs d(ATGGCGXGCTAT), where X designates O^4 -alkylidT with the alkyl group being Me, Et, *n*Pr, *n*Bu, *i*Pr, *i*Bu, (*R*)-*s*Bu, or (*S*)-*s*Bu (Figure 1), were previously synthesized.³⁰ The 20-mer lesion-containing ODNs were generated by ligating the aforementioned 12-mer ODN to an 8-mer ODN d(GATCCTAG) in the presence of a 27-mer scaffold d(GTAGCTAGGATCATAGCACGCCATTAG), as described

previously.²⁷ All ligation products were then purified by polyacrylamide gel electrophoresis (PAGE) and annealed to an 11-, 13-, or 14-mer (with a dG or dA paired with the lesion) ³²P-labeled primer (10 nM) to yield the primer–template complex for *in vitro* primer extension and steady-state kinetics experiments (Figures 1, S4, and S8).

Primer Extension Assay

Primer extension assays under standing-start conditions were performed by incubating the primer–template complex (10 nM) at 37 °C for 1 h with various concentrations of human Pol η (Figure 2), κ (Figure S1), and ι (Figure S2) or for 5 h with Pol ζ (Figure S3), all four dNTPs (250 μ M each), MgCl₂ (5 mM), and reaction buffer, which contained 25 mM potassium phosphate (pH 7.0), 5 mM MgCl₂, 5 mM DTT, 100 μ g/mL BSA, and 10% glycerol. 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] was added to the samples to terminate the reaction. The reaction mixtures were subsequently resolved on a 20% (19:1) denaturing polyacrylamide gel, and the intensities of the gel bands were analyzed by phosphorimaging using a Typhoon 9410 variable mode imager (Amersham Biosciences Co.).

The 20-mer lesion-containing and control templates were also annealed individually with a 14-mer primer with the correct nucleotide, dA, or incorrect nucleotide, dG, being placed opposite the lesion, and the primer extension assays were then carried out with these primer–template complexes under conditions otherwise identical to those described above.

Primer extension assays under running-start conditions were performed as described above for standing-start experiments except that an 11-mer primer was used and the reactions were conducted at room temperature for 2 h.

Steady-State Kinetic Assay

Standing-start steady-state kinetic assays were performed following previously published procedures.³¹ The lesion-containing or the control primer–template complexes (10 nM each) were incubated with 5 nM human Pol η in the aforementioned reaction buffer with various concentrations of individual dNTPs at 37 °C for 10 min (Figures 3 and S5–S7). The reactions were again terminated by adding an equal volume of formamide gel-loading buffer, the reaction mixtures were resolved on a 20% (19:1) denaturing PAGE, and intensities of the gel bands were quantified by phosphorimaging analysis as described above.

The observed rate for nucleotide incorporation, V_{obs} , was first determined by dividing the quantified amount of product formed by the incubation time (i.e., 10 min).^{32,33} The kinetic parameters (i.e., V_{max} and K_{m}) for nucleotide incorporation were then determined by plotting V_{obs} as a function of dNTP concentration and fit to the Michaelis–Menten equation using Origin 6.0 software (Origin-Lab).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 used. The efficiency of nucleotide incorporation was determined by the $k_{\text{cat}}/K_{\text{m}}$ ratio, and the frequency of incorrect nucleotide insertion (f_{inc}) was calculated from the $k_{\text{cat}}/K_{\text{m}}$ value obtained for the insertion of incorrect nucleotide over that for the correct nucleotide incorporation.³¹

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

RESULTS

We sought to investigate how the structures of the alkyl group in O^4 -alkyldT lesions influence the efficiency and fidelity of nucleotide incorporation mediated by TLS DNA polymerases.

Primer Extension Assay

We first conducted primer extension assays under standing-start conditions to assess the ability of human Pol η , κ , and ι and yeast Pol ζ to extend a 13-mer primer in the presence of a 20-mer template containing a site-specifically inserted O^4 -alkyldT lesion or unmodified dT. The results showed that, in the presence of all four dNTPs, human Pol η was capable of successfully bypassing all eight O^4 -alkyldT lesions and extending the primer to the end of the template (Figure 2). Pol κ was able to bypass O^4 -MedT and O^4 -EtdT, but it was blocked by the other O^4 -alkyldT lesions (Figure S1). Pol ι could insert a nucleotide opposite each of the O^4 -alkyldT lesions but was unable to generate full-length extension products (Figure S2). Yeast Pol ζ , on the other hand, was blocked by all O^4 -alkyldT lesions (Figure S3). Thus, Pol η constitutes the most efficient TLS polymerase involved in bypassing the O^4 -alkyldT lesions *in vitro*.

Subsequently, we conducted primer extension assays under running-start conditions to assess the ability of human Pol η to bypass the O^4 -alkyldT lesions in the aforementioned 20-mer templates and generate full-length extension products. Our results showed that human Pol η is capable of replicating across all O^4 -alkyldT lesions and producing full-length extension products comparable to what we found from standing-start primer extension experiments (Figure S4). In addition, we observed that human Pol η was partially stalled at the nucleoside prior to the lesion site, as reflected by the presence of a 13-mer product in the reaction mixtures for the lesion-containing substrates that was not found with the control substrate (Figure S4). These results suggest that O^4 -alkyldT lesions exhibit a slight blocking effect on human Pol η -mediated primer extension under running-start conditions.

Steady-State Kinetic Analysis

We next conducted steady-state kinetic assays to assess the efficiency and fidelity with which human Pol η inserts nucleotides opposite O^4 -alkyldT lesions (Table 1 and Figures 3 and S5–S7). These results revealed that human Pol η was more efficient at incorporating the correct nucleotide, i.e., dAMP, opposite O^4 -MedT, O^4 -dBudT, O^4 -(*R*)-sBudT, and O^4 -(*S*)-sBudT (2.9, 4.4, 4.1, and 1.9%, respectively, relative to the corresponding insertion opposite

dT substrate) than the remaining O^4 -alkyldT lesions investigated (i.e., O^4 -EtdT, O^4 -*n*PrdT, O^4 -*i*PrdT, and O^4 -*n*BudT, at 0.36, 0.15, 0.05, and 0.20%, respectively; Figure 4), where the relative incorporation rates were calculated based on k_{cat}/K_m values. In this vein, it is worth noting that the insertion of the correct dAMP opposite O^4 -(*R*)-*s*BudT occurred at an efficiency that is approximately 2-fold higher than the corresponding incorporation opposite O^4 -(*S*)-*s*BudT. This result underscores the effect that subtle variations in the stereochemistry of the alkyl functionality have on the efficiency of nucleotide incorporation.

Our results also unveiled differences in the human Pol η -mediated incorporation of incorrect nucleotides opposite various O^4 -alkyldT lesions. The incorrect nucleotide, dGMP, was found to be preferentially incorporated opposite O^4 -MedT and O^4 -EtdT (175 and 265%, respectively, relative to the incorporation of the correct dAMP). This result is in line with a previous finding made from liquid chromatography-mass spectrometry (LC-MS) analysis of the full-length primer extension products, which revealed that human Pol η inserted dGMP opposite O^4 -EtdT 2–3-fold more preferentially over dAMP.³⁴ In this vein, a previous steady-state kinetic study by Andersen et al.³⁵ showed that *Saccharomyces cerevisiae* Pol η incorporated dGMP opposite O^4 -MedT with an efficiency that is markedly higher (by 75-fold) than that for dAMP insertion. This difference is likely ascribed to the difference between the yeast and human enzymes. Preferential misincorporation of dGMP over other incorrect nucleotides (i.e., dTMP and dCMP) was also found for two other O^4 -alkyldT lesions with a straight-chain alkyl group (i.e., O^4 -*n*PrdT and O^4 -*n*BudT). In contrast, all O^4 -alkyldT lesions with a branched-chain alkyl group except O^4 -(*S*)-*s*BudT directed primarily promiscuous nucleotide misincorporation, where no marked preference for misincorporation of dCMP, dGMP, or dTMP was found (Table 1 and Figure 5).

Primer Extension Assays Using Primers with dA or dG Placed Opposite the Lesion

Our results from steady-state kinetic assays indicated that human Pol η misincorporated dGMP opposite O^4 -alkyldT lesions with high frequency. Thus, we next conducted primer extension assays to determine whether primers with dA or dG placed opposite the lesions are better extended (Figure S8). Our results showed that, for some O^4 -alkyldT lesions (i.e., O^4 -MedT, O^4 -*n*BudT and, to a lesser extent, O^4 -*i*BudT), more full-length products were obtained with a dG-containing primer than with a dA-bearing primer. The opposite is true for O^4 -(*S*)-*s*BudT, whereas substrates housing other O^4 -alkyldT lesions displayed similar extension efficiencies with the two primers (Figure S9). Thus, the preferential extension of primers with dA or dG situated opposite the lesion is highly dependent on the identity of the O^4 -alkyldT lesion, although the rationale behind these differences remains unclear.

DISCUSSION

Previous *in vitro* replication studies showed that O^4 -MedT, O^4 -EtdT, and O^4 -*i*PrdT are highly mutagenic and induce T $\rightarrow$ C mutations.^{36,37} In the present study, we were able to expand this line of investigation to O^4 -*n*PrdT, O^4 -*n*BudT, O^4 -*i*BudT, O^4 -(*R*)-*s*BudT, and O^4 -(*S*)-*s*BudT. We found that the structures of the alkyl group attached to the O^4 position of thymidine influence the frequencies of human Pol η -mediated nucleotide misinsertion *in vitro*.

Our results showed that, when all four dNTPs are present, human Pol η is capable of bypassing all O^4 -alkylidT lesions and generating full-length replication products under both running- and standing-start conditions. Human Pol κ was able to generate full-length extension products for substrates harboring O^4 -MedT and O^4 -EtdT lesions, but the polymerase was incapable of bypassing other O^4 -alkylidT lesions. Neither Pol ι nor yeast Pol ζ was able to extend the primer to the end of a template containing any of the O^4 -alkylidT lesions. These results indicate that human Pol η is the main TLS polymerase involved in bypassing O^4 -alkylidT lesions. Human Pol η is unique among Y-family polymerases in its ability to accommodate two template bases in its active site by slightly shifting the little finger domain relative to the finger domain.^{24,38} Thus, a lack of strong blocking effects of O^4 -alkylidT lesions on Pol η -mediated replication may be due to the flexibility of the active site of the polymerase, which is capable of accommodating O^4 -alkylidT lesions and supporting the bypass of these lesions. In this vein, it is worth noting that O^4 -alkylidT lesions exhibit negligible blocking effects on DNA replication in wild-type *E. coli* cells.³⁰

Our steady-state kinetic assay results revealed that the efficiency and fidelity of human Pol η -mediated nucleotide insertion opposite O^4 -alkylidT lesions are modulated by the structure of the alkyl group. We observed that human Pol η preferentially incorporates the correct nucleotide, dAMP, opposite O^4 -dBudT and O^4 -(*R*)-sBudT lesions. We also determined the efficiency with which human Pol η incorporates the incorrect nucleotide opposite these major-groove lesions. We found that the incorrect nucleotide, dGMP, was incorporated opposite O^4 -MedT, O^4 -EtdT, O^4 -nPrdT, O^4 -nBudT, and O^4 -(*S*)-sBudT at relatively high frequencies. These results are consistent with the previous finding that these lesions direct DNA polymerases to misincorporate dGMP.^{36,37} Human Pol η also misincorporates dGMP opposite O^4 -nPrdT, O^4 -nBudT, O^4 -iPrdT, and O^4 -(*S*)-sBudT at a higher frequency than dCMP or dTMP insertion. Thus, the preferential misincorporation of dGMP likely arises from human Pol η 's recognition of these lesions as cytosine in lieu of thymine.

Interestingly, human Pol η is rather promiscuous in nucleotide insertion opposite most O^4 -alkylidT lesions bearing a branched-chain alkyl group, which is reflected by the similar frequencies of misincorporation of dTMP, dCMP, and dGMP opposite O^4 -iPrdT, O^4 -iBudT, and O^4 -(*R*)-sBudT (Figure 5). These branched-chain alkyl groups may make it difficult for human Pol η to recognize the hydrogen-bonding property of an alkylated nucleobase and result in a low efficiency of and lack of selectivity for nucleotide incorporation.

Together, the results from the present study provide important insights into the role of human Pol η in replication across major-groove O^4 -alkylidT lesions. A more complete understanding of the molecular mechanisms underlying the differences in efficiency and fidelity of nucleotide incorporation opposite various O^4 -alkylidT lesions requires future structural studies of human Pol η in complex with a lesion-containing primer-template. In addition, it is important to define the role of Pol η and other TLS polymerases in bypassing O^4 -alkylidT lesions in human cells in the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

NOCs	N-nitroso compounds
<i>O</i>⁴-alkylTdT	<i>O</i> ⁴ -alkylthymidine
ROS	reactive oxygen species
UV	ultraviolet
TLS	translesion synthesis
Pol	polymerase
ODN	oligodeoxyribonucleotide
PAGE	polyacrylamide gel electrophoresis
XPV	xeroderma pigmentosum variant

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Figure 1.

(a) Structures of the O⁴-alkyldT lesions examined in this study and (b) primer–template complex used for the *in vitro* primer extension and steady-state kinetic assays; X= dT, O⁴-MedT, O⁴-EtdT, O⁴-*n*PrdT, O⁴-*n*BudT, O⁴-*i*PrdT, O⁴-*i*BudT, O⁴-(*R*)-sBudT, or O⁴-(*S*)-sBudT.

Figure 2.

Representative gel images from primer extension assays under standing-start conditions for templates containing an unmodified dT, O^4 -MedT, O^4 -EtdT, O^4 -*n*PrdT, O^4 -*n*BudT, O^4 -*i*PrdT, O^4 -*i*BudT, O^4 -(*R*)-sBudT, or O^4 -(*S*)-sBudT with human Pol η .

Figure 3.

Representative gel images for steady-state kinetic assays measuring the individual nucleotide incorporation opposite unmodified dT (a), O⁴-nPrdT (b), and O⁴-iPrdT (c) with human Pol η . The highest concentrations of individual dNTPs used are indicated, and the concentration ratio between neighboring lanes was 0.50. The concentration of human Pol η was 5 nM.

Figure 4.

Efficiencies of human Pol η -catalyzed insertion of the correct nucleotide, dAMP, opposite unmodified dT, O^4 -MedT, O^4 -EtdT, O^4 -nPrdT, O^4 -nBudT, O^4 -iPrdT, O^4 -(R)-sBudT, O^4 -(S)-sBudT, and O^4 -iBudT (relative to dT). The results are shown as the mean $\pm$ standard deviation from at least three independent measurements.

Figure 5. Relative efficiencies of Pol η -mediated nucleotide incorporation opposite O^4 -MedT, O^4 -EtdT, O^4 -nPrdT, O^4 -nBudT, O^4 -iPrdT, O^4 -iBudT, O^4 -(R)-sBudT, and O^4 -(S)-sBudT.

Table 1

Steady-State Kinetic Parameters for Human Pol γ -Mediated Incorporation of Individual dNTPs Opposite O^4 -MedT, O^4 -EtIdT, O^4 -*n*PrdT, O^4 -*n*BudT, O^4 -*i*PrdT, O^4 -*i*BudT, O^4 -(*R*)-*s*BudT, O^4 -(*S*)-*s*BudT, and Unmodified dT Substrates^a

	dNTP	k_{cat} (min ⁻¹)	K_m (μM)	k_{cat}/K_m (mM ⁻¹ min ⁻¹)	f_{inc}
Undamaged dT Substrate
dTTP	$1.1 \times 10^{-2} \pm 2 \times 10^{-3}$	11 ± 2	1.0 ± 0.2	2.1×10^{-3}
	dGTP	$5.5 \times 10^{-2} \pm 5 \times 10^{-3}$	9.3 ± 2	6.0 ± 1	1.2×10^{-2}
	dCTP	$2.5 \times 10^{-2} \pm 5 \times 10^{-3}$	25 ± 3	1.0 ± 0.2	2.1×10^{-3}
	dATP	$1.5 \times 10^{-2} \pm 3 \times 10^{-3}$	$3.0 \times 10^{-2} \pm 9 \times 10^{-4}$	500 ± 80	1.0
O^4 -MedT					
	dTTP	$1.3 \times 10^{-2} \pm 6 \times 10^{-4}$	16 ± 2	0.82 ± 0.01	5.2×10^{-2}
dGTP	$1.9 \times 10^{-2} \pm 2 \times 10^{-3}$	$7.0 \times 10^{-1} \pm 9 \times 10^{-2}$	28 ± 6	1.8
	dCTP	$1.7 \times 10^{-2} \pm 5 \times 10^{-3}$	50 ± 2	0.54 ± 0.08	3.4×10^{-2}
	dATP	$1.5 \times 10^{-2} \pm 2 \times 10^{-3}$	$9.6 \times 10^{-1} \pm 2 \times 10^{-1}$	16 ± 1	1.0
O^4 -EtIdT					
	dTTP	$9.9 \times 10^{-3} \pm 7 \times 10^{-4}$	$24 \pm 6 \times 10^{-1}$	0.42 ± 0.03	2.2×10^{-1}
dGTP	$5.5 \times 10^{-2} \pm 1 \times 10^{-2}$	11 ± 1	5.1 ± 0.7	2.7
	dCTP	$1.4 \times 10^{-2} \pm 1 \times 10^{-3}$	34 ± 7	0.41 ± 0.07	2.1×10^{-1}
	dATP	$2.6 \times 10^{-2} \pm 3 \times 10^{-3}$	14 ± 2	1.9 ± 0.2	1.0
O^4 - <i>n</i> PrdT					
	dTTP	$2.1 \times 10^{-2} \pm 4 \times 10^{-3}$	$5.1 \times 10^{-2} \pm 70$	0.042 ± 0.002	5.3×10^{-2}
dGTP	$5.6 \times 10^{-2} \pm 1 \times 10^{-2}$	$1.2 \times 10^{-2} \pm 2$	0.48 ± 0.09	6.1×10^{-1}
	dCTP	$9.3 \times 10^{-3} \pm 2 \times 10^{-3}$	$2.6 \times 10^{-2} \pm 40$	0.036 ± 0.002	4.7×10^{-2}
	dATP	$2.4 \times 10^{-2} \pm 4 \times 10^{-3}$	32 ± 4	0.78 ± 0.10	1.0
O^4 - <i>i</i> PrdT					
	dTTP	$2.5 \times 10^{-2} \pm 5 \times 10^{-3}$	$1.2 \times 10^{-2} \pm 10$	0.22 ± 0.03	8.1×10^{-1}
dGTP	$9.7 \times 10^{-3} \pm 2 \times 10^{-3}$	53 ± 9	0.19 ± 0.03	7.0×10^{-1}
	dCTP	$2.7 \times 10^{-2} \pm 1 \times 10^{-3}$	$1.1 \times 10^{-2} \pm 10$	0.25 ± 0.02	9.3×10^{-1}

dNTP	k_{cat} (min^{-1})	K_{m} (μM)	$k_{\text{cat}}/K_{\text{m}}$ ($\text{mM}^{-1}\text{min}^{-1}$)	f_{inc}
<i>O</i>⁴-<i>n</i>BudT				
dATP	$1.6 \times 10^{-2} \pm 1 \times 10^{-3}$	60 ± 4	0.27 ± 0.03	1.0
$7.7 \times 10^{-2} \pm 6 \times 10^{-3}$	$1.7 \times 10^{-3} \pm 2 \times 10^{-2}$	0.046 ± 0.004	4.3×10^{-2}
dTTP	$2.1 \times 10^{-2} \pm 1 \times 10^{-3}$	23 ± 2	0.9 ± 0.2	8.7×10^{-1}
dGTP	$2.3 \times 10^{-2} \pm 5 \times 10^{-3}$	74 ± 10	0.32 ± 0.05	2.9×10^{-1}
dCTP	$3.8 \times 10^{-2} \pm 8 \times 10^{-3}$	36 ± 5	1.1 ± 0.1	1.0
<i>O</i>⁴-(<i>R</i>)-<i>s</i>BudT				
dTTP	$4.5 \times 10^{-2} \pm 8 \times 10^{-3}$	9.9 ± 2	4.6 ± 0.5	2.0×10^{-1}
dGTP	$9.4 \times 10^{-2} \pm 2 \times 10^{-2}$	22 ± 2	4.3 ± 0.5	1.9×10^{-1}
dCTP	$1.1 \times 10^{-1} \pm 2 \times 10^{-2}$	35 ± 7	3.3 ± 0.4	1.4×10^{-1}
dATP	$1.9 \times 10^{-2} \pm 3 \times 10^{-3}$	$8.1 \times 10^{-1} \pm 9 \times 10^{-2}$	23 ± 3	1.0
<i>O</i>⁴-(<i>S</i>)-<i>s</i>BudT				
dTTP	$7.3 \times 10^{-2} \pm 2 \times 10^{-2}$	57 ± 2	1.3 ± 0.3	1.3×10^{-1}
dGTP	$1.4 \times 10^{-1} \pm 3 \times 10^{-2}$	21 ± 4	6.5 ± 0.7	6.5×10^{-1}
dCTP	$4.4 \times 10^{-2} \pm 4 \times 10^{-3}$	63 ± 10	0.71 ± 0.08	7.0×10^{-2}
dATP	$5.5 \times 10^{-2} \pm 5 \times 10^{-4}$	$5.5 \pm 6 \times 10^{-1}$	10 ± 0.9	1.0
<i>O</i>⁴-<i>i</i>BudT				
dTTP	$1.8 \times 10^{-2} \pm 1 \times 10^{-3}$	$5.3 \pm 9 \times 10^{-1}$	3.4 ± 0.4	1.4×10^{-1}
dGTP	$9.4 \times 10^{-2} \pm 1 \times 10^{-2}$	17 ± 3	5.8 ± 0.4	2.3×10^{-1}
dCTP	$1.8 \times 10^{-2} \pm 3 \times 10^{-3}$	$3.4 \pm 7 \times 10^{-1}$	5.3 ± 0.4	2.2×10^{-1}
dATP	$4.6 \times 10^{-2} \pm 5 \times 10^{-3}$	$1.9 \pm 3 \times 10^{-1}$	25 ± 3	1.0

^dThe results are shown as the mean $\pm$ standard deviation from at least three independent measurements. f_{inc} , relative misinsertion efficiency = $[k_{\text{cat}}/K_{\text{m}}(\text{incorrect nucleotide})]/[k_{\text{cat}}/K_{\text{m}}(\text{correct nucleotide})]$.