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Accurate DNA Synthesis Across 8-Oxoadenine by Human PrimPol

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Abstract: PrimPol is a human DNA primase and DNA polymerase involved in DNA damage tolerance both in nuclei and mitochondria. PrimPol restarts stalled replication forks by synthesizing DNA primers *de novo* and also possesses DNA translesion activity (TLS activity). PrimPol efficiently and relatively accurately bypasses several DNA lesions including 8-oxoguanine and 5-formyluracil as well as carries out synthesis on DNA with thymine glycol in an error-prone manner. In this work, we showed that PrimPol possesses efficient and accurate TLS activity across 8-oxoadenine, another common DNA lesion caused by oxidative stress. The accuracy of PrimPol on DNA with 8-oxoA was significantly higher compared to DNA containing 8-oxoG. Replacement of Mg^{2+} ions with Mn^{2+} stimulated activity of PrimPol on DNA with 8-oxoA and 8-oxoG as well as undamaged A in a sequence-dependent manner by lesion skipping (or template scrunching) mechanism. Altogether, our data support the idea that PrimPol possesses efficient TLS activity across a wide range of DNA lesions caused by oxidative stress.

Keywords: PrimPol; 8-oxoadenine; DNA translesion synthesis

1. Introduction

Human PrimPol possesses DNA primase and DNA polymerase activities and is present in both nucleus and mitochondria [1–3]. It is involved in DNA damage tolerance by restart of replication forks at the sites of DNA damage and non-B DNA structures such as G-quadruplexes [4–9]. Cells deficient in PrimPol are sensitive to DNA-damaging agents [3,10,11].

PrimPol also demonstrates DNA translesion activity (TLS activity) and can bypass a variety of small DNA lesions [1,12]. PrimPol is blocked on DNA with bulky N^2-dG adducts [13] and a DNA-protein and AP site–peptide cross-links [14,15] but can bypass and incorporate complementary nucleotides opposite the cisplatin GG cross-link [15].

Previously, we and others demonstrated that PrimPol carries out efficient and relatively accurate synthesis past DNA lesions caused by oxidation such as 8-oxoguanine (8-oxoG) and 5-formyluracil [1,12,16]. Substitution of Mg^{2+} with Mn^{2+} ions also stimulated the synthesis on DNA with thymine glycol in an error-prone manner, likely by lesion skipping (or template scrunching) mechanism [12]. Such efficient TLS activity might facilitate PrimPol-mediated repriming on severely damaged DNA, e.g. containing clustered DNA damage.

Along with 8-oxoG, 8-oxoadenine (8-oxoA) is the most abundant oxidative lesion [17–20] with dual miscoding properties. These modified bases readily adopt the syn conformation: 8-oxoG forms stable 8-oxoG(syn):A(anti) Hoogsteen mispair while 8-oxoA efficiently forms the 8-oxoA(syn):G(anti) pair [21,22]. The majority of DNA polymerases preferentially incorporates non-complementary dAMP opposite 8-oxoG (so-called "A-rule") leading to G:C→T:A transversions [22,23]. While 8-oxoA is not mutagenic in *Escherichia coli* [24], its mutagenic effect was demonstrated in mammalian cells

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[25,26]. The 8-oxoA lesion placed in the *HRAS* oncogene sequence stimulated A:T → C:G transversions and A:T → G:C transitions at a relatively high frequency of mutagenesis comparable [25] or 4-fold reduced [27] to that caused by 8-oxoG.

In this work, we for the first time report activity of PrimPol on DNA with 8-oxoA. We showed that PrimPol accurately bypasses 8-oxoA *in vitro*. We also analyzed the effect of metal ions and DNA sequence context on the TLS activity of PrimPol on DNA with 8-oxoA and compared these data with those obtained for 8-oxoG.

Altogether, these findings further support the possible role of PrimPol in replication of DNA with oxidative damage.

2. Results

2.1. Efficient and Accurate Bypass of 8-oxoA in Reactions in the Presence of Mg^{2+}

First, we analyzed activity of PrimPol in reactions in the presence of Mg^{2+} on DNA substrates with the *HRAS* oncogene sequence context 5`-CCXA-3` containing A, G, 8-oxoA or 8-oxoG in the +1 position. Both DNA lesions, 8-oxoA and 8-oxoG, only slightly inhibited activity of PrimPol (Figure 1). Unlike many other DNA polymerases, PrimPol was more accurate and incorporated opposite 8-oxoG complementary dCMP with slight preference over non-complementary dAMP (Figure 1A, Table 1). However, PrimPol almost exclusively incorporated complementary dTMP opposite A and 8-oxoA (Figure 1, Table 1). PrimPol incorporated non-complementary dGMP with 3- to 4-fold reduced efficiency on DNA template with 8-oxoA and with a 10-fold reduction in efficiency on DNA template with undamaged A compared to dTMP (Figure 1C, Table 1). Interestingly, incorporation of dGMP was observed as a ladder in reactions in the presence of DNA templates containing 8-oxoA, 8-oxoG or undamaged A but not in reactions with template G (Figure 1A, lanes 4, 10, 16, 22). This activity can be a result of dGMP incorporation opposite C in the +2 and +3 template positions during template scrunching (or lesion skipping) leading to small deletions.

Table 1. Steady-state kinetics analysis of dNMP incorporation opposite A, G, 8-oxoA and 8-oxoG.

Template	dNMP	V_{max}, % per min	K_M, μM	V_{max}/K_M	F_{inc}
Mg^{2+}					
Template A	dT^{radio}	12.9 ± 0.7	420 ± 37	0.031 ±0.003	
	dT	12.2 ± 0.7	400 ± 42	0.031 ± 0.002	1
	dG	0.4 ± 0.004	184 ± 46	0.003 ± 0.0005	0.09
Template oxoA	dT^{radio}	3.6 ±0.3	610 ± 11	0.006 ±0.001	
	dT	5.3 ± 0.1	677 ± 16	0.008 ± 0.0001	1
	dG	0.4 ± 0.005	182 ± 33	0.002 ± 0.0001	0.25
Template G	dC	9 ± 0.9	73 ± 5	0.123 ± 0.019	
	dA	ND			
Template oxoG	dC	3.8 ± 0.3	128 ± 3	0.029 ± 0.004	1
	dA	2.9 ± 0.3	137 ± 9	0.021 ± 0.0005	0.72
Mn^{2+}					
	dT^{radio}	50 ± 1.5	11 ± 1	4.5 ± 0.3	

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Template A	**dT**	40 ± 0.9	12.2 ± 0.3	3.3 ± 0.01	1
	dG	2.7 ± 0.5	4 ± 0.3	0.7 ± 0.2	0.2
Template oxoA	**dT^{radio}**	28 ± 1.4	10 ± 0.5	2.7 ± 0.1	
	dT	22.5 ± 0.02	14.6 ± 1.9	1.6 ± 0.2	1
	dG	2.4 ± 0.2	3.9 ± 0.8	0.4 ± 0.1	0.25
Template G	**dC**	41.5 ± 0.1	5.1 ± 0.2	8.2 ± 0.3	
	dA	ND			
Template oxoG	**dC**	23.5 ± 1	4 ± 0.5	6 ± 1	1
	dA	15.9 ± 0.6	2.5 ± 0.3	6.4 ± 0.9	1.1

dT^{radio} – data calculated for ^{32}P-labelled DNA substrate. F_{inc} = $V_{max}^{non\text{-}complementary}/K_M^{non\text{-}complementary}/V_{max}^{complementary}/K_M^{complementary}$. *ND – not detected.

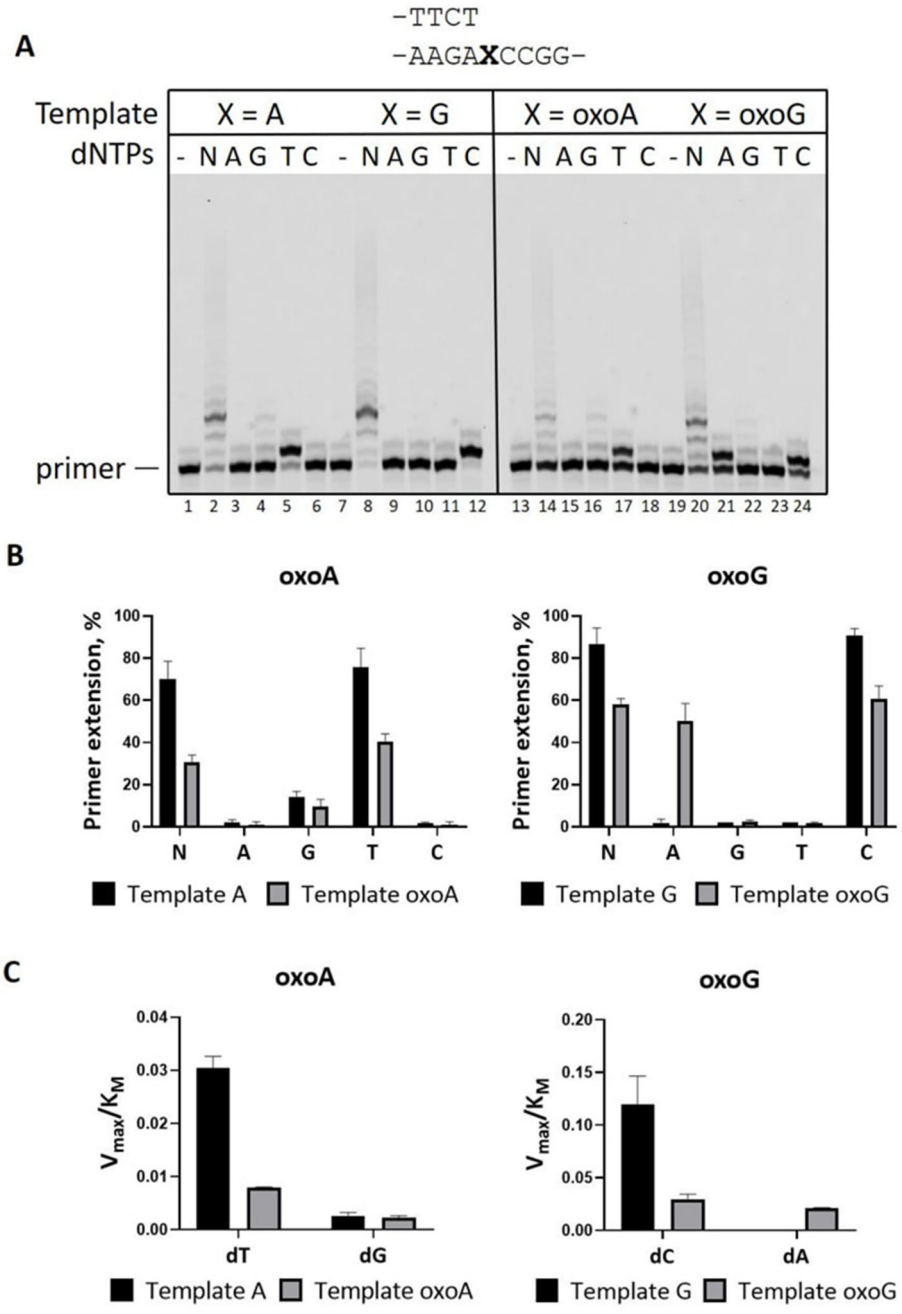

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Figure 1. DNA polymerase activity of PrimPol on DNA with 8-oxoA or 8-oxoG in the presence of Mg^{2+}. (a). Primer extension reactions on DNA containing 8-oxoA or 8-oxoG with Mg^{2+}. Reactions were carried out in the presence of 10 mM $MgCl_2$, 200 nM PrimPol, 100 nM Cy5-DNA-substrate and 200 μM of all four dNTP (N) or individual nucleotide substrates (A – dATP, G – dGTP, T – dTTP, C – dCTP) for 4 min. (b). Diagram showing the percent of primer extension on DNA with 8-oxoA or 8-oxoG in reactions in the presence of Mg^{2+} (Figure 1A). The mean values of primer extension and standard errors are indicated. (c). Diagram showing the V_{MAX}/K_M ratio calculated for DNA containing 8-oxoA or 8-oxoG in reactions in the presence of Mg^{2+} (Table 1).

2.2. Mn^{2+} Ions Decrease Accuracy of PrimPol on DNA Substrates with 8-Oxopurines

The DNA polymerase activity of PrimPol as well as the template scrunching mechanism is stimulated by Mn^{2+} ions [12,13,28]. Indeed, replacement of Mg^{2+} with Mn^{2+} ions stimulated activity of PrimPol and reduced its accuracy on all DNA templates (Figure 2). PrimPol carried out error-prone synthesis on DNA with 8-oxoG. Enzyme incorporated dAMP slightly more efficient than complementary dCMP (Figure 2A, lane 21, Table 1).

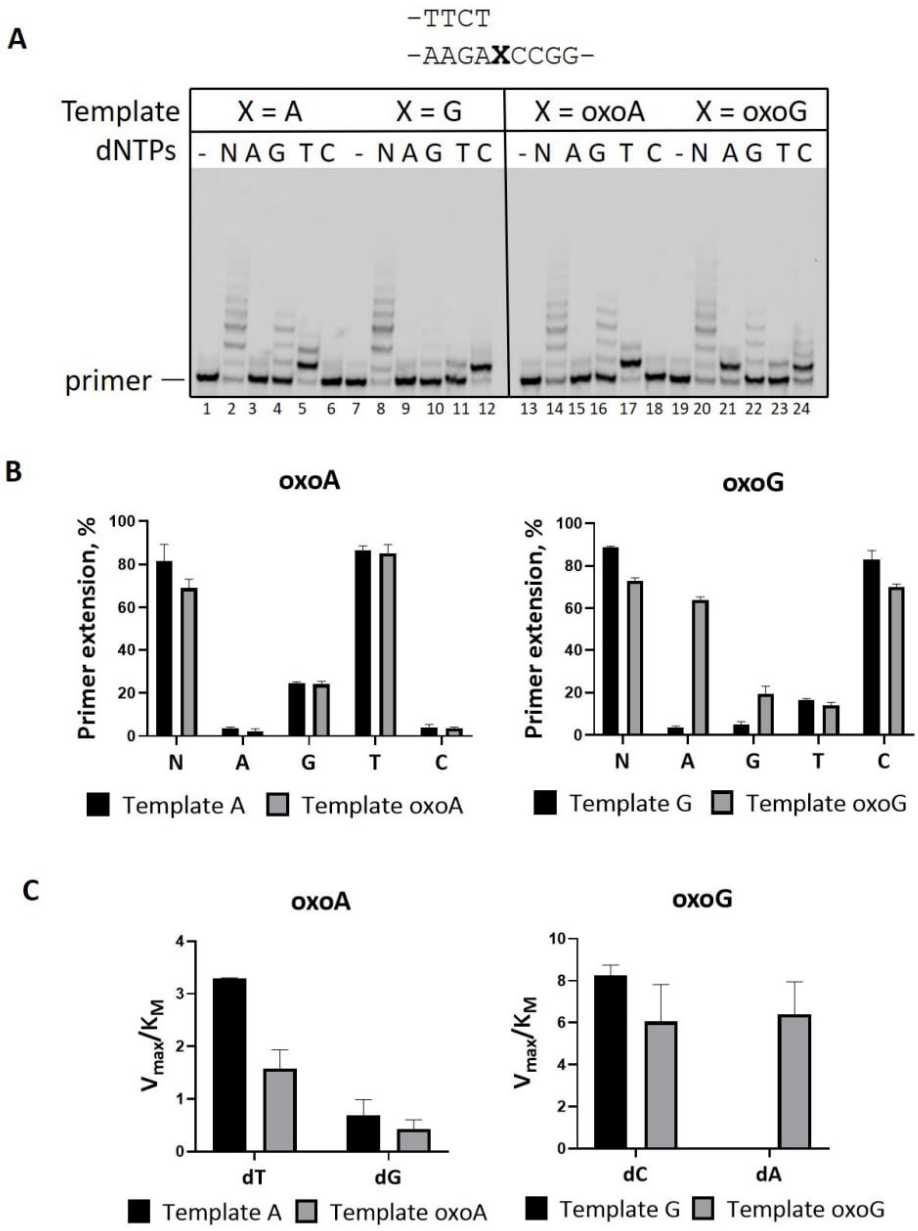

Figure 2. DNA polymerase activity of PrimPol on DNA with 8-oxoA or 8-oxoG in the presence of Mn^{2+}. (a). Primer extension reactions on DNA containing 8-oxoA or 8-oxoG with Mn^{2+}. Reactions were carried out in the

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presence of 1 mM $MnCl_2$, 200 nM PrimPol, 100 nM Cy5-DNA-substrate and 200 μM of all four dNTP (N) or individual nucleotide substrates (A – dATP, G – dGTP, T – dTTP, C – dCTP) for 1 min. (b). Diagram showing the percent of primer extension on DNA with 8-oxoA or 8-oxoG in reactions in the presence of Mn^{2+} (Figure 2A). The mean values of primer extension and standard errors are indicated. (c). Diagram showing the V_{MAX}/K_M ratio calculated for DNA containing 8-oxoA or 8-oxoG in reactions with Mn^{2+} (Table 1).

Mn^{2+} ions also facilitated incorporation of dGMP and dTMP on DNA with 8-oxoG (Figure 2A, lanes 22, 23). Mn^{2+} stimulated the incorporation of dGMP on both templates A and 8-oxoA (Figure 2A, lanes 4 and 16, Table 1). PrimPol was slightly more accurate on DNA with A compared to 8-oxoA in the presence of Mg^{2+} (Table 1). In reactions in the presence of Mn^{2+}, PrimPol demonstrated similar accuracy by incorporating dGMP on both templates A and 8-oxoA with 4- to 7-fold reduced efficiency (Table 1). The dGMP incorporation was observed as prominent ladders on DNA with A, 8-oxoA and 8-oxoG and is likely a result of indels following alternative alignments with short +2-+3 CC template microhomology region.

2.3. The Effect of DNA Sequence Context on A and 8-oxoA Bypass

To test the effect of DNA sequence context on nucleotide incorporation, we replaced C in the +2 position of the *HRAS* oncogene sequence with A, G or T. All replacements increased the accuracy of PrimPol and reduced the incorporation of dGMP on DNA substrates with template undamaged A or 8-oxoA lesion in the presence of Mg^{2+} (Figure 3A,B). In reaction with Mn^{2+}, PrimPol also incorporated dGMP on DNA templates with A and 8-oxoA in the *HRAS* 5`-CCXAG-3` sequence context only (Figure 3C,D). Moreover, PrimPol incorporated dCMP on both A- and 8-oxoA-containing DNA substrates after replacement +2 template C with G. Altogether, these data suggest that PrimPol carries out efficient and accurate DNA synthesis across 8-oxoA. PrimPol also induce microdeletions with low efficiency in the sequence-dependent manner.

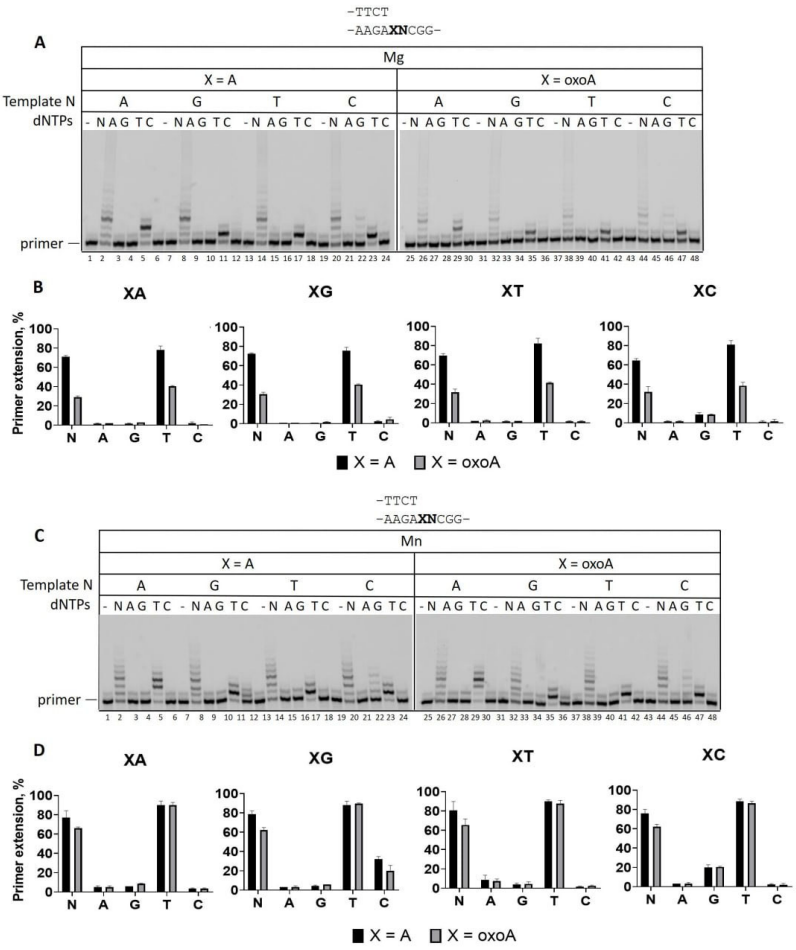

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Figure 3. DNA polymerase activity of PrimPol on DNA with A or 8-oxoA in different sequence context. (a). Primer extension reactions on DNA containing A or 8-oxoA with Mg^{2+}. DNA substrates contain A, G, T or C at the +2 position of the template (N). Reactions were carried out in the presence of 10 mM $MgCl_2$, 200 nM PrimPol, 100 nM Cy5-DNA-substrate and 200 μM of all four dNTP (N) or individual nucleotide substrates (A – dATP, G – dGTP, T – dTTP, C – dCTP) for 4 min. (c). Primer extension reactions on DNA containing A or 8-oxoA with Mn^{2+}. DNA substrates contain A, G, T or C at the +2 position of the template. Reactions were carried out in the presence of 1 mM $MnCl_2$, 200 nM PrimPol, 100 nM Cy5-DNA-substrate and 200 μM of all four dNTP (N) or individual nucleotide substrates (A – dATP, G – dGTP, T – dTTP, C – dCTP) for 1 min. (b, d). Diagrams showing the percent of primer extension on DNA with A or 8-oxoA in reactions in the presence of Mg^{2+} (Figure 3A) and Mn^{2+} (Figure 3C). The mean values of primer extension and standard errors are indicated.

3. Discussion

8-oxoG and 8-oxoA are the most common lesions caused by reactive oxygen species. 8-oxoG is ambiguously read by DNA polymerases leading to mutations after the next round of replication rather than showing blocking effects. The available evidence suggests that 8-oxoA in mammalian cells also has a moderate mutagenic potential and induces A:T → C:G transversions and A:T → G:C transitions in the *HRAS* 5`-CCXAG-3` sequence [25–27].

In contrast to 8-oxoG, the activity of eukaryotic DNA polymerases opposite 8-oxoA remains poorly characterized. Only three DNA polymerases, namely Pol α, Pol β and Pol η, have been studied to date [25,29,30]. These DNA polymerases preferentially incorporated opposite 8-oxoA dTMP and small amounts of dGMP. Pol α carried out quite accurate DNA synthesis by incorporating complementary dTMP 10-fold more efficiently compared to non-complementary dGMP [29], while translesion Pol η incorporated dTMP and dGMP with almost similar efficiency [30]. Accuracy of Pol β varied from 4- to 18-fold preference for dTMP over dGMP on DNA substrates with different sequence context [29,30].

Human PrimPol is a unique DNA primase involved in DNA damage tolerance pathways both in nuclei and mitochondria [1,16,31]. PrimPol can encounter DNA lesions during repriming events and also possesses the DNA translesion activity. PrimPol is also known by template scrunching activity which can generate small deletions [28,32]. This activity is stimulated by Mn^{2+} and DNA lesions (lesion skipping mechanism) [12,28].

In this work, we studied PrimPol bypass of 8-oxopurine lesions in the *HRAS* sequence context. We demonstrated that PrimPol bypasses 8-oxoA with high efficiency and relatively high fidelity. PrimPol preferentially incorporated complementary dTMP opposite 8-oxoA in reactions in the presence of both Mg^{2+} and Mn^{2+} cofactors. PrimPol also incorporated dGMP on DNA substrates with 8-oxoA with 3- to 4-fold reduced efficiency compared to the complementary dTMP. Unlike other DNA polymerases, dGMP-incorporation by PrimPol was observed on DNA with both 8-oxoA and undamaged A. It was sequence-dependent and was stimulated by short CC nucleotide repeats in the *HRAS* CCXAG sequence. Replacement of C in the +2 template position abrogated the dGMP incorporation suggesting that it is mediated by template scrunching mechanism and causes deletions.

The error-prone dGMP incorporation was observed in reactions with both Me^{2+} cofactors but the efficiency of dGMP incorporation was higher with Mn^{2+}. In particular, PrimPol incorporated dGMP on DNA substrate with 8-oxoA ~ 3-fold less efficient compared to undamaged A in the presence of Mg^{2+} ions and with equal efficiencies for both templates in reactions with Mn^{2+}. Another type of errors – dCMP incorporation on DNA templates with A or 8-oxoA – was exclusively stimulated by Mn^{2+} ions and was observed only in reactions with the CGXAG sequence context and guided by G in the +2 templates position. Altogether, these data are in agreement that PrimPol misincorporates nucleotides in a sequence-dependent manner utilizing the Mn^{2+}-stimulated template scrunching mechanism which is not specific to the 8-oxoA lesion. Our data also suggest that the rate of PrimPol-mediated errors is relatively low in all tested sequence contexts and PrimPol unlikely contributes to the 8-oxoA-induced mutagenesis in living cells.

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The 5`-flanking nucleotide near 8-oxoG affects the accuracy of TLS by Pol η [33]. Interestingly, in our work (in the *HRAS* oncogene sequence context 3'-A8-oxoGC-5'), PrimPol demonstrated lower accuracy on DNA with 8-oxoG than in previous studies and incorporated dCMP and dAMP with almost equal efficiencies. In contrast, PrimPol incorporated dCMP almost exclusively or about 6- to 8-fold more efficiently than non-complementary dAMP in other sequence contexts such as 3'-C8-oxoGT-5' [1], 3'-G8-oxoGC-5' [34], 3'-G8-oxoGA-5' [12] and 3'-G8-oxoGT-5' [16]. Crystallographic PrimPol studies demonstrated that 8-oxoG in DNA containing the 3'-C8-oxoGA-5' sequence in complex with both incoming dCTP and dATP adopts the *anti* or *syn* conformation, respectively, without significant structural hindrance within the active site which supports the relatively low accuracy of PrimPol opposite 8-oxoG [35]. Our results corroborate these observations. It is possible that stacking interactions of 8-oxoG with flanking nucleobases contribute to its positioning in the *anti* or *syn* conformation.

Since PrimPol efficiently bypasses several DNA lesions including 8-oxoA, 8-oxoG, 5-fU and thymine glycol, we suggest that PrimPol carries out efficient TLS across a wide range of DNA lesions caused by oxidative stress. Indeed, PrimPol attenuates response of A549 cells to oxidative damage [36]. Also PrimPol as a component of the MUS81-LIG4 axis takes part in replication fork restart during transcription-dependent replication stress under excessive reactive oxygen species action [37]. Recent study demonstrated that PrimPol can contribute to SBS-A mutational signature resembling the mutagenic effect of 8-oxoG due to PrimPol ability to bypass oxidized damage [38].

4. Materials and Methods

4.1. DNA Templates and Enzymes

PrimPol was purified from *E. coli* as described [15]. DNA oligonucleotides used in the study (Table 1) were synthetized as described previously [39]. 8-oxoA and 8-oxoG lesions were placed in the sequence context similar with the *HRAS* CCXAG mutagenesis hot spot [25] in TemplateXA. Templates TemplateXG, TemplateXT and TemplateXC differ from TemplateXA by the single substitution at the +2 position. To prepare DNA substrates, the 5′-Cy5 labeled primer Pr18-Cy5 of ^{32}P-labelled primer Pr18 was annealed to the corresponding unlabeled template oligonucleotides at a molar ratio of 1:1.1 in 100 mM NaCl by heating to 97°C and slowly cooling to 4°C.

Table 1. Oligonucleotides used in the study.

Oligonucleotide	Sequence 5`-3`
Pr18-Cy5	Cy5-AGGGCAGAGTATTCTTCT
Pr18	AGGGCAGAGTATTCTTCT
TemplateXA	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTATACCGCAGGCA **X**AGAAGAATACTCTGCCCT
TemplateXG	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTATACCGCAGGCG **X**AGAAGAATACTCTGCCCT
TemplateXT	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTATACCGCAGGCT **X**AGAAGAATACTCTGCCCT
TemplateXC	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTATACCGCAGGCC **X**AGAAGAATACTCTGCCCT

X = A, 8-oxoA, G or 8-oxoG.

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4.2. DNA Polymerase Reactions for the Primer Extension Assay

Primer extension reactions were performed in 20 μl containing 100 nM DNA substrate, 200 μM dNTP, 30 mM HEPES pH 7.0, 10 mM $MgCl_2$ or 1 mM $MnCl_2$, 100 μg/ml BSA, 1 mM DTT, 4% glycerol and 100 – 200 nM PrimPol. Reactions were incubated at 37°C for 1 – 4 min, stopped by the addition of an equal volume of 2x loading buffer (20 mM EDTA, 0.001% bromophenol blue, 96% formamide) and heated for 5 min at 95°C. The reaction products were resolved on 21% polyacrylamide gels with 8 M urea, visualized on Typhoon 9400 (GE Healthcare, USA) and analyzed with ImageQuant software. All experiments were repeated three times. The percent of the extended primer (PrExt) was calculated for each reaction and the mean values of PrExt with the standard errors are shown in figures.

4.3. Steady-State Kinetics Analysis of dNMP Incorporation

To quantify the incorporation of individual dNMP opposite DNA lesions, we varied the dNTP concentration from 2.5 to 6000 μM in reactions in the presence of 10 nM PrimPol and 10 mM $MgCl_2$ and from 0.25 to 1000 μM in reactions with 5 nM PrimPol and 1 mM $MnCl_2$. Depending on the lesion, the reactions were incubated for 3–20 min with $MgCl_2$ and for 1–10 min with $MnCl_2$ to ensure that less than 40% of the primer is utilized. Calculations were made using GraFit software (Erithacus Software, UK). The data were fit to the Michaelis-Menten equation $V = V_{max} \times [dNTP])/(K_M + [dNTP])$, where V and V_{max} are the observed and the maximum rates of the reaction (in percentages of utilized primer per minute), respectively, and K_M is the apparent Michaelis constant.

Author Contributions: methodology E.O.B., A.A.K., P.N.K., investigation, E.O.B., A.A.K., P.N.K..; resources, A.V.M., A.V.A..; data curation, E.O.B., A.A.K.; writing—original draft preparation, A.V.M..; writing—review and editing, A.V.A.; visualization, E.O.B..; supervision, A.V.M., A.V.A.; funding acquisition, A.V.M.

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Data Availability Statement: All data are available under request.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

8-oxoA	8-oxoadenine
8-oxoG	8-oxoguanine

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