DNA Damage

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Discrimination Between 8-Oxo-2′-Deoxyguanosine and 2′-Deoxyguanosine in DNA by the Single Nucleotide Primer Extension Reaction with Adap Triphosphate**

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Abstract: *The adenosine derivative of 2-oxo-1,3-diazaphenoxazine (Adap) exhibits a superb ability to recognize and form base pairs with 8-oxo-2′-deoxyguanosine (8-oxo-dG) in duplex DNA. In this study, the triphosphate of Adap (dAdapTP) was synthesized and tested for single nucleotide incorporation into primer strands using the Klenow Fragment. The efficiency of dAdapTP incorporation into 8-oxo-dG-containing templates was more than 36-fold higher than with dG-containing templates, and provides better discrimination than does the incorporation of natural 2′-deoxyadenosine triphosphate (dATP). The selective incorporation of dAdapTP into 8-oxo-dG templates was therefore applied to the detection of 8-oxo-dG in human telomeric DNA sequences extracted from H_2O_2-treated HeLa cells. The enzymatic incorporation of dAdapTP into 8-oxo-dG-containing templates may provide a novel basis for sequencing oxidative DNA damage in the genome.*

8-Oxo-2′-deoxyguanosine (8-oxo-dG), which is formed when 2′-deoxyguanosine (dG) reacts with reactive oxygen species (ROS), is one of the most common lesions resulting from oxidative DNA damage.[1] 8-Oxo-dG induces genotoxicity by causing replication errors and transcription interference,[2,3] and cells express several DNA repair enzymes to remove 8-oxo-dG from the genome and the nucleotide pool.[4,5] A number of studies have shown that the intracellular level of 8-oxo-dG is related to diseases and aging[6,7] and that 8-oxo-dG is a biomarker for oxidative damage in cells.[8] Currently, 8-oxo-guanine and the corresponding nucleosides (8-oxo-G and 8-oxo-dG) are typically analyzed by HPLC-ECD, HPLC-MS, etc.[9] Our group previously found that 8-oxoG-clamp acts as a selective fluorescence quenching probe for 8-oxo-G in solution.[10–12] The initial step in the formation of 8-oxo-dG is widely thought to involve the one-electron oxidation of the guanine moiety to form the guanine radical cation, and subsequent electrophilic attack on this guanine residue, with the hole caused by the oxidizing agent migrating to the GG stacks.[13–15] Theoretical studies support a distinctive non-random pattern for the genome-wide distribution of 8-oxo-dG in DNA,[16] along with sequence-specific DNA damage in the -GGG- context of synthesized telomeric repeat sequences.[17,18] Because of the biological importance of 8-oxo-dG sequencing, the ability to discriminate between 8-oxo-dG and dG within DNA is an important issue. Methods for detecting 8-oxo-dG in DNA have previously been developed using antibodies,[19] small molecules,[20] and nanopore systems,[21] among others,[22] but these methods are not suitable for analyzing the sequences of intact DNA samples. Recently, we found that the novel adenosine nucleoside analogue Adap (adenosine-1,3-diazaphenoxazine) an hybridize with 8-oxo-dG and that this could be detected by the fluorescence quenching effect in duplex DNA.[23] This detection method has successfully been applied to the FRET (fluorescence resonance energy transfer)-based strand exchange reaction, and to SYBR Green detection using oligonucleotide (ODN) probes containing Adap and the Adap derivative 2-amino-Adap, respectively.[24,25] Because the accuracy of these methods is based on the melting temperature of ODN probes containing Adap derivatives, precise molecular design, along with substantial trial and error, is necessary to develop useful probes consisting of the correct sequence element and length for the sequence-specific recognition of 8-oxo-dG. In the meantime, single-base extension technology is a useful tool for performing genotyping,[26] and unnatural nucleosides, including universal nucleobase analogues, are being applied to the development of DNA sequencing technologies.[27] We therefore attempted to apply Adap to the enzymatic primer extension reaction to selectively identify 8-oxo-dG amidst a large amount of dG in a DNA sequence. Herein, we report the first demonstration of the selective detection of 8-oxo-dG over dG within synthesized or intact DNA sequences by the single nucleotide primer extension reaction using Adap triphosphate and the Klenow Fragment (see Scheme S1 in the Supporting Information and Figure 1). Our results indicate that these nucleoside analogues are candidates for novel tools in the identification of the position of the oxidative damage in DNA.

To identify the base preference of dAdapTP, single nucleotide primer extension reactions were performed using a 25-mer Template(**X**) (X = oxodG, dG, dA, dC or T) and a FAM-labeled 15-mer primer, Primer**1**, and the Klenow

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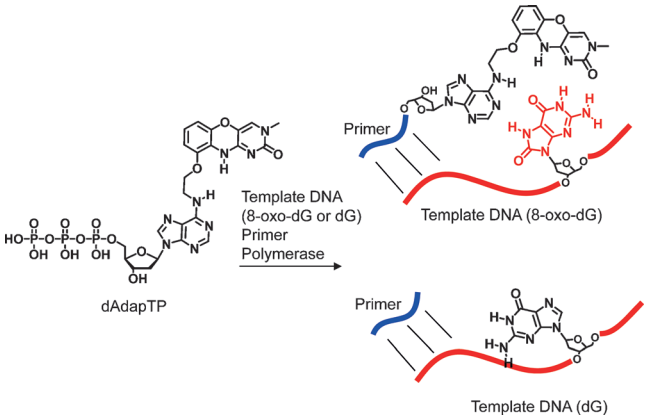


Figure 1. Schematic illustration of the discrimination between 8-oxo-dG and dG in template DNA through an enzymatic primer extension reaction using Adap triphosphate.

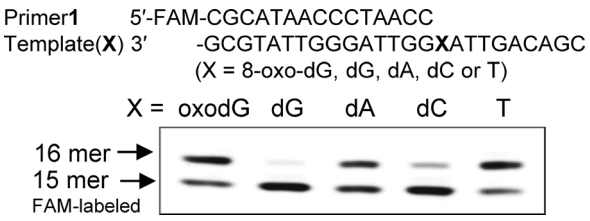
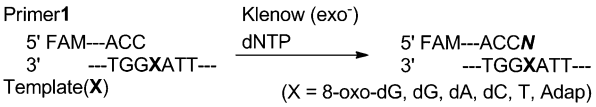


Figure 2. Single nucleotide primer extension reaction of dAdapTP for the Template(**X**) (X = 8-oxo-dG, dG, dA, dC and T). Conditions: 1.0 μM 15-mer/25-mer FAM-labeled Primer**1**-Template(**X**) duplex, 0.1 unit/μL Kf(exo⁻), 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 50 μM dAdapTP incubated at 37 °C for 10 min in a 10 μL reaction volume.

fragment (Kf exo⁻). The T_m value of the template-primer DNA duplex was 59.2 °C in the buffer conditions used. The reaction was performed in buffer containing 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$, and 1 mM DTT, and the progress of the reaction was analyzed by a gel-shift assay (Figure 2). Preferential extension was observed for the templates containing 8-oxo-dG or T, a somewhat lower efficiency was found for the dA template, and no significant extension occurred for the template ODNs which included dG or dC. Unfortunately, dAdapTP was incorporated opposite T in the template strand even better than 8-oxo-dG because Adap has the dA skeleton. However, it completely discriminated between 8-oxo-dG and dG in the template ODNs. For quantitative comparisons of the extension efficiency, we obtained steady-state kinetic data for the primer extension reactions using dAdapTP against template ODNs containing 8-oxo-dG, dG, dA, dC, and T, and compared the results with those obtained using either dATP or dCTP as a control. The results of the steady-state kinetics (V_{max}: the maximum rate of the enzyme reaction. K_M: the Michaelis constant.) are summarized in Table 1 and Table S1 (see the Supporting Information). Interestingly, the extension efficiency (V_{max}/K_M) of dAdapTP for the 8-oxo-dG template was similar to that of dATP (Table 1, entry 1 versus 6). The dAdapTP reaction had a more favorable K_M value, whereas dATP had a higher V_{max} value, thus resulting in similar V_{max}/K_M values. Given that the adenine base forms a Hoogsteen base pair with 8-oxo-dG, it is reasonable to assume that the Adap:8-oxo-dG base pair is stabilized by an additional Watson–Crick-type base pair as expected. Molecular models, in which the adenine base of the adenosine:8-oxo-dG mismatch base pair in the DNA polymerase I/DNA complex (PDB 1U49) was modified to attach phenoxazine with an ethoxy linker, show that the phenoxazine moiety may be located in the space of the polymerase active site to form a Watson–Crick-type base pair with 8-oxo-dG in a *syn*-conformation in the template ODN (see Figure S1 in the Supporting Information). The fact that the V_{max} value of dAdapTP was much lower than that of dATP suggests that the presence of phenoxazine leads to unfavorable effects in the primer extension reaction for the 8-oxo-dG template using Kf polymerase. In contrast, the V_{max} value of dAdapTP for the T template was reduced compared with that of dATP, with slightly lower K_M values (Table 1, entry 5 versus 10). As

Table 1: Steady-state kinetics for primer extension of dNTP opposite to nucleoside in the template ODNs by the Klenow fragment (exo⁻).[a]



Entry	dNTP	Template X=	V_{max} [% min^{-1}]	K_M [μM]	V_{max}/K_M [% min^{-1} M^{-1}]	Relative [%]
1		oxo-dG	2.35 (0.37)	0.80 (0.02)	3.02×10^6	100
2		dG	0.26 (0.01)	3.11 (0.25)	0.08×10^6	2.75
3	dAdapTP	dA	0.99 (0.26)	6.35 (0.34)	0.16×10^6	5.17
4		dC	0.79 (0.01)	5.40 (0.47)	0.18×10^6	4.87
5		T	5.23 (0.43)	0.48 (0.14)	10.9×10^6	360
6		oxo-dG	14.9 (0.97)	4.42 (0.39)	3.30×10^6	100
7		dG	0.90 (0.06)	3.21 (0.29)	0.28×10^6	8.44
8	dATP	dA	0.85 (0.05)	2.75 (0.17)	0.31×10^6	9.37
9		dC	0.86 (0.13)	3.83 (0.74)	0.23×10^6	6.82
10		T	13.8 (1.42)	0.73 (0.06)	18.9×10^6	572
11	oxo-dGTP		4.72 (0.34)	1.78 (0.76)	2.50×10^6	100
12	dGTP		0.22 (0.02)	2.24 (0.62)	0.10×10^6	3.87
13	dATP	Adap	0.50 (0.08)	1.64 (0.03)	0.31×10^6	12.3
14	dCTP		0.44 (0.05)	0.60 (0.07)	0.74×10^6	29.6
15	TTP		8.02 (1.72)	3.17 (0.04)	2.53×10^6	101
16	oxo-dGTP		6.43 (0.13)	2.16 (0.98)	2.98×10^6	100
17	dGTP		0.53 (0.04)	0.95 (0.37)	0.55×10^6	18.6
18	dATP	dA	0.85 (0.05)	2.75 (0.17)	0.31×10^6	10.4
19	dCTP		0.55 (0.06)	0.58 (0.08)	0.95×10^6	32.0
20	TTP		8.85 (0.13)	0.72 (0.04)	12.3×10^6	411

[a] Conditions: 1.0 μm 15-mer/25-mer FAM-labeled Primer**1**-Template(**X**) duplex, 0.01–0.04 unit/μL Kf(exo⁻), 10 mm Tris-HCl (pH 7.9), 50 mm NaCl, 10 mm $MgCl_2$, 1 mm DTT, and dNTPs incubated at 37 °C for 1–20 min in a 10 μL reaction volume. Standard deviations are given within parentheses (three independent experiments). Velocity is normalized for the lowest enzyme concentration used.

a result, the relative efficiency for T over 8-oxo-dG with either dATP or dAdapTP was improved from 5.7 to 3.6. It is noteworthy that the V_{max} value of dAdapTP for the template was also decreased compared with that of dATP, though the K_M values were similar, thereby increasing the selectivity 8-oxo-dG over dG from 12-fold for dATP to 36-fold for dAdapTP (Table 1, entry 2 versus 7). The V_{max}/K_M value of dCTP for the dG template is larger than that for 8-oxo-dG (relative efficiency = 7.2; see entries 21 and 22 in Table S1 in the Supporting Information). The extension efficiencies of dAdapTP for the dA and dC templates were comparable to those for the mismatch base pair (Table 1, entries 3 and 4). Thus, dAdapTP shows the greatest distinction between 8-oxo-dG and dG in an enzymatic single nucleotide primer extension reaction.

To determine whether the presence of Adap in a DNA template would affect the primer extension reaction, we investigated the enzymatic incorporation of each dNTP into Primer**1** using a template ODN containing either Adap or dA (Table 1, entries 11–15 versus 16–20). The V_{max}/K_M values of the Adap template were almost identical to those of the dA template, with the exception of dGTP and TTP. The latter reduction was mainly attributed to the higher K_M values of the Adap template compared with those of the dA template (Table 1, entriy 12 versus 17 and 15 versus 20). These results indicated that it is difficult to form the complex between the Adap template and either dGTP or TTP because of the interference from the phenoxazine moiety in the enzyme active site. Fortunately, the K_M values for 8-oxo-dGTP indicated that the phenoxazine unit contributed to the interactions with the *syn*-conformer of 8-oxo-dGTP as discussed above (Table 1, entry 11 versus 16). The resulting relative efficiency (101) of the Adap template for TTP over 8-oxo-dGTP was improved compared with that (411) of the dA template (Table 1, entries 15 and 20). Furthermore, the discrimination ability for 8-oxo-dGTP versus dGTP increased from 5.4-fold in the dA template to 26-fold in the Adap template. The incorporation efficiency of dATP and dCTP for the Adap template also showed the levels of misincorporation. These results indicated that dAdapTP and the Adap template are highly capable of discriminating between 8-oxo-dG and dG in the 2′-deoxynucleoside triphosphate and the template ODN in enzymatic single nucleotide primer extension reactions.

Further studies were then performed to carry out the sequence-specific detection of 8-oxo-dG in telomeric DNA using dAdapTP and Kf (exo$^-$). The oxidation of dG in telomeric DNA is well known to be related to aging,[17a] and telomeric DNA is more susceptible to oxidative base damage than nontelomeric regions in vivo.[28] First, we used three types of synthetic 25-mer template ODNs based on a $(TTAGGG)_4$ repeat sequence: Template**1** (X = Y = dG), Template**2** (X = dG, Y = 8-oxo-dG), and Template**3** (X = 8-oxo-dG, Y = dG), as well as two different types of primers, as shown in Figure 3a. One primer consisted of an 18-mer oligonucleotide which targeted the middle of the -GGG- sequence and was labeled with Cy5, and the other primer consisted of a 17-mer oligonucleotide which was directed against the 5′-side of the -GGG- sequence and was labeled

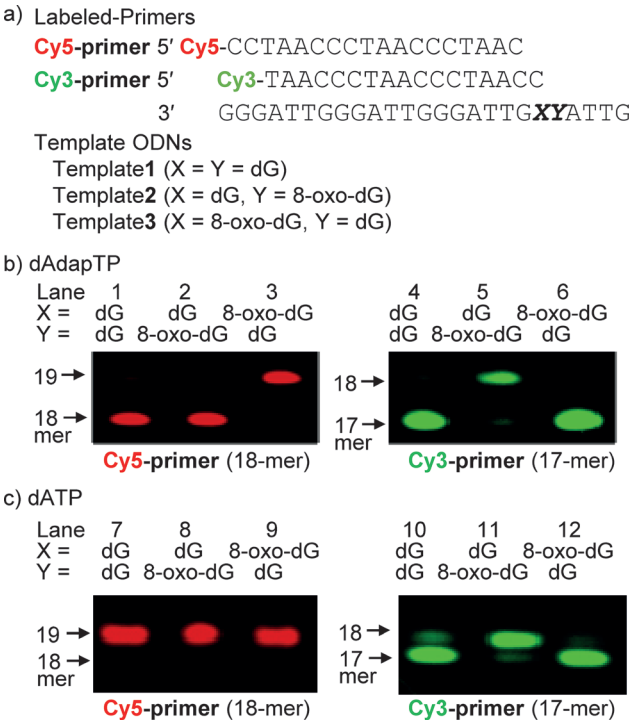


Figure 3. Sequence-specific incorporation of dAdapTP for 8-oxo-dG. a) The sequences of the template ODNs and primers. b) The gel results of primer extension reaction with dAdapTP. c) The gel results with dATP. Conditions: 1.0 μM 18-mer/25-mer Cy5-labeled primer-template duplex or 1.0 μM 17-mer/25-mer Cy3-labeled primer-template duplex, 0.1 unit/μL Kf(exo$^-$), 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT and 62.5 μM either dAdapTP or dATP incubated at 37 °C for 10 min in a 10 μL reaction volume.

with Cy3. The T_m values of the DNA duplexes containing Template**1** and either Cy5-primer or Cy3-primer were confirmed to be 63.6 and 61.7 °C, respectively. (see Figure S2 in the Supporting Information). The single nucleotide primer extension reactions were run on gels and visualized using a fluoroimager to detect the corresponding color bands (Figure 3b and c). In the case of the Cy5-primer, a slower migrating band was clearly observed with Template**3**, which contained 8-oxo-dG on the strand opposite the incorporation site (Figure 3b, Lane 3), whereas there were no extended bands for either Template**1** or Template**2** (Figure 3b, Lanes 1 or 2). In contrast, the Cy3-primer was elongated in the presence of Template**2**, dAdapTP, and Kf (Figure 3b, Lane 5). The Adap-incorporated band was not observed in the template ODNs containing dG (Figure 3b, Lanes 4 or 6). In contrast to the ability of dAdapTP to discriminate between 8-oxo-dG and dG, dATP was misincorporated in both the 8-oxo-dG and dG templates (Figure 3c, Lanes 7–12). In the case of the Cy5-primer, dATP was also incorporated into the dG template (Figure 3c, Lanes 7 and 8), even with a shorter incubation time (see Figure S3 in the Supporting Information; Lane 1–4). Moreover, dATP was slightly incorporated into the Cy3-primer using the control dG template, Template**1** (Figure 3c, Lane 10). Importantly, this single nucleotide primer extension reaction is easier and more useful than the

fluorescence quenching effect or light-up system used in our previous studies to detect and sequence 8-oxo-dG in DNA with Adap probes.[23–25]

Finally, DNA was prepared from H_2O_2-treated HeLa cells to test the recognition of 8-oxo-dG in telomeric DNA. HeLa cells were cultured in a medium containing H_2O_2 concentrations ranging from 0 to 1000 μM for 30 minutes at 37 °C under 5% CO_2, and DNA was extracted using an established procedure in the presence of an oxidation inhibitor to reduce the excessive oxidation of nucleosides. After the polymerase reactions were analyzed by gel electrophoresis, since the primers are present in excess, the percentage of the incorporated band was found to depend on the amount of DNA (see Figure S4 in the Supporting Information). Therefore, we used a low amount of each primer and a constant amount of each H_2O_2-treated DNA sample (Figure 4). A low signal intensity

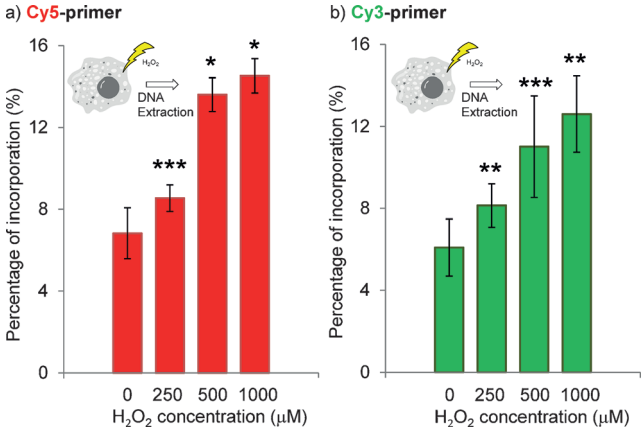


Figure 4. Single nucleoside primer extension reactions in DNA (10 μg) from HeLa cells treated with various concentrations of H_2O_2 (0–1000 μM). a) 0.25 μM 18-mer Cy5-labeled primer. b) 0.25 μM 17-mer Cy3-labeled primer, 0.1 unit/μL Kf(exo$^-$), 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, and 50 μM dAdapTP, incubated at 37 °C for 60 min in a 10 μL reaction volume. Error bars indicate standard deviations from three independent experiments. $*p < 0.01$, $**p < 0.02$, $***p < 0.05$ compared with 0 μM H_2O_2-containing medium.

was detected in the primer elongation area using H_2O_2-untreated HeLa DNA as a control (Figure 4 and Figure S4), thus suggesting that 8-oxo-dG residues are either present endogenously in telomeric DNA or were generated during the extraction process, or dAdapTP was misincorporated into the primer strands, but the details remain unclear. However, the intensity of the incorporated band increased in a dose-dependent manner along with the concentration of the H_2O_2 solution in the media (Figure 4). As shown in the bar graph, dAdapTP was significantly incorporated into the Cy5-primer for the target in the middle of the -GGG- context (Figure 4a). In contrast, Adap incorporation into the Cy3-primer increased gradually (Figure 4b). The experimental and theoretical studies have shown that the selectivity of oxidation site in the -GGG- context depends on the oxidation methods in the test tube.[14,15,17,18] Although no information is available about the selectivity of the oxidation of dG in telomeric DNA in the cells, our results might reflect the selectivity of the generation of the 8-oxo-dG of -GGG- context in the cells. It is difficult to estimate the absolute amount of 8-oxo-dG by this method, nevertheless, these results show the possibility of the sequence-specific recognition of 8-oxo-dG in telomeric DNA extracted from H_2O_2-treated HeLa cells.

In summary, we have synthesized dAdapTP and evaluated its use in the single nucleotide primer extension reaction with the Klenow fragment. dAdapTP was incorporated opposite to not only the 8-oxo-dG template but also the T template. However, our results show that dAdapTP was the most capable of discriminating between 8-oxo-dG and dG among the natural nucleoside triphosphate in the template ODN. In the case of template ODN, 8-oxo-dGTP and TTP were inserted opposite Adap with the same incorporation efficiency. Most importantly, Adap can discriminate between 8-oxo-dG and dG in enzymatic primer extension reaction at the level of both triphosphate and template ODN. Finally, this system can be used to ascertain the level of 8-oxo-dG in template DNA extracted from H_2O_2-treated HeLa cells. Future experiments will focus on assessing the activity and fidelity of various polymerases to apply dAdapTP to a novel DNA sequencing technology.

Keywords: DNA damage · enzymes · nucleotides · oxidation · sequence determination

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