

Comparison of the mutagenic properties of 8-oxo-7,8-dihydro-2'-deoxyadenosine and 8-oxo-7,8-dihydro-2'-deoxyguanosine DNA lesions in mammalian cells

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The comparative mutagenicity of 8-oxo-7,8-dihydro-2'-deoxyadenosine (8-oxodA) and 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) was explored using simian kidney (COS-7) cells. Oligodeoxynucleotides [5'-TCCTCCT-G₁X₂CCTCTC or 5'-TCCTCCTX₁G₂CCTCTC (X = dA, dG, 8-oxodA or 8-oxodG)] containing 8-oxodA or 8-oxodG positioned within codon 60 or 61 of the non-coding strand of human c-Ha-ras1 gene were inserted into a single-stranded phagemid shuttle vector. The vector was replicated in COS-7 cells and the progeny plasmids were used to transform *Escherichia coli* DH10B. The transformants were analyzed by oligodeoxynucleotide hybridization and DNA sequence analysis to establish the mutation frequency and specificity. When 8-oxodA was positioned at X₁, targeted A^{oxo}→C transversions were detected; the mutation frequency was 1.2%. When 8-oxodA was positioned at X₂, one targeted mutant among 416 colonies screened (an A^{oxo}→G transition) was detected. Thus, the mutation frequency and spectrum of 8-oxodA depend on the sequence context of the lesion. The mutation frequency of 8-oxodG at X₁ and X₂ was 5.2 and 6.8%, respectively. G^{oxo}→T transversions dominated the spectrum, accompanied by small numbers of G^{oxo}→A transitions and G^{oxo}→C transversions. We conclude that 8-oxodA has mutagenic potential in mammalian cells, generating A→C transversions. However, when tested under similar conditions, the mutation frequency of 8-oxodA is at least four times lower than that of 8-oxodG.

Introduction

Reactive oxygen species (ROS) arise in living cells as byproducts of cellular metabolism and from exogenous sources (1). ROS react with DNA, generating a variety of structural modifications including base damage, sugar damage and DNA–protein crosslinks (2,3). Oxidized purines in DNA, including 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) and 8-oxo-7,8-dihydro-2'-deoxyadenosine (8-oxodA), have been implicated in mutagenesis, carcinogenesis and aging (1–3).

8-OxodG is a commonly found base modification in mammalian DNA and is known to be mutagenic *in vitro* and *in vivo* (4–9). The level of 8-oxodG in DNA increases with oxidative stress (10). Prokaryotic and eukaryotic DNA

Abbreviations: 8-oxodA, 8-oxo-7,8-dihydro-2'-deoxyadenosine; 8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; ds vector, double-stranded vector; HPLC, high-performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; ss vector, single-stranded vector.

polymerases misincorporate dAMP opposite 8-oxodG (4). G→T transversion is the principal mutagenic event observed in *Escherichia coli* and mammalian cells (5–9).

8-OxodA has been recovered from DNA of γ -irradiated mice and from human cancer tissues (11–13). Using the Klenow fragment of *E. coli* DNA polymerase I and mammalian DNA polymerases α and β , Shibutani *et al.* showed that dTMP, the correct base, is incorporated almost exclusively opposite 8-oxodA (14,15). With pol β , small amounts of dGMP were inserted opposite 8-oxodA (14,15). Kamiya *et al.* reported similar results, using a polymerase chain reaction-restriction enzyme (PCR-RE) method (16). This group observed that pol α and pol β facilitated misincorporation of dGMP and/or dAMP *in vitro*; however, misincorporation of dAMP was not detected in mutagenesis studies in cells (16).

In *E. coli*, the mutagenic potential of 8-oxodA is reported to be at least an order of magnitude less than that of 8-oxodG (17). Using NIH 3T3 cells and a double-stranded vector containing 8-oxodA, Kamiya *et al.* reported that ~1.0% of mutants contained targeted A→G transitions and A→C transversions (16). In a double-stranded (ds) vector, 8-oxodA could be removed by DNA repair enzymes; in this study, a single-stranded (ss) vector was used to minimize such repair.

A 15mer oligodeoxynucleotide containing a single 8-oxodA or 8-oxodG adduct positioned at codon 60 and 61 of the non-coding strand of human c-Ha-ras1 gene was inserted into a ss pMS2 vector. To explore the mutagenicity of the oxidized bases, the vector was transfected into mammalian COS-7 cells and progeny plasmid used to transform *E. coli* DH10B. We conclude from this study that 8-oxodA is only weakly mutagenic, generating A→C transversions in mammalian cells. When positioned within the same sequence context, the mutational frequency of 8-oxodA was four to 28 times less than that of 8-oxodG.

Materials and methods

Bacteria, mammalian cells and plasmids

Escherichia coli DH10B was purchased from Gibco BRL. Simian kidney (COS-7) cells were obtained from the tissue culture facility of SUNY Stony Brook. ss phagemid vector, pMS2, was isolated from *E. coli* JM109 harboring the helper phage VCSM13 (Stratagene, La Jolla, CA) as described previously (7).

Synthesis and purification of oligodeoxynucleotides

Unmodified and modified oligodeoxynucleotides (5'-TCCTCCT-G₁G₂-CTCTC, 5'-TCCTCCTA₁G₂CCTCTC and 5'-TCCTCCTG₁A₂CCTCTC) (18) in which G₁, G₂, A₁ or A₂ were replaced by 8-oxodG and 8-oxodA (19), were prepared by solid-state synthesis on a Dupont Coder 300 automated synthesizer and purified on a reverse-phase μ Bondapak C₁₈ column (0.39 30 cm; Waters, Milford, MA), eluted over 60 min at a flow rate of 1.0 ml/min with a linear gradient of 0.05 triethylammonium acetate, pH 7.0, containing 10–15% acetonitrile (20). Oligodeoxynucleotides were further purified by electrophoresis on 20% polyacrylamide gels in the presence of 7 M urea. Bands were extracted by soaking in distilled water overnight. Samples were concentrated using Centricon no. 3 molecular filter (Amicon, Beverly, MA) and precipitated with ethanol to remove urea.

Construction of circular ssDNA containing single 8-oxodA or 8-oxodG residues
Following a published procedure (8), ssDNA vectors containing 8-oxodG or 8-oxodA were constructed as shown in Figure 1. Briefly, ss pMS2 was

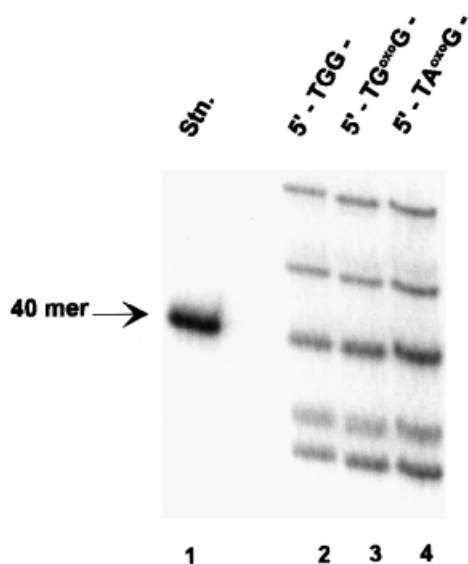


Fig. 2. Confirmation of insertion of 8-oxodG into the constructed ss vector. A portion of the constructed ss vector annealing with the 61mer scaffold was digested with *BanI* and *HaeIII*, and subjected to 12% denaturing gel, as described in Materials and methods.

between the unmodified and modified oligodeoxynucleotide was observed (data not shown).

The final concentration of ssDNA vector was quantified by Southern blot hybridization (data not shown). The S13 probe was hybridized to the ligation site of the ss vector (Figure 1). Using the β -phosphorimager, the net production of ccDNA of each construct was estimated by comparison with pMS2 DNA standards. The concentration of unmodified and modified cc vector was 24 ng/ μ l for 5'-TG₁G₂, 40 ng/ μ l for 5'-TG^{oxo}₁G₂, 29 ng/ μ l for 5'-TG₁G^{oxo}₂, 25 ng/ μ l for 5'-TG₁A₂, 13 ng/ μ l for 5'-TA₁G₂, 27 ng/ μ l for 5'-TG₁A^{oxo}₂ and 15 ng/ μ l for 5'-TA^{oxo}₁G₂.

Mutagenicity of 8-oxodA and 8-oxodG in COS-7 cells

An aliquot containing 500 ng of ccDNA was used to transfect COS-7 cells. Progeny plasmid obtained were used to transform *E. coli* DH10B. The DNA sequence of randomly selected colonies was determined by oligodeoxynucleotide hybridization and/or nucleotide sequence analysis. The transformation efficiency (88–90%) of the 8-oxodA-modified vector was slightly less than that of unmodified ssDNA (Table I) and higher than that (64–78%) of the 8-oxodG-modified cc vector (Table II). Thus, 8-oxodA does not represent a significant block to DNA synthesis in mammalian cells.

When 8-oxodA was positioned at the position X₁ (5'-TX₁GC-), four targeted mutants showing A→C transversion were detected among 337 colonies recovered; the mutation frequency was 1.2% (Table I). No targeted mutations were observed in the control experiment. However, when 8-oxodA was at X₂ (5'-TGX₂C-), only one targeted mutant showing A→G transition was observed among 416 colonies. This mutation frequency (0.24%) does not differ significantly from the unmodified control.

Positioning 8-oxodG in a similar sequence context, G→T transversions (4.0%, Table II) were generated opposite 8-oxodG in 5'-TX₁GC-, accompanied by G→C transversions and G→A transitions. When 8-oxodG was in the 5'-TGX₂C-sequence, G→T transversions were preferentially observed (Table II). Small numbers of G→A transitions also were

detected. No mutations were detected in the control experiment. Thus, mutational frequencies for 8-oxodG at X₁G and GX₂ were 5.2 and 6.8%, respectively; this difference is not statistically significant.

Discussion

8-OxodA or 8-oxodG, inserted into codon 60 and 61 of the non-coding strand of human *c-Ha-ras1* gene, were used to investigate the mutagenic potential of these prominent oxidized bases in mammalian cells. Targeted A→C transversions were found in 5'-TA^{oxo}GC-; the mutation frequency was 1.2%. However, in 5'-TGA^{oxo}C-, only one A→G transition in over 400 colonies screened was detected. Thus, the mutational frequency and spectra of 8-oxodA varies depending on the sequence context of the lesion.

Using a PCR-RE method, Kamiya *et al.* reported that 8-oxodA led to A→G transitions, along with lesser number of A→C and A→T mutations (16). The mutational spectra reported here differ from that reported by Kamiya *et al.* (16) but are fully consistent with results obtained *in vitro* (14,15). Thus, DNA pol α , a mammalian replicative enzyme, was shown to direct incorporation of dGMP opposite 8-oxodA in reactions containing a single dNTP (14). In addition, incorporation of dCMP and dAMP was not detected in fully-extended products formed on 8-oxodA-modified templates (14,15). Kamiya *et al.* also reported misincorporation of dGMP catalyzed by pol α (16). Based on these experiments, A→C transversions are expected to occur in cells; the mutational spectra reported here reflect the miscoding specificities observed *in vitro*. Parenthetically, we note that PCR-RE requires highly specific and selective restriction enzymes; this method may not be ideal for quantitative analysis of mutations.

8-OxodG was inserted into an oligodeoxynucleotide having same sequence context as experiments performed with 8-oxodA (Table II). When 8-oxodG was at X₁ in 5'-TX₁GC-, 4.0% of the progeny contained targeted G→T transversions. G→C and G→A mutations were also observed. In 5'-TGG^{oxo}C-, preferential G→T transversions (6.0%) were detected, along with lesser amounts of G→A transitions (0.8%). Thus, the mutational spectra of 8-oxodG also may depend on sequence context. The overall mutational frequency in the two sequences tested were similar (5.2 versus 6.8%). The mutational frequencies of 8-oxodA in the same sequence context were 4.3 and 28.3 times less, respectively, than that of 8-oxodG. Thus, 8-oxodA is significantly less mutagenic than 8-oxodG in simian kidney cells.

Mutational spectra and frequencies of 8-oxodG reported from several laboratories are summarized in Table III. When 8-oxodG was positioned in codon 12 of the *c-Ha-ras1* gene (5'-CG^{oxo}GC-), only G^{oxo}→T mutations were detected (9,22). These results are consistent with our experiments showing that G^{oxo}→T mutations are exclusively detected when 8-oxodG is similarly positioned (5'-TG^{oxo}GC-) in codon 61 of the *c-Ha-ras1* gene. When 8-oxodG was placed 3' in codon 12 (5'-CGG^{oxo}C-), G^{oxo}→T mutations also were observed (9,22). Thus, G^{oxo}→T transversions are the principal mutagenic events generated by 8-oxodG in almost all previous reports (8,9,22) and in the present study.

Kamiya *et al.* reported significant numbers of G^{oxo}→A mutations using NIH 3T3 host cells and the PCR-RE method (22). Using the same sequence context, COS-7 cells and a ss vector, Le Page *et al.* did not observe this mutation (9). In our

Table I. Mutational specificity of 8-oxodA in COS-7 cells

Plasmid	Experiment	No. of transformants	No. of colonies analyzed	No. of targeted mutants					Targeted mutation frequency	Others
				A ^{oxo} →	C	A	G	T		
5'-TA ₁ G ₂ C-	1	2467	174	0	174	0	0	0	0	
	2	3142	138	0	138	0	0	0	0	
	Total	5609 (100%)	312	0	312	0	0	0	<0.32%	
5'-TA ^{oxo} ₁ G ₂ C-	1	1939	145	1	141	0	0	0	3	
	2	3095	192	3	188	0	0	0	1	
	Total	5034 (89.7%)	337	4	329	0	0	0	1.20% ^a 4 ^b	
5'-TG ₁ A ₂ C-	1	2662	227	0	0	224	0	0	3	
	2	4231	230	0	228	0	0	0	2	
	Total	6893 (100%)	457	0	452	0	0	0	<0.22% 5 ^c	
5'-TG ₁ A ^{oxo} ₂ C-	1	2230	225	0	223	1	0	0	1	
	2	3866	191	0	191	0	0	0	0	
	Total	6096 (88.4%)	416	0	414	1	0	0	0.24% 1 ^d	

Aliquots of 500 ng close circular ss vector were used to transfect COS-7 cells in duplicate. Progeny phagemid was recovered after 48 h and used to transform *E.coli* DH10B for mutation analysis. Mutation frequency is calculated by: [(no. of G + C + T)/total analyzed colonies]×100.

^aSignificantly different from the control value; *P* < 0.05.

^bNon-targeted mutations (mutation indicated by lowercase letter): 3× 5'-TcCTAGCCTCTC (C→A); 1× 5'-TCCTCTAGCATCTC (C deletion).

^cNon-targeted mutations (mutation indicated by lowercase letter): 3× 5'TCCTCTGACCTgTC (C→G); 1× 5'TCCTACTGACCTCTC (C deletion); 1× 5'TCCTCΔTGACCTCTC (C deletion).

^dNon-targeted mutations (mutation indicated by lowercase letter): 1× 5'TCCTCTcACCTCTC (G →C).

Table II. Mutational specificity of 8-oxodG in COS-7 cells

Plasmid	Experiment	No. of transformants	No. of colonies analyzed	No. of targeted mutants					Targeted mutant frequency	Others
				G ^{oxo} →	C	A	G	T		
5'-TG ₁ G ₂ C-	1	7313	127	1	0	126	0	0	0	
	2	6546	113	0	0	113	0	0	0	
	Total	13859 (100%)	240	1	0	239	0	0	0.42%	
5'-TG ^{oxo} ₁ G ₂ C-	1	4322	126	1	1	117	5	5	2	
	2	4518	125	1	0	118	5	5	1	
	Total	8840 (63.8%)	251	2	1	235	10	10	5.2% ^a 3 ^b	
5'-TG ₁ G ₂ C-	1	7313	127	0	0	126	0	0	0	
	2	6546	113	0	0	113	0	0	0	
	Total	13859 (100%)	239	0	0	239	0	0	<0.42%	
5'-TG ₁ G ^{oxo} ₂ C-	1	5747	126	0	1	111	13	13	1	
	2	5004	124	0	0	120	3	3	1	
	3	-	116	0	2	110	6	6	1	
	Total	10751 ^c (77.6%)	366	0	3	341	22	22	6.8% ^d 3 ^e	

Aliquots of 500 ng close circular ss vector were used to transfect COS-7 cells in duplicate. Progeny phagemid was recovered after 48 h and used to transform *E.coli* DH10B for mutation analysis. Mutation frequency is calculated by: [(no. of A + C + T)/total analyzed colonies]×100.

^aSignificantly different from the control value; *P* < 0.0001.

^bNon-targeted mutations (mutation indicated by lowercase letter): 1× 5'-TCCTcTGGCCTCTC (C→T); 1× 5'-TtCTCTGGCCTCTC (C→T); 1× 5'-TCgTCCTGGCCTCTC (C→G).

^cNumber of experiment 3 is not included.

^dSignificantly different from the control value; *P* < 0.05.

^eNon-targeted mutations (mutation indicated by lowercase letter): 3× 5'-TCCTCTcGCCTCTC (G→C).

Table III. Mutational spectrum and frequency induced by 8-oxodG with different sequence context and experimental system

Sequence context (X = 8-oxodG)	Host cell	Targeted mutation					Targeted mutation frequency	Reference
		G ^{oxo} →	C	A	T	Δ		
5'-CXTA-	COS-7	1	0	15	0	0	3.7%	(8)
5'-CXGC-	NIH 3T3	0	1	14	0	0	-	(22)
5'-CGXC-	NIH 3T3	1	8	22	0	0	1.0% ^a	
5'-TXGC-	NIH 3T3	0	0	35	0	0	-	
5'-CXGC-	COS-7	0	0	12	0	0	4.0%	(9)
5'-CGXC-	COS-7	0	0	12	1	0	4.0%	
5'-TXGC-	COS-7	2	1	10	0	0	5.2%	This study
5'-TGXC-	COS-7	0	3	22	0	0	6.8%	

^aThe mutation frequency is calculated on relative focus formation of that of activated c-Ha-ras gene.

experiments, using a similar system in which the 5' flanking base, dC (5'-CGG^{oxo}C-) was replaced by dT (5'-TGG^{oxo}C-), a small number of G^{oxo}→A mutations were detected. Thus, the frequency of this transition may depend on the sequence context of the lesion and/or the host cell used for the experiment. We conclude that 8-oxodG generates primarily G→T transversions together with a much smaller number of G→A transitions.

When ds vectors containing 8-oxodG were used for mutagenesis studies, G→T transversions also were observed; however, mutational frequencies were ~1.0% (22). With ss vectors, mutational frequencies range between 3.7 and 6.8% (8,9; this study). A DNA glycosylase that excises 8-oxodG (Ogg1) has been identified in mammalian cells (23–25); thus, observed differences may reflect the contribution of DNA repair. So far, repair activities that excise 8-oxodA from DNA have not been reported.

The presence of an oxygen atom at position 8 of deoxyguanine and deoxyadenine alters the electronic and steric properties of these DNA bases and leads to miscoding during replication of DNA (4,14). Structural studies reveal that 8-oxodG can assume the *syn* conformation to form a stable Hoogsteen base pair with dA (26,27). This pair also is resistant to the proofreading exonuclease activity associated with certain DNA polymerases (4), enhancing the mutagenic potential of 8-oxodG. Based on its weak mutagenicity, 8-oxodA is assumed to pair preferentially with dT under physiological conditions (28); the targeted A→C transversions reported in the present paper indicate that the A^{oxo}:dG pair also is formed. Since the DNA duplex is expected to retain a B conformation, one of the two purines is expected to assume the *syn* conformation (29).

In the *p53* gene of human tumors and cell lines, G→A transitions are major mutations (41–65%) in colon, breast, bladder and brain tumors; the frequency of G→T transversions is 3–7 times less than G→A transitions (30). In lung tumors, the frequency of G→T mutations is slightly higher than G→A (30). In addition, G→A and G→T mutations were frequently detected as spontaneous mutations in mammalian cells (31). However, mutations occur infrequently at A:T pairs in human tumors (30) and spontaneous A→C transversions are rare in mammalian cells (31). Thus, 8-oxodG contributes significantly to the pool of G→T mutations in *p53* while the potential of 8-oxodA for mutations associated with human cancers appears to be quite low.

We conclude from this study that 8-oxodA induces mainly A→C transversions in simian kidney cells but, in contrast to 8-oxodG, the mutational potential of this modified base is very low and unlikely to contribute significantly to cellular mutagenesis resulting from oxidative DNA damage in mammalian cells.

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