DNA Damage

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Replication of N^2,3-Ethenoguanine by DNA Polymerases**

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DNA is prone to attack by physical and chemical agents generated endogenously and exogenously, producing modified DNA bases (i.e. DNA adducts/lesions), abasic sites, and inter- and intrastrand DNA crosslinks. DNA adducts, if not properly repaired, can lead to blocked replication, misincorporation, and mutation, potentially causing gene deregulation and cancer. Etheno (ε) DNA adducts are exocyclic adducts that, in addition to their use as fluorescent nucleotide derivatives,[1] were first recognized as reaction products of DNA with reactive metabolites of the occupational carcinogen vinyl chloride (VC).[2] Endogenous etheno-DNA adducts, arising from lipid peroxidation-derived DNA damage, were also detected in rats[3] and humans[4] without VC exposure. VC is a known carcinogen that induces hepatic angiosarcomas.[5] The major DNA adduct formed by VC, N^7-(2-oxoethyl)guanine,[3,6] is generally not considered to be mutagenic, because in vitro experiments showed that it did not cause detectable miscoding in an assay with modified poly(GC).[7] However, etheno adducts formed by VC (e.g. 1,N^6-ethenoadenine, 3,N^4-ethenocytosine, N^2,3-ethenoguanine (N^2,3-εG), and 1,N^2-ethenoguanine (1,N^2-εG)) have all been shown to be mutagenic in vitro and in bacteria (see N^2,3-εG and 1,N^2-εG structures in Figure 1 a).[8] N^2,3-εG is the most abundant endogenous etheno adduct, with levels estimated to be approximately 36 N^2,3-εG lesions/cell in livers of untreated rats or humans.[9] A common assumption is that N^2,3-εG is highly mutagenic; N^2,3-εG is considered to contribute to the carcinogenesis of VC and inflammation-driven malignancies.[10] The dominance of a GC to AT transition in five of six K-*ras* (oncogene) tumors from VC workers[9] suggests the importance of a G adduct, but the misincorporation characteristics of 1,N^2-εG are not consistent with this transition.[8a,f] Little repair of N^2,3-εG occurs in VC-exposed rats, since the half-life of this lesion in rat liver and lung (150 days) and in rat kidney (75 days) is quite long.[11] The lability of the glycosidic bond of N^2,3-ε-deoxyguanosine (N^2,3-ε-dG) makes it difficult

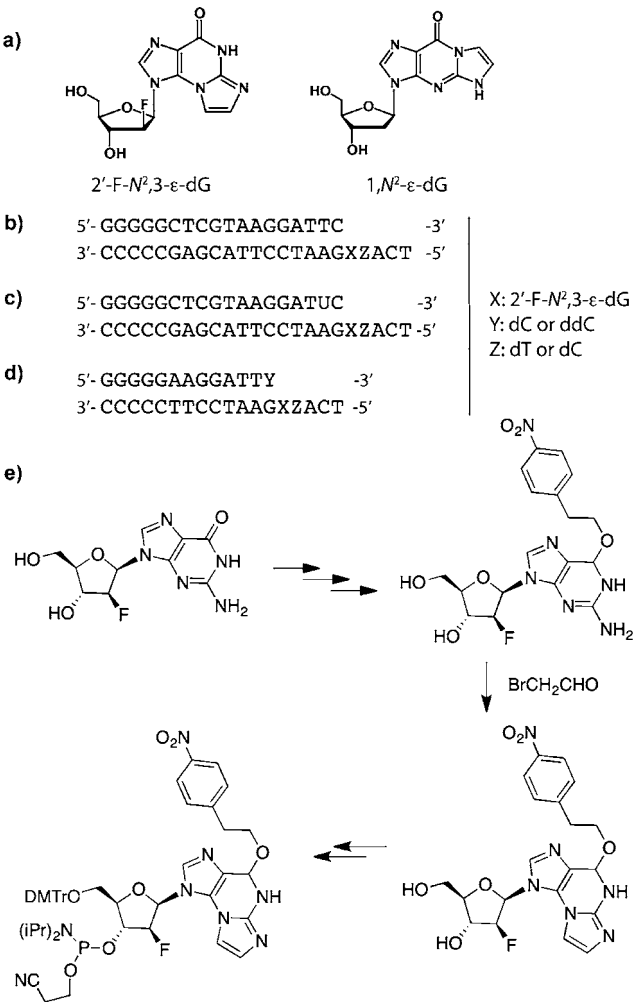

Figure 1. Oligonucleotides used in this study. a) Structural formulas of 2′-F-N^2,3-ε-dG and 1,N^2-ε-dG. Primer-template DNA sequences used for b) steady-state kinetic analysis, c) primer extension analysis, and d) crystallography. e) Summary of 2′-F-N^2,3-ε-2′-deoxyarabinoguanosine phosphoramidite synthesis. The complete procedure is given in Scheme S1 of the Supporting Information. DMTr = dimethoxytrityl.

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to adequately discern its mutagenic potential.[12] Both C and T were incorporated opposite N^2,3-εG in a polyribo(G/N^2,3-εG) template by avian myeloblastosis virus (AMV) reverse transcriptase.[13] N^2,3-ε-Deoxyguanosine triphosphate is inserted opposite T by several polymerases (pols).[8d]

A corrected mutation frequency of 13% was calculated for N^2,3-εG in an indirect assay, resulting in G to A transitions in *Escherichia coli*.[8b] Recently, theoretical calculations were used to predict the preferred base-pairing partner of N^2,3-εG in the order G > T > A > C.[14] These results may partially explain the miscoding potential of N^2,3-εG; however, kinetic and mechanistic details of the interaction of N^2,3-ε-dG with replication enzymes are still missing.

2′-Fluoro substitution in nucleosides slows cleavage of the *N*-glycosidic bond, presumably by destabilization of the transition state and an oxocarbenium ion intermediate.[15] Recently 2′-fluoroarabinose was used to stabilize the glycosidic bond of an established, labile DNA adduct, N^7-methylguanine.[16] We hypothesized that such a strategy could be utilized to retard the glycosidic cleavage of N^2,3-ε-dG. Here we report a synthetic strategy for the site-specific incorporation of 2′-F-N^2,3-ε-2′-deoxyarabinoguanosine (2′-F-N^2,3-ε-dG) into oligonucleotides (Figure 1e and Scheme S1 of Supporting Information). This strategy, based on the use of fluorine as a non-classical isostere (one atom substituting for another) of hydrogen, greatly increased the stability of the glycosidic bond and allowed detailed biochemical and structural studies to be performed. Kinetic and mechanistic details of the replication of N^2,3-εG by five representative DNA polymerases were investigated. Three crystallographic structures of *Sulfolobus solfataricus* P2 DNA polymerase IV (Dpo4) with DNA reveal, for the first time, base-pairing characteristics of N^2,3-εG:C and N^2,3-εG:T, the two major base pairs identified in single-base insertion and primer-extension assays.

A protected phosphoramidite reagent of 2′-F-N^2,3-ε-dG was synthesized from the 2′-fluoro-2′-deoxyarabinoguanine derivative and is described in Figure 1e and the Supporting Information. Protection of the O6 atom is necessary to drive the reaction with bromoacetaldehyde to form N^2,3-εG instead of 1,N^2-εG.[1b] Two 23-mer oligomers (Figure 1b,c) containing N^2,3-εG were utilized in biochemical assays, and two 18-mer oligomers (Figure 1d) were designed based on the existing Dpo4 crystal structures[17] for use in crystallographic studies. The synthetic oligomers were characterized by MALDI-TOF (Figures S9–S14 of Supporting Information), and the presence of N^2,3-εG was confirmed by enzymatic digestion (Figures S15, S16 of Supporting Information).

The $t_{1/2}$ for glycosidic cleavage of 2′-F-N^2,3-ε-dG at pH 7.0 and 37 °C was 23 ± 4 days in a single-stranded oligonucleotide and 33 ± 6 days in a duplex (Figure S1 of Supporting Information), which is comparable to the $t_{1/2}$ (around 600 h) reported for sequestering N^2,3-εG in a poly(GC/N^2,3-ε-dGC) template.[12] The stability of 2′-F-N^2,3-ε-dG permitted careful biochemical assays and crystallographic studies.

The miscoding potential of 2′-F-N^2,3-ε-dG was examined with steady-state kinetic assays using a survey of prokaryotic and eukaryotic DNA polymerases with different functions, including the replicative bacteriophage pol T7 DNA exonuclease$^-$ (pol T7$^-$), the moderately replicative *E. coli* pol I Klenow fragment exonuclease$^-$ (both 5′ to 3′ exo and 3′ to 5′ exo deficient, KF$^-$), and the translesion pols Dpo4, human pol κ, and yeast pol η. A preference for inserting the correct base, C, opposite N^2,3-εG was detected with four of the five polymerases (i.e. $f < 1$; Table 1). Misincorporation of a T residue was seen for all DNA polymerases, with frequencies ranging from 0.22 to 1.0 (Table 1 and Table S1 and S2 of Supporting Information), and some misincorporation of an A residue was also seen for pol T7$^-$. Translesion pols are considered important for processing damaged DNA, although some of them also promote the generation of mutations, in certain cases. As expected, these pols (Dpo4, pol κ, and pol η) showed lower miscoding tendency than the more replicative pols (KF$^-$ and pol T7$^-$), indicating poor discrimination of the incoming dNTP with replicative pols when N^2,3-εG is present. Catalytic efficiencies ($k_{cat}/K_{M,dNTP}$) of C residue insertion opposite N^2,3-εG (N^2,3-εdG:C) showed at least tenfold attenuation compared to insertion opposite an unmodified-G residue (dG:C), with the most significant decrease (over 200-fold) seen for pol T7$^-$. Only small changes in catalytic efficiency were seen for C residue insertion opposite 2′-F-2′-deoxyarabinoguanosine (2′-F-dG), ensuring that the 2′-fluoro modification does not markedly perturb polymerase catalysis.

Table 1: Steady-state kinetic analysis of polymerase-catalyzed single-base insertion opposite X in a template sequence of 3′-CCCCCGAG-CATTCCTAAGXTACT-5′.[a]

Polymerase/ base pairing	k_{cat} [min^{-1}]	$K_{M,dNTP}$ [μM]	$k_{cat}/K_{M,dNTP}$ [min^{-1} μM^{-1}]	f[b]
Dpo4				
2′-F-N^2,3-εdG:T	0.52 ± 0.03	96 ± 16	0.0054	0.22
2′-F-N^2,3-εdG:C	0.37 ± 0.04	15 ± 5	0.025	
2′-F-dG:C	0.63 ± 0.08	1.0 ± 0.02	0.63	
dG:C	1.41 ± 0.03	7.7 ± 1.0	0.18	
Human pol κ				
2′-F-N^2,3-εdG:T	0.90 ± 0.04	111 ± 14	0.0082	0.37
2′-F-N^2,3-εdG:C	1.6 ± 0.1	73 ± 13	0.022	
2′-F-dG:C	1.9 ± 0.1	2.8 ± 0.3	0.68	
dG:C	1.8 ± 0.1	20 ± 1	0.090	
Yeast pol η				
2′-F-N^2,3-εdG:T	0.38 ± 0.015	3300 ± 480	0.00012	0.29
2′-F-N^2,3-εdG:C	0.38 ± 0.05	931 ± 210	0.00041	
2′-F-dG:C	0.53 ± 0.02	26 ± 6	0.020	
dG:C	0.26 ± 0.02	45 ± 8	0.0058	
Pol T7$^-$				
2′-F-N^2,3-εdG:T	0.29 ± 0.03	120 ± 20	0.0024	0.57
2′-F-N^2,3-εdG:A	0.74 ± 0.06	1000 ± 130	0.00074	0.17
2′-F-N^2,3-εdG:C	0.27 ± 0.02	62 ± 9	0.0044	
2′-F-dG:C	0.44 ± 0.03	12 ± 2	0.037	
dG:C	1.1 ± 0.04	1.1 ± 0.2	1.0	
KF$^-$				
2′-F-N^2,3-εdG:T	3.4 ± 0.2	14 ± 3	0.24	1.0
2′-F-N^2,3-εdG:C	5.4 ± 0.4	24 ± 5	0.23	
2′-F-dG:C	4.3 ± 0.4	1.5 ± 0.6	2.9	
dG:C	3.6 ± 0.4	1.5 ± 0.6	2.4	

[a] X is 2′-F-N^2,3-ε-2′-deoxyarabinoguanosine (2′-F-N^2,3-ε-dG), 2′-fluoro-2′-deoxyarabinoguanosine (2′-F-dG), or 2′-deoxyguanosine (dG) (complete data are given in Tables S1 and S2 of Supporting Information). [b] f (misinsertion frequency) = $(k_{cat}/K_{M,dNTP})_{incorrect}/(k_{cat}/K_{M,dNTP})_{correct}$.

To gain insight into the capability of reading and extending beyond N^2,3-εG by polymerases, Dpo4 was characterized in terms of its ability to catalyze full-length extension reactions. Sequences of the products were determined and relative yields were estimated (summarized in Table 2 and Table S3 of Supporting Information) from LC-MS/MS results

Table 2: Products of the extension of template–primer complexes by Dpo4.[a]

3′-CCCCCGAGCATTCCTAAG**X**TACT 5′-GGGGGCTCGTAAGGATUC		Yield [%]	Base added
X: 2′-F-N^2,3-ε-dG	C**C**ATGA	45	C
	C**C**ATGAA	7	
	C**T**ATGA	35	T
	C**T**ATGAA	8	
	C**A**ATGA	4	A
	C**G**ATGA	<1	G
	CATGA	1	deletion
2′-F-dG	C**C**ATGA	100	C
dG	C**C**ATGA	100	C

[a] **X** is 2′-F-N^2,3-ε-2′-deoxyarabino-guanosine (2′-F-N^2,3-ε-dG), 2′-fluoro-2′-deoxyarabinoguanosine (2′-F-dG), or (unmodified) 2′-deoxyguanosine (dG). Mass spectrometry data used to derive these results are presented in Figures S2–S7 and Tables S4–S16 of Supporting Information.

(Tables S4–S17 and Figures S2–S7 of Supporting Information). The primer was readily extended by Dpo4, bypassing N^2,3-ε-dG, similar to that seen for 2′-F-dG and unmodified G templates. With the T-containing template (3'-εGT-5'; Z = T from Figure 1), Dpo4 produced a higher yield of extension products with C incorporated opposite the lesion (52%, Table 2) compared to T (43%, Table 2). Similar results were seen with the C-containing template (3'-εGC-5'; Z = C from Figure 1) shown in Table S3 of the Supporting Information. Thus, the insertion of T opposite N^2,3-εG underscores the mutagenic potential of this lesion. A general trend of T misinsertion observed for the five polymerases studied herein is in concert with reports by Singer et al. for catalysis by AMV reverse transcriptase in a polyribo(GC) template containing N^2,3-εG,[13] but the results (pairing with C > T > A) are at considerable variance with model calculations.[14]

To understand the base-pairing mechanisms of N^2,3-εG with C and T residues (see above), we determined crystal structures of two ternary complexes Dpo4·DNA·dCTP (Dpo4-1, 3'-εGC-5'; Dpo4-2, 3'-εGT-5) at 2.3 Å resolution and a binary complex of Dpo4·DNA (Dpo4-3, with ddT opposite N^2,3-εG) at 3.5 Å resolution (Figure 2, refinement statistics summarized in Table S17 of Supporting Information). The active sites of all three structures resemble the reported configuration of the so-called "type I" Dpo4–DNA complex,[17b] where one base pair is accommodated at the active site, and the 5′ base in the template is rotated over 90° away (Figure 2a,c and Figure S8 of Supporting Information). Base pairing of N^2,3-εG with dCTP in ternary complexes (templates: 3′-εGC-5′ and 3′-εGT-5′) showed both N^2,3-εG and dCTP in an *anti* conformation. Interestingly, electron density suggested that the G residue 3′ to the lesion is most likely in the *syn* conformation to form a better stacking

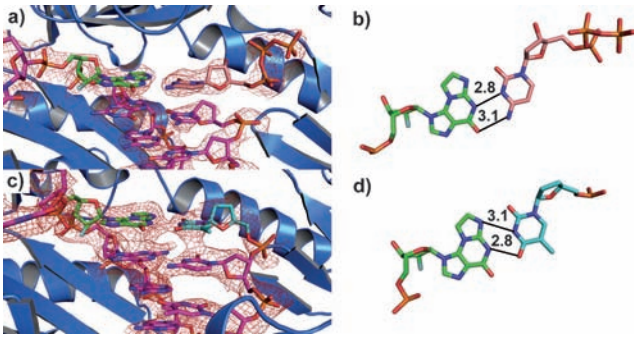

Figure 2. Crystal structures of Dpo4·N^2,3-εG-DNA complexes (Z = C in the template). a) Ternary complex of Dpo4 with dCTP and N^2,3-εG-containing duplex DNA, (Dpo4-1) and b) the orientation of the bases with proposed hydrogen-bonding mechanism (distances shown in Å). c) Binary complex of Dpo4 with ddT across from N^2,3-εG in the DNA duplex (Dpo4-3) and d) the orientation of the bases with proposed hydrogen-bonding mechanism. The quality of the data is demonstrated using non-biased omit electron density maps, displayed as red mesh, at 1.0 σ in (a) and (c). Colors of the atoms: O, red; N, blue; P, orange; F, gray.

interaction with εG. A Watson–Crick-like configuration was seen for N^2,3-εG:C base pairing (Figure 2b), whereas N^2,3-εG:T mispairing resembles a sheared base pair (Figure 2d). Interestingly, Singer et al.[13] had suggested "wobble" pairing but of a very different type.

Significant differences in the replication patterns and mechanisms exist when comparing current results to our previously reported 1,N^2-εG, an isomer of N^2,3-εG formed through similar pathways.[6b] Differences in catalytic efficiencies and miscoding frequencies for the two lesions are summarized in Table S18 of the Supporting Information. Overall, 1,N^2-εG has a much higher miscoding potential, with potential base pairing with A, T, or G by different pols.[17c,18] Extension beyond 1,N^2-εG by Dpo4 yields mainly deletion products (−1 and −2),[17c] whereas these were rare for N^2,3-εG (approximately 1%). Crystal structures of Dpo4 with 1,N^2-εG resemble "type II" structures,[17b] where the 5′ base in the template is oriented in the active site to pair with the incoming nucleotide, which explains the deletion products observed in primer extension reactions.

In summary, we have successfully used a non-classical isostere approach to stabilize an important, labile DNA lesion, N^2,3-εG.[19] Kinetic assays using representative DNA polymerases allow quantitative assessment of the miscoding tendency of this lesion and underscore the diversity of biological effects that can result from isomeric DNA adducts. Structural insights reveal the base-pairing mechanisms of the correct base C and miscoded base T with one of the DNA polymerases (Dpo4). The most common mispairing is consistent with the reported GC to AT transition mutations observed in the second base of codon 13 of the K-*ras* gene in five out of six human VC-induced angiosarcomas,[9,20] which are not explained by known C or G adducts (3,N^4-ethenocytosine, N^7-(2-oxoethyl)G, or 1,N^2-εG).[8f,18,19,21] Thus, this adduct (N^2,3-εG) may be more relevant to the VC-induced tumors, and its presence in unexposed humans may be an issue in disease, in that the misincorporation patterns (N^2,3-

εG:T) appear very consistently throughout DNA polymerases (Table 1) and have also been detected with human pol ι.[22] The stability of the 2′-fluoro-modified lesion is adequate for more complex biological studies, for example, cellular site-specific mutagenesis and DNA repair.

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