Nucleic Acids Research, 2005, Vol. 33, No. 5 ***1637–1643***
doi:10.1093/nar/gki305

The effect of the 2-amino group of 7,8-dihydro-8-oxo-2′-deoxyguanosine on translesion synthesis and duplex stability

Natsuhisa Oka and Marc M. Greenberg*

Department of Chemistry, Johns Hopkins University, 3400 N. Charles St, Baltimore, MD 21218, USA

Received January 14, 2005; Revised and Accepted February 25, 2005

ABSTRACT

Replication of DNA containing 7,8-dihydro-8-oxo-2′-deoxyguanosine (OxodG) gives rise to G → T transversions. The *syn*-isomer of the lesion directs misincorporation of 2′-deoxyadenosine (dA) opposite it. We investigated the role of the 2-amino substituent on duplex thermal stability and in replication using 7,8-dihydro-8-oxo-2′-deoxyinosine (OxodI). Oligonucleotides containing OxodI at defined sites were chemically synthesized via solid phase synthesis. Translesion incorporation opposite OxodI was compared with 7,8-dihydro-8-oxo-2′-deoxyguanosine (OxodG), 2′-deoxyinosine (dI) and 2′-deoxyguanosine (dG) in otherwise identical templates. The Klenow exo⁻ fragment of *Escherichia coli* DNA polymerase I incorporated 2′-deoxyadenosine (dA) six times more frequently than 2′-deoxycytidine (dC) opposite OxodI. Preferential translesion incorporation of dA was unique to OxodI. UV-melting experiments revealed that DNA containing OxodI opposite dA is more stable than when the modified nucleotide is opposed by dC. These data suggest that while duplex DNA accommodates the 2-amino group in *syn*-OxodG, this substituent is thermally destabilizing and does not provide a kinetic inducement for replication by Klenow exo⁻.

INTRODUCTION

7,8-Dihydro-8-oxo-2′-deoxyguanosine (OxodG, Figure 1) is a commonly formed DNA lesion that misdirects polymerases to incorporate 2′-deoxyadenosine (dA) opposite it and gives rise to G → T transversions *in vivo* (1–3). OxodG has been described as a major mutagenic oxidative lesion (4). Although the absolute amount of OxodG found in cells is controversial, its promutagenic effect and relatively high formation frequency have encouraged its use as a biomarker (5,6). Furthermore, the implied biological importance of OxodG has motivated extensive *in vitro* studies concerning its effects on DNA structure, as well as its interactions with polymerases and repair enzymes (7–9). We report steady-state polymerase kinetic experiments and UV-melting studies in which we used 7,8-dihydro-8-oxo-2′-deoxyinosine (OxodI) to probe the interaction between the 2-amino substituent of OxodG and the phosphate backbone when the lesion is in the *syn*-conformation employed for pairing with dA. The observations made using OxodI are calibrated by comparing the results to similar experiments carried out using OxodG, 2′-deoxyinosine (dI) and 2′-deoxyguanosine.

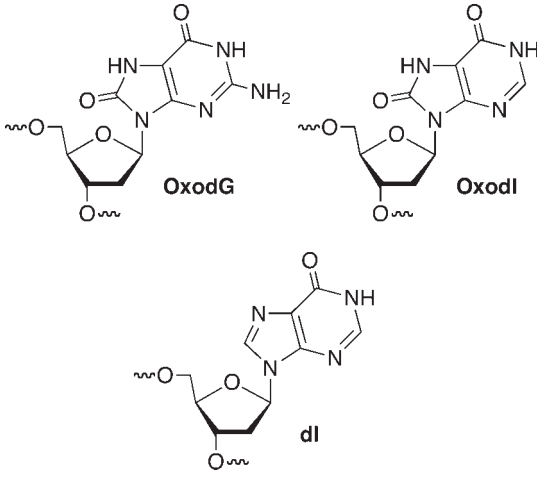


Figure 1. Structure of nucleotides 7,8-dihydro-8-oxo-2′-deoxyguanosine (OxodG), 7,8-dihydro-8-oxo-2′-deoxyinosine (OxodI) and 2′-deoxyinosine (dI).

Translesion incorporation of dA opposite OxodG was originally rationalized by the propensity for 8-substituted purines to adopt the *syn*-conformation in solution due to steric repulsion between the C8-substituent and 4′-oxygen atom in

*To whom correspondence should be addressed. Tel: +1 410 516 8095; Fax: +1 410 516 7044; Email: mgreenberg@jhu.edu

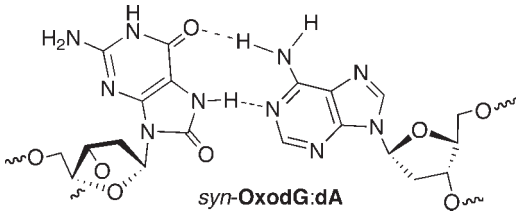


Figure 2. Hoogsteen base-pairing in *syn*-OxodG:dA.

the *anti*-isomer (10,11). *Syn*-OxodG was proposed to form a Hoogsteen base pair with dA (Figure 2). This hypothesis was born out in structural studies on duplex DNA containing OxodG:dA base pairs (12,13). Aside from the *syn*-OxodG:dA base pair, the duplex retained the overall structure of unmodified DNA. One subtle difference recognized by our research group, as well as Grollman, Wilson and possibly others, is that the 2-amino group of *syn*-OxodG is ~1 Å closer to the 5′-phosphate oxygen than the respective amine in a *syn*-dG:dA base pair (14). This suggests that there could be a weak interaction between the phosphate backbone and the 2-amino group of *syn*-OxodG. Grollman and Wilson carried out a molecular modeling study of DNA containing a *syn*-OxodG complexed to DNA polymerase β (14). The modeling was based upon two relevant crystal structures. A favorable interaction between the 2-amino group and phosphate backbone was predicted when the modified DNA is complexed to the polymerase. A recent co-crystal structure of a duplex containing a *syn*-OxodG:dA base pair complexed by a DNA polymerase I fragment (thermostable *Bacillus stearothermophilus*) does not mention any stabilizing interaction between the exocyclic amine and the phosphate backbone (4).

7,8-Dihydro-8-oxo-2′-deoxyinosine (OxodI) should be useful for probing the role of the 2-amino group in OxodG. OxodI differs from OxodG by lacking a 2-amino substituent, and has been used to probe the interactions between damaged DNA and repair enzymes (15–17). Removing the 2-amino substituent from OxodG was not detrimental to repair when the lesion was part of a DNA template (15,17). In addition, removing the C2-amine from OxodG was not expected to adversely affect the nucleotide's interaction with dC or increase its preference for pairing with dA when present in the *anti*-conformation. When OxodI is in the *anti*-conformation, the molecule presents a shape and hydrogen bonding pattern to the polymerase that is identical to that of dI. Experiments using 2′-deoxyinosine (dI) indicate that this molecule behaves very much like dG with respect to its thermodynamic base-pairing preferences and effect on replication (18,19).

MATERIALS AND METHODS

General methods

Oligonucleotides were synthesized on an Applied Biosystems Incorporated 394 DNA synthesizer using standard protocols. DNA synthesis reagents, including phosphoramidites for the incorporation of OxodG and dI, were purchased from Glen Research (Sterling, VA). DNA was purified by 20% polyacrylamide denaturing gel electrophoresis (5% cross-link, 45% urea by weight). DNA manipulation, including enzymatic labeling, was carried out using standard procedures. T4 polynucleotide kinase and the Klenow exo$^-$ fragment of DNA polymerase I were obtained from New England Biolabs (Beverly, MA). Adenosine deaminase from calf intestine mucosa (Type II) was obtained from Sigma (St Louis, MO). [γ-^{32}P]ATP was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Quantification of radiolabeled oligonucleotides was carried out using a Molecular Dynamics Storm 840 Phosphorimager equipped with ImageQuant Version 5.1 software. ESI-MS spectra of oligonucleotides were obtained using a Finnigan LCQ-Deca. UV-melting studies were carried out using a Beckman 640 spectrophotometer equipped with a Peltier temperature controller and six sample cell transporter.

Preparation of 7,8-dihydro-8-oxo-2′-deoxyinosine (2) (16)

This compound was synthesized according to the procedure for deamination of 2′-deoxyadenosine with minor modification (20). A solution of 7,8-dihydro-8-oxo-2′-deoxyadenosine (**1**, 3.87 g, 14.5 mmol) and adenosine deaminase (0.35 g) in H_2O (145 ml) was kept at room temperature for 10 h and concentrated under reduced pressure. The residue was triturated with MeOH (30 ml) under reflux and the resultant solid was collected by suction filtration and dried under vacuum over P_2O_5 to afford **2** (3.17 g, 12 mmol, 81%) as an off-white solid. ^{1}H NMR (DMSO-d_6) δ 7.94 (s, 1H), 6.12 (dd, J = 8.0, 6.8 Hz, 1H), 4.36 (m, 1H), 3.78 (m, 1H), 3.58 (dd, J = 11.8, 4.6 Hz, 1H), 3.44 (dd, J = 11.8, 4.6 Hz, 1H), 2.94 (m, 1H), 1.99 (ddd, J = 12.9, 6.5, 2.8 Hz, 1H). ^{13}C NMR (DMSO-d_6) δ 152.5, 151.6, 145.8, 144.2, 108.4, 87.5, 81.5, 71.3, 62.4, 36.2.

5′-(4,4′-Dimethoxytrityl)-7,8-dihydro-8-oxo-2′-deoxyinosine (3)

7,8-Dihydro-8-oxo-2′-deoxyinosine (**2**, 2.68 g, 10 mmol) was dissolved in dry DMF (50 ml), dried by repeated co-evaporations with dry pyridine (3 × 50 ml) and then dissolved in dry pyridine (100 ml). 4,4′-Dimethoxytrityl chloride (3.73 g, 11 mmol) was added to the stirred mixture at 0°C, and the mixture was stirred at the same temperature for 3 h and at room temperature for 12 h under Ar. The mixture was poured into saturated $NaHCO_3$ solution (400 ml) and extracted with CH_2Cl_2 (500 ml). The organic layer was washed with saturated $NaHCO_3$ aqueous solution (400 ml) again and extracted with CH_2Cl_2 (500 ml). The combined organic layers were dried over Na_2SO_4, filtered and concentrated under reduced pressure. The residue was triturated with CH_2Cl_2–hexane (1:1, v/v) (50 ml) under reflux. The resultant solid was collected by suction filtration and dried under vacuum to afford **3** (4.86 g, 8.5 mmol, 85%) as a white solid. ^{1}H NMR (DMSO-d_6) δ 12.6 (brs, 1H), 11.4 (brs, 1H), 7.78 (s, 1H), 7.35 (d, J = 7.2 Hz, 2H), 7.22–7.17 (m, 7H), 6.82 (d, J = 9.2 Hz, 2H), 6.78 (d, J = 8.4 Hz, 2H), 6.13 (t, J = 6.6 Hz, 1H), 5.22 (m, 1H), 4.43 (m, 1H), 3.88 (m, 1H), 3.72 (s, 3H), 3.71 (s, 3H), 3.17–3.07 (m, 2H), 2.95 (m, 1H), 2.09 (m, 1H). ^{13}C NMR (DMSO-d_6) δ 158.0, 157.9, 151.5, 150.9, 145.1, 144.2, 144.1, 135.7, 129.6, 127.7, 127.6, 126.5, 113.0, 112.9, 108.6, 85.3, 85.2, 80.9, 70.9, 64.3, 55.0, 36.1. IR (KBr) 3400, 3090, 1685, 1508, 1177, 1032, 832 cm^{-1}. ESI-MS calculated for $C_{31}H_{30}N_4NaO_7^+$ (M + Na^+) 593.20, found 593.20.

OxodI-phosphoramidite (4)

Dimethoxytritylated OxodI (**3**, 0.143 g, 0.25 mmol) was dried by repeated co-evaporations with dry pyridine and dry toluene, and dissolved in freshly distilled THF (5 ml). Diisopropylethylamine (87.1 µl, 64.6 mg, 0.50 mmol) was added, and the solution was cooled to −78°C. Chloro(2-cyanoethyloxy)-diisopropylaminophosphine (83.7 µl, 88.8 mg, 0.375 mmol) was added to the stirred solution and the mixture was stirred for 12 h at room temperature under Ar. The mixture was then poured into saturated $NaHCO_3$ solution (50 ml) and extracted with ethyl acetate (2 × 50 ml). The combined organic layers were dried with Na_2SO_4, filtered and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel [CH_2Cl_2–MeOH–pyridine (100:0:2 to 96:4:2, v/v)]. The fractions containing the desired product were combined and washed with saturated $NaHCO_3$ solution (200 ml) before being concentrated. The organic layer was dried with Na_2SO_4, filtered, toluene was added (50 ml), and the mixture was concentrated to dryness to afford **4** (0.130 g, 0.17 mmol, 68%) as a white foam (∼1:1 mixture of *P*-diastereomers). ^{1}H NMR (DMSO-d_6) δ 12.50 (brs, 1H), 11.33 (brs, 1H), 7.78, 7.77 (s, s, 1H), 7.35–7.32 (m, 2H), 7.23–7.19 (m, 7H), 6.81–6.75 (m, 4H), 6.13 (m, 1H), 4.72 (m, 1H), 4.00 (m, 1H), 3.71, 3.71, 3.70, 3.69 (s, s, s, s, 6H), 3.67–3.45 (m, 4H), 3.22–3.01 (m, 3H), 2.74, 2.63 (t, J = 6.0 Hz, t, J = 6.0 Hz, 2H), 2.35–2.22 (m, 1H), 1.12 (d, J = 6.8 Hz, 3H), 1.10 (d, J = 6.8 Hz, 3H), 1.09 (d, J = 6.8 Hz, 3H), 0.99 (d, J = 6.8 Hz, 3H). ^{13}C NMR ($CDCl_3$) δ 158.3, 153.7, 152.6, 145.0, 144.8, 143.3, 136.0, 135.9, 130.0, 128.2, 128.1, 127.5, 126.6, 117.5, 117.4, 112.8, 109.2, 85.9, 85.3, 85.2, 81.9, 81.7, 74.3 (d, JPC = 16.7 Hz), 73.6 (d, JPC = 19.0 Hz), 63.9, 63.7, 58.3 (d, JPC = 14.4 Hz), 58.2 (d, JPC = 14.4 Hz), 55.1, 43.1, 43.0, 35.7, 24.6, 24.5, 24.4, 24.4, 20.3 (d, JPC = 7.6 Hz), 20.1 (d, JPC = 7.6 Hz). ^{31}P NMR ($CDCl_3$) δ 149.1, 148.9. IR (film) 3052, 2966, 1683, 1613, 1509, 1445, 1375, 1251, 1178, 1075, 1034, 975, 829 cm^{-1}. ESI-MS calculated for $C_{40}H_{47}N_6NaO_8P^+$ ($M + Na^+$) 793.31, found 793.27.

Oligonucleotide synthesis, deprotection and purification

The oligonucleotides were assembled on a 1.0 µmol scale using **4** and commercially available phosphoramidites. The coupling yield for **4** was ∼80% with an extended coupling time of 5 min. All other phosphoramidites were coupled using standard preprogrammed coupling cycles. A portion of the resin from a synthesis was treated with 28% NH_4OH at 55°C for 17 h in the presence or absence of 0.25 M β-mercaptoethanol. The released oligonucleotides were purified by denaturating 20% PAGE, and isolated using the crush and soak method (0.2 M NaCl, 1 mM EDTA), followed by desalting on a C-18 Sep Pak cartridge. Oligonucleotides were characterized by ESI-MS. The purity of the resultant oligonucleotides (**5**,**6**) was independent of the presence of β-mercaptoethanol.

Extension kinetics

Duplex-enzyme cocktails (2×) were prepared in the following manner. Klenow exo$^-$ (3.68 µM, 5 U/µl, 0.340 µl, 1.25 pmol) was treated with inorganic pyrophosphatase (40 U/ml) (8.5 µl, 0.34 U) at room temperature for 10 min in order to prevent degradation of the template. After reaction with pyrophosphatase, 100× BSA (2.5 µl), 10× Klenow buffer (100 mM Tris–HCl, pH 7.5, 50 mM $MgCl_2$, 75 mM DTT) (25 µl), H_2O (82.4 µl), and the stock solution of ^{32}P-labeled duplex (2 µM, 6.25 µl, 12.5 pmol) were added. The 2× duplex-enzyme cocktail (125 µl) consisted of the appropriate template (**5a–c**)/primer (100 nM), Klenow exo$^-$ (20 nM), BSA (0.2 µg/µl) in a 2× Klenow buffer (20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 15 mM DTT). The 2× duplex-enzyme cocktail was incubated for 5 min at room temperature, prior to mixing with the appropriate 2× dNTP solution. The 2× dNTP solutions were prepared by diluting 100 mM stock solutions of the corresponding dNTP before use. Extension reactions were carried out by adding a 2× duplex-enzyme cocktail (5 µl) to a 2× dNTP solution. The reactions were run for 4.5 min and quenched with 95% formamide loading buffer (20 µl) containing 20 mM EDTA. The mixtures were then denatured by heating at 90°C for 5 min and cooled immediately to 0°C. Aliquots (5 µl) of the mixtures were subjected to 20% denaturing PAGE and analyzed with a Storm 840 phosphor imager. Kinetic parameters were calculated using Hanes–Woolf plots. Nonlinear regression analysis of velocity versus [dNTP] yielded Michaelis constants that were not statistically different from those obtained from linear Hanes–Woolf plots. All values are the averages of three independent experiments. Each experiment was carried out using three replicates. The concentrations of dNTPs in the reactions were chosen such that the maximum amount of extension was ⩽30% (21). The dNTP concentrations used were as follows: 1, 2.5, 5, 10, 15, 20 µM for X = OxodI, dNTP = dC; 0.05, 0.1, 0.25, 0.5, 1, 2 µM for X = OxodI, dNTP = dA; 0.5, 1, 2.5, 5, 25, 50 nM for X = dI, dNTP = dC; 5, 12.5, 25, 50, 80, 120 µM for X = dI, dNTP = dA; 0.25, 0.5, 2.5, 5, 12.5, 25 µM for X = OxodG, dNTP = dC; 0.5, 5, 25, 50, 100, 150 µM for X = OxodG, dNTP = dA.

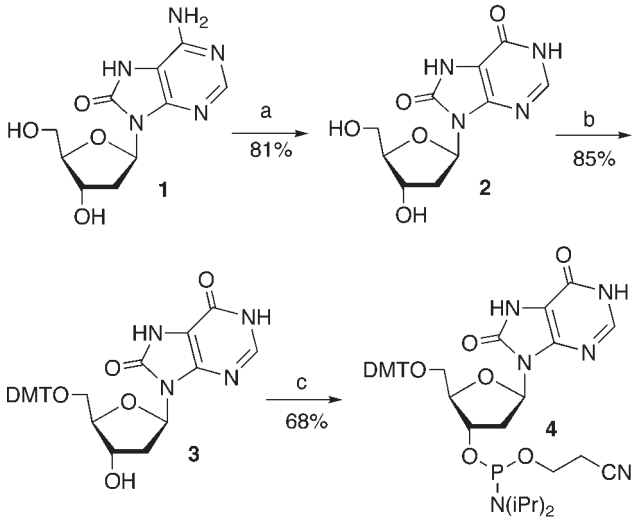
DNA melting experiments

Samples for the thermal denaturation studies contained a one-to-one ratio of complementary oligonucleotides. The concentrations of each oligonucleotide were 5, 4, 2.2, 1 and 0.5 µM. Solutions of the complementary oligonucleotides (5 µM) in PIPES buffer (10 mM PIPES, pH 7.0, 10 mM $MgCl_2$, 100 mM NaCl) were hybridized by heating at 90°C for 5 min, followed by gradual cooling to room temperature over 2.5 h, whereupon they were placed in a refrigerator at 4°C for an additional 2.5 h period. Duplex solutions (4–5 µM) were prepared from the 5 µM solution by diluting with PIPES buffer. Melting experiments were carried out in 1 cm pathlength quartz cells using 100 µl of the duplex solution covered with 200 µl of mineral oil to prevent evaporation. UV absorbance values were recorded while the temperature was ramped at a rate of 0.5°C/min from 25 to 70°C. Reported parameters are the average of three independent experiments.

RESULTS AND DISCUSSION

Synthesis of oligonucleotides containing OxodI

Although oligonucleotides containing OxodI have been used in biochemical studies, we could not find a description of their



Scheme 1. Synthesis of OxodI phosphoramidite. (a) Adenosine deaminase, H_2O; (b) DMTCl, pyridine, 0–25°C; (c) chloro-(2-cyanoethyloxy) diisopropylaminophosphine, diisopropylethylamine, THF, −78 to 25°C.

synthesis in the literature (15,17). The β-cyanoethyl phosphoramidite (**4**) was prepared from the free nucleoside (**2**) (16). Nucleoside **2** was prepared from 7,8-dihydro-8-oxo-2′-deoxyadenosine (**1**, Scheme 1), which was synthesized using exact literature procedures when available, or corresponding methods for analogous compounds when specific details were unavailable (22–24). OxodI was prepared by enzyme-mediated deamination of **1** in H_2O (20). The polar dimethoxytrityl compound (**3**) was purified without chromatography. However, the final product, phosphoramidite **4** required chromatographic purification. It was necessary to add methanol (4%) and pyridine (2%) to elute **4**. After pooling the appropriate fractions, any silica gel that dissolved in the protic solvent was removed by washing with aqueous bicarbonate solution. In addition, toluene was added prior to concentrating the pooled fractions containing **4**, in order to prevent decomposition induced by possible traces of acid associated with the protic solvent upon concentration.

The stability of OxodI to routine oligonucleotide deprotection conditions (concentrated aqueous ammonia, 55°C, 12 h) was examined before incorporating it into oligonucleotides. The effect of thiol on the stability of **2** during DNA deprotection conditions was examined because of its importance for protecting against OxodG decomposition during aqueous ammonia treatment (22). No decomposition was detected using thin layer chromatography and ^{1}H NMR analysis, indicating that the free nucleoside (**2**) was stable to concentrated aqueous ammonia (55°C, 17 h) in the absence or presence of β-mercaptoethanol (0.25 M). That thiol is unnecessary during oligonucleotide deprotection was verified when **5b** was synthesized. The oligonucleotide was prepared using standard conditions, except that **4** was coupled for 5 min (80% coupling yield via dimethoxytrityl cation analysis). ESI-MS analysis of **5b** showed only the desired ion whether or not thiol was present during deprotection (see Supplementary Information). The 36 nt oligonucleotide (**6b**) used as a template in Klenow exo$^-$ experiments was synthesized, deprotected (no thiol) and characterized by ESI-MS in an identical manner (see Supplementary Material).

Table 1. Comparison of melting temperatures and duplex stability

5′-d(TGC AGT **N**AC AGC)
3′-d(ACG TCA **Y**TG TCG)

N:Y	T_m (°C)[a]	$\Delta H°$ (kcal/mol)	$\Delta S°$ (cal/K·mol)	$\Delta G°_{298}$ (kcal/mol)
OxodG:dC	54.5 ± 0.5	90.2	247.8	16.3
OxodG:dA	50.3 ± 0.0	83.2	229.6	14.7
OxodI:dC	48.7 ± 0.2	89.1	249.6	14.7
OxodI:dA	51.5 ± 0.9	86.4	239.0	15.2
dI:dC	52.1 ± 0.3	84.1	231.4	15.1
dI:dA	49.2 ± 0.6	86.7	241.6	14.7
dG:dC[b]	57.1	106.1	294.4	18.4
dG:dA[b]	45.5	83.0	233.7	13.3

Conditions: PIPES (pH 7.0), 10 mM; $MgCl_2$, 10 mM; NaCl, 100 mM.
[a][Duplex] = 2.2 μM.
[b]Data taken from reference (30).

5′-d(TGC AGT **N**AC AGC)
5a-d

5′-d(AGG CGT TCA ACG TGC AGT **N**AC AGC ACG TCC CAT GGT)
6a-d

N
a OxodG
b OxodI
c dI
d dG

Substituent effects on duplex thermal stability

UV-melting studies were used to compare the ability of a duplex to accommodate dG, dI, OxodG and OxodI base-paired with dA or dC. The experiments were carried out on dodecameric duplexes that differed at a single position and contained dG, OxodG, OxodI or dI at the variable position (Table 1 and Figure 3). The qualitative trends observed here are consistent with available data in the literature on duplexes containing OxodG or dI (18,25). No data is available for DNA containing OxodI. These data reveal that the 2-amino substituent stabilizes the duplex when the respective purine is opposite dC and presumably in the *anti*-conformation. This is evident when one compares the melting of duplexes containing a dG:dC or OxodG:dC base pair to those containing dI:dC or OxodI:dC base pairs, respectively. In contrast, the 2-amino group destabilizes the duplex when the purine is opposed by dA, and presumably adopts the *syn*-conformation. This point is illustrated by comparing the thermodynamics for melting an OxodG:dA duplex to a comparable one containing an OxodI:dA base pair.

Direct quantitative comparisons between duplexes in which the 2-amino group is the only variable are consistent with the above interpretations in all instances. DNA containing a dI:dC base pair is more than 3 kcal/mol less stable than one

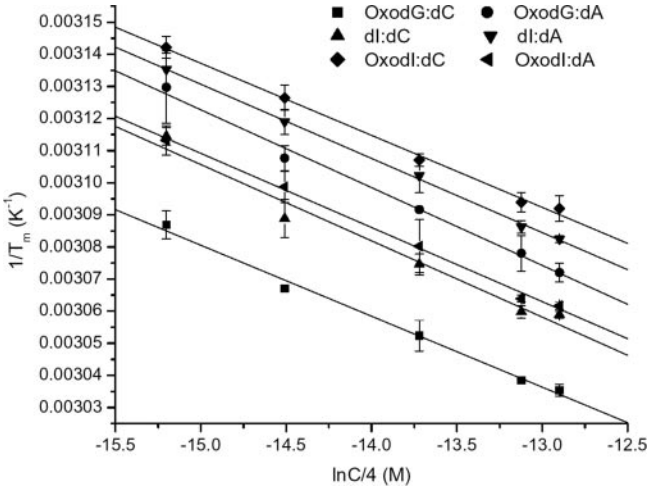


Figure 3. Van't Hoff plot of UV-melting experiments for dodecamers prepared from **5a–5c**.

containing the native dG:dC pair. Furthermore, placing OxodI opposite dC is 1.6 kcal/mol less favorable than incorporating an OxodG:dC base pair in duplex DNA. OxodG is known to populate the *anti*-conformation when opposite dC. The presence of the C8-substituent decreases duplex stability (25). The comparison between duplexes containing OxodI:dC and dI:dC base pairs are consistent with this. The UV-melting data are consistent with the OxodI:dC base pair containing the OxodI nucleotide in its *anti*-conformation. It is not possible to draw a donor–acceptor hydrogen bonding pattern for an OxodI:dC base pair when the purine is in the *syn*-conformation, regardless of the 5-member ring tautomer present.

Comparisons between duplexes indicate that the 2-amino group is destabilizing when the appropriate nucleotides (dG, dI, OxodG, OxodI) are opposite dA. Most noteworthy with respect to this study is that the duplex containing OxodI:dA is 0.5 kcal/mol more stable than one containing OxodG:dA. The OxodG:dA base pair is known to exist with the lesion in the *syn*-conformation (12). Although one could draw *anti*-OxodI hydrogen bonded to dA, this would be sterically unfavorable compared to the duplex in which the modified purine is present in the *syn*-conformation (26). Finally, one should note that a duplex containing OxodG opposite dC is more stable than one in which dA opposes the lesion. However, an OxodI:dA base pair is preferred over a duplex containing dC opposite the modified nucleotide. The OxodI:dA base pair can relieve the steric interaction of the 8-oxo substituent with the deoxyribose by adopting a *syn*-conformation, without introducing an amino group in its place. We conclude that the comparison between the melting thermodynamics for OxodG:dA and OxodI:dA containing duplexes suggest that the 2-amino group does not form a stabilizing interaction with the phosphate backbone.

Substituent effects on translesion incorporation

Steady-state kinetic experiments were carried out using templates containing dG, dI, OxodG or OxodI (21,27). The method used provides 'apparent' K_m values and V_{max} are typically reported in terms of % reaction (21,27–29). Nucleotide

Table 2. Kinetic parameters for nucleotide incorporation opposite purines by Klenow exo$^-$

5'-d(AGG CGT TCA ACG TGC AGT **N**AC AGC ACG TCC CAT GGT)
TG TCG TGC AGG GTA CCA)

dN'TP
Klenow exo$^-$

5'-d(AGG CGT TCA ACG TGC AGT **N**AC AGC ACG TCC CAT GGT)
N'TG TCG TGC AGG GTA CCA)

N	dN′TP	V_{max} (%·min^{-1})	K_m (μM)	V_{max}/K_m (%·min^{-1}·μM^{-1})
OxodG	C	1.7 ± 0.3	2.4 ± 0.3	$7.2 \pm 0.6 \times 10^{-1}$
OxodG	A	6.6 ± 1.9	41.3 ± 7.3	$1.6 \pm 0.3 \times 10^{-1}$
OxodI	C	7.5 ± 1.5	4.5 ± 1.7	2.0 ± 1.1
OxodI	A	11.7 ± 3.3	$9.7 \pm 0.7 \times 10^{-1}$	12.0 ± 2.9
dI	C	3.2 ± 0.3	$5.7 \pm 2.3 \times 10^{-3}$	$6.1 \pm 1.7 \times 10^{2}$
dI	A	2.8 ± 0.7	52.3 ± 13.1	$5.3 \pm 0.3 \times 10^{-2}$
dG[a]	C	5.1	5.2×10^{-3}	9.8×10^{2}
dG[a]	A	14.0	2.6×10^{2}	5.4×10^{-2}

[a]Data taken from reference (30).

insertion frequencies (V_{max}/K_m) are measured using the same enzyme concentration. Consequently, relative insertion frequencies [$(V_{max}/K_m)_{\cdot correct}/(V_{max}/K_m)_{\cdot incorrect}$] provide the relative specificity constants and are independent of active enzyme concentration. Kinetic parameters were obtained for dA and dC incorporation by the Klenow exo$^-$ fragment of DNA polymerase I (Table 2 and Figure 4). Incorporation of dT or dG opposite OxodI was much slower. Only a small amount of primer extension (2–3%) was observed when concentrations of these nucleotide triphosphates were as high as 500 μM, and reaction times were extended to 9 min. Hence, we did not determine kinetic constants for incorporation of these nucleotides opposite dG or any of the modified nucleotides. Parameters for nucleotide incorporation opposite dG are those reported by us previously (30). Efficient incorporation of dC opposite dI is consistent with previous reports showing that this lesion codes like dG (19,31). This modified nucleotide also discriminates strongly against translesion incorporation of dA, as does dG. The ratio of specificity constants for nucleotide incorporation opposite OxodG ($(V_{max}/K_m)_{dCTP}/(V_{max}/K_m)_{dATP}$ = 4.5) by Klenow exo$^-$ determined here is comparable to the respective ratio reported (4.0) in a different sequence context (32). These data provide confidence that the kinetic parameters are precise and accurately reflect the physical process that we are interested in probing.

2′-Deoxycytidine incorporation opposite OxodI is comparable, and perhaps slightly more efficient than when OxodG is in the template. However, the specificity of dA incorporation opposite OxodI is the most efficient of any nucleotide whose incorporation was measured opposite OxodG or this lesion. The relatively high specificity was ascribable to the lowest K_m and the highest V_{max} of nucleotides incorporated opposite OxodI or OxodG. Furthermore, dA incorporation opposite OxodI was 75 times more efficient than opposite OxodG, indicating that removing the 2-amino substituent from OxodG actually accelerates the translesional incorporation of dA by Klenow exo$^-$.

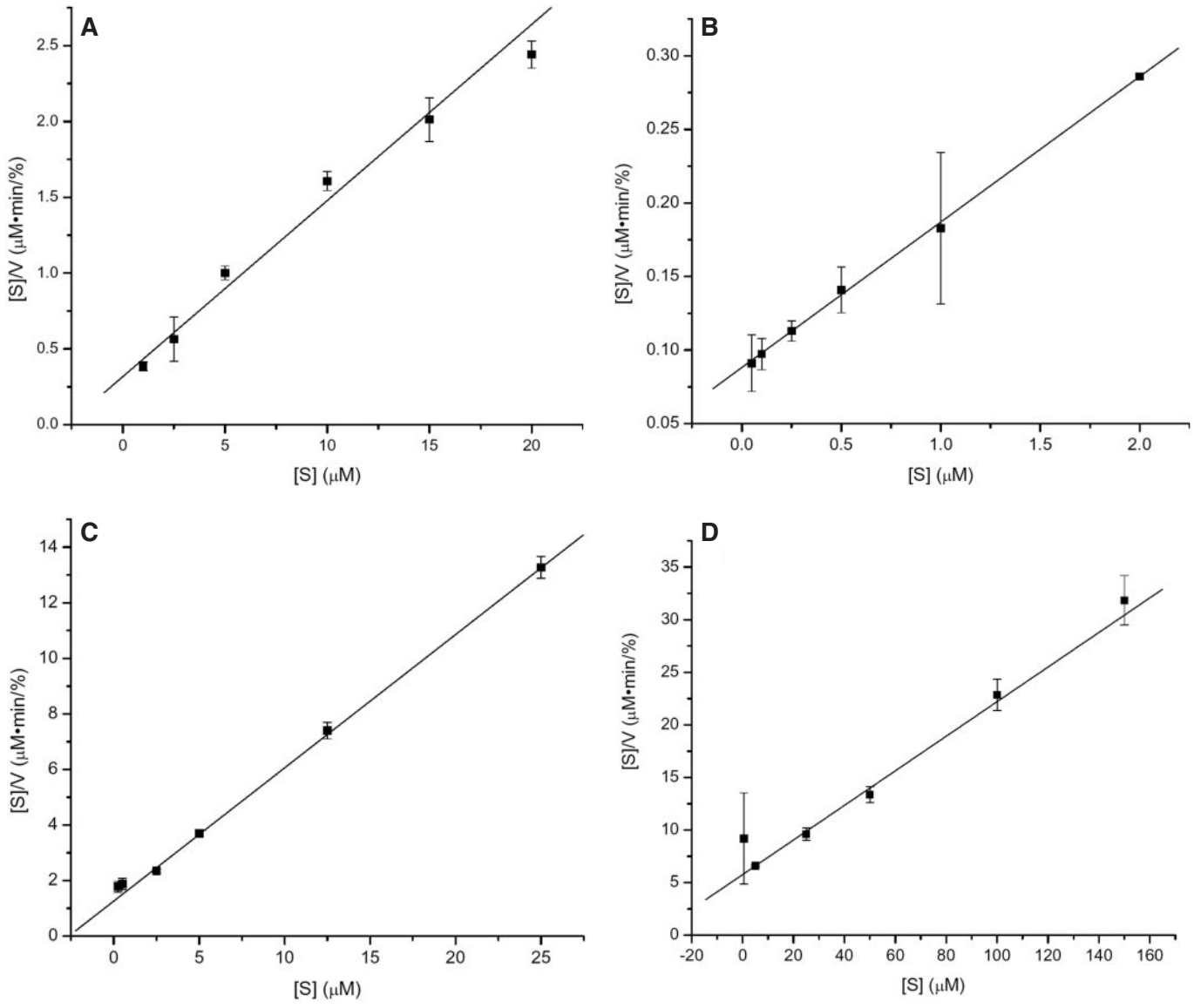


Figure 4. Sample Hanes–Woolf plots describing translesional synthesis for the incorporation of dC or dA opposite OxodI or OxodG. (**A**) dC opposite OxodI; (**B**) dA opposite OxodI; (**C**) dC opposite OxodG; and (**D**) dA opposite OxodG.

These kinetic data alone do not rule out a stabilizing interaction between the phosphate backbone and the 2-amino group of *syn*-OxodG. However, if there is a favorable interaction between the 2-amino substituent of OxodG and the phosphate backbone during replication by Klenow exo$^-$ then it must position the nucleotide in a conformation that is less productive for translesion synthesis. Given that the UV-melting studies do not provide any evidence for a favorable interaction between the nucleic acid backbone, application of Ockham's razor requires that we propose that the 2-amino substituent in OxodG does not facilitate misincorporation of 2′-deoxyadenosine by Klenow exo$^-$ (33).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

ACKNOWLEDGEMENTS

We are grateful for support of this research from the National Cancer Institute (CA-74954). Funding to pay the Open Access publication charges for this article was provided by CA-74954.

Conflict of interest statement. None declared.

REFERENCES

1. Wood,M.L., Esteve,A., Morningstar,M.L., Kuziemko,G.M. and Essigmann,J.M. (1992) Genetic effects of oxidative DNA damage: comparative mutagenesis of 7,8-dihydro-8-oxoguanine and 7,8-dihydro-8-oxoadenine in *Escherichia coli*. *Nucleic Acids Res*., **20**, 6023–6032.
2. Tan,X., Grollman,A.P. and Shibutani,S. (1999) 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. *Carcinogenesis*, **20**, 2287–2292.

3. Kamiya,H. (2003) Mutagenic potentials of damaged nucleic acids produced by reactive oxygen/nitrogen species: approaches using synthetic oligonucleotides and nucleotides. *Nucleic Acids Res*., **31**, 517–531.
4. Hsu,G.W., Ober,M., Carell,T. and Beese,L.S. (2004) Error-prone replication of oxidatively damaged DNA by a high-fidelity DNA polymerase. *Nature*, **431**, 217–221.
5. Collins,A.R., Cadet,J., Moeller,L., Poulsen,H.E. and Vina,J. (2004) Are we sure we know how to measure 8-oxo-7,8-dihydroguanine in DNA from human cells? *Arch. Biochem. Biophys*., **423**, 57–65.
6. Spitz,M.R., Wei,Q., Dong,Q., Amos,C. and Wu,X. (2003) Genetic susceptibility to lung cancer: the role of DNA damage and repair. *Cancer Epidemiol. Biomarkers Prevention*, **12**, 689–698.
7. Wang,D., Kreutzer,D.A. and Essigmann,J.M. (1998) Mutagenicity and repair of oxidative DNA damage: insights from studies using defined lesions. *Mutat. Res*., **400**, 99–115.
8. Dizdaroglu,M. (2003) Substrate specificities and excision kinetics of DNA glycosylases involved in base-excision repair of oxidative DNA damage. *Mutat. Res*., **531**, 109–126.
9. David,S.S. and Williams,S.D. (1998) Chemistry of glycosylases and endonucleases involved in base-excision repair. *Chem. Rev*., **98**, 1221–1261.
10. Uesugi,S. and Ikehara,M. (1977) Carbon-13 magnetic resonance spectra of 8-substituted purine nucleosides. Characteristic shifts for the Syn conformation. *J. Am. Chem. Soc*., **99**, 3250–3253.
11. Shibutani,S., Takeshita,M. and Grollman,A.P. (1991) Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-OxodG. *Nature*, **349**, 431–434.
12. McAuley-Hecht,K.E., Leonard,G.A., Gibson,N.J., Thomson,J.B., Watson,W.P., Hunter,W.N. and Brown,T. (1994) Crystal structure of a DNA duplex containing 8-hydroxydeoxyguanine-adenine base pairs. *Biochemistry*, **33**, 10266–10270.
13. Kouchakdjian,M., Bodepudi,V., Shibutani,S., Eisenberg,M., Johnson,F., Grollman,A.P. and Patel,D.J. (1991) NMR structural studies of the ionizing radiation adduct 7-dihydro-8-oxodeoxyguanosine (8-oxo-7H-dG) opposite deoxyadenosine in a DNA duplex. 8-oxo-7H-dG(syn).dA(anti) alignment at lesion site. *Biochemistry*, **30**, 1403–1412.
14. Krahn,J.M., Beard,W.A., Miller,H., Grollman,A.P. and Wilson,S.H. (2003) Structure of DNA polymerase β with the mutagenic DNA lesion 8-oxodeoxyguanine reveals structural insights into its coding potential. *Structure*, **11**, 121–127.
15. Zharkov,D.O., Rosenquist,T.A., Gerchman,S.E. and Grollman,A.P. (2000) Substrate specificity and reaction mechanism of murine 8-oxoguanine-DNA glycosylase. *J. Biol. Chem*., **275**, 28607–28617.
16. Kamiya,H., Yakushiji,H., Dugue,L., Tanimoto,M., Pochet,S., Nakabeppu,Y. and Harashima,H. (2004) Probing the substrate recognition mechanism of the human MTH1 protein by nucleotide analogs. *J. Mol. Biol*., **336**, 843–850.
17. Tchou,J., Bodepudi,V., Shibutani,S., Antoshechkin,I., Miller,J., Grollman,A.P. and Johnson,F. (1994) Substrate specificity of Fpg protein. Recognition and cleavage of oxidatively damaged DNA. *J. Biol. Chem*., **269**, 15318–15324.
18. Martin,F.H., Castro,M.M., Aboul-Ela,F. and Tinoco,I.,Jr. (1985) Base pairing involving deoxyinosine: implications for probe design. *Nucleic Acids Res*., **13**, 8927–8938.
19. Kamiya,H., Sakaguchi,T., Murata,N., Fujimuro,M., Miura,H., Ishikawa,H., Shimizu,M., Inoue,H., Nishimura,S., Matsukage,A., Masutani,C., Hanaoka,F. and Ohtsuka,E. (1992) In vitro replication study of modified bases in ras sequences. *Chem. Pharm. Bull*, **40**, 2792–2795.
20. Ciuffreda,P., Casati,S. and Santaniello,E. (1999) Lipase-catalyzed protection of the hydroxy groups of the nucleosides inosine and 2′-deoxyinosine: a new chemoenzymatic synthesis of the antiviral drug 2′,3′-dideoxyinosine. *Bioorg. Med. Chem. Lett*., **9**, 1577–1582.
21. Goodman,M.F., Creighton,S., Bloom,L.B. and Petruska,J. (1993) Biochemical basis of DNA replication. *Crit. Rev. Biochem. Mol. Biol*., **28**, 83–126.
22. Bodepudi,V., Shibutani,S. and Johnson,F. (1992) Synthesis of 2′-deoxy-7,8-dihydro-8-oxoguanosine and 2′-deoxy-7,8-dihydro-8-oxoadenosine and their incorporation into oligomeric DNA. *Chem. Res. Toxicol*., **5**, 608–617.
23. Eason,R.G., Burkhardt,D.M., Phillips,S.J., Smith,D.P. and David,S.S. (1996) Synthesis and characterization of 8-methoxy-2′-deoxyadenosine-containing oligonucleotides to probe the syn glycosidic conformation of 2′-deoxyadenosine within DNA. *Nucleic Acids Res*., **24**, 890–897.
24. Koizume,S., Kamiya,H., Inoue,H. and Ohtsuka,E. (1994) Synthesis and thermodynamic stabilities of damaged DNA involving 8-hydroxyguanine (7,8-dihydro-8-oxoguanine) in a ras-gene fragment. *Nucleosides Nucleotides*, **13**, 1517–1534.
25. Plum,G.E., Grollman,A.P., Johnson,F. and Breslauer,K.J. (1995) Influence of the oxidatively damaged adduct 8-oxodeoxygunosine on the conformation energetics, and thermodynamic stabilty of a DNA duplex. *Biochemistry*, **34**, 16148–16160.
26. Kool,E.T. (2002) Active site tightness and substrate fit in DNA replication. *Ann. Rev. Biochemistry*, **71**, 191–219.
27. Creighton,S., Bloom,L.B. and Goodman,M.F. (1995) Gel fidelity assay measuring nucleotide misinsertion, exonucleolytic proofreading, and lesion bypass efficiencies. *Methods Enzymol*., **262**, 232–256.
28. Mendelman,L.V., Petruska,J. and Goodman,M.F. (1990) Base mispair extension kinetics. *J. Biol. Chem*., **265**, 2338–2345.
29. Randall,S.K., Eritja,R., Kaplan,B.E., Petruska,J. and Goodman,M.F. (1987) Nucleotide insertion kinetics opposite abasic lesions in DNA. *J. Biol. Chem*., **262**, 6864–6870.
30. Wiederholt,C.J. and Greenberg,M.M. (2002) Fapy·dG instructs Klenow exo$^-$ to misincorporate deoxyadenosine. *J. Am. Chem. Soc*., **124**, 7278–7279.
31. Hill-Perkins,M., Jones,M.D. and Karran,P. (1986) Site-specific mutagenesis in vivo by single methylated or deaminated purine base. *Mutat. Res*., **162**, 153–163.
32. Lowe,L.G. and Guengerich,F.P. (1996) Steady-state and pre-steady-state kinetic analysis of dNTP insertion opposite 8-oxo-7,8-dihydroguanine by *E. coli*: polymerases I exo- and II exo-. *Biochemistry*, **35**, 9840–9849.
33. Hoffmann,R., Minkin,V.I. and Carpenter,B.K. (1996) Ockham's razor and chemistry. *Bull. Soc. Chim. France*, **133**, 117–130.