The Efficiency and Fidelity of Translesion Synthesis past Cisplatin and Oxaliplatin GpG Adducts by Human DNA Polymerase β*

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DNA polymerase β (pol β) is the only mammalian DNA polymerase identified to date that can catalyze extensive bypass of platinum-DNA adducts in vitro. Previous studies suggest that DNA synthesis by pol β is distributive on primed single-stranded DNA and processive on gapped DNA. The data presented in this paper provide an analysis of translesion synthesis past cisplatin- and oxaliplatin-DNA adducts by pol β functioning in both distributive and processive modes using primer extension and steady-state kinetic experiments. Translesion synthesis past Pt-DNA adducts was greater with gapped DNA templates than with single-stranded DNA templates. In the processive mode pol β did not discriminate between cisplatin and oxaliplatin adducts, while in the distributive mode it displayed about 2-fold increased ability for translesion synthesis past oxaliplatin compared with cisplatin adducts. The differentiation between cisplatin and oxaliplatin adducts resulted from a Kₘ-mediated increase in the efficiency of dCTP incorporation across from the 3′-G of oxaliplatin-GG adducts. Rates of misincorporation across platinated guanines determined by the steady-state kinetic assay were higher in reactions with primed single-stranded templates than with gapped DNA and a slight increase in the misincorporation of dTTP across from the 3′-G was found for oxaliplatin compared with cisplatin adducts.

Decreased DNA synthesis during replication is responsible in part for the chemotherapeutic effect of drugs which form bulky adducts on the DNA. Mammalian cells contain a post-replication repair pathway that allows cells to overcome replication blocks caused by these adducts. Post-replication repair can be either error-free, which would increase cellular tolerance of bulky adducts, or error-prone, which would increase the mutagenicity of bulky adducts. Previous studies have shown that mammalian cells possess the ability to replicate DNA containing cisplatin (cis-diaminedichloroplatinum(II))¹ adducts and that cisplatin-resistant cell lines have a greater ability to replicate past Pt-DNA adducts than their parental cisplatin-sensitive cell lines (1–4). This suggests that some cellular DNA polymerases must be able to replicate Pt-contain-

ing DNA relatively well and tolerant cells might have either elevated levels of the polymerases responsible for lesion bypass or mutant DNA polymerases with enhanced translesion replication activity.

DNA polymerase β has been suggested as a candidate for the role of a DNA polymerase that performs error-prone translesion synthesis of cisplatin-DNA adducts (5, 6). Pol β is the only mammalian DNA polymerase identified to date that has been shown to catalyze extensive replicative bypass of Pt-DNA adducts in vitro (5–8). In addition, pol β is able to elongate the arrested replication products of polymerases α and δ, thus showing its capacity to successfully complete stalled replication (5). The full-length DNA products of translesion synthesis by pol β contain a high frequency of mutations in the vicinity of the Pt-DNA adducts (6). On the basis of these data, Hoffmann et al. (5, 6) have suggested that pol β could be involved in error-prone translesion replication past Pt-DNA adducts in vivo. In support of this hypothesis, increased cisplatin resistance (9–12) and an increased rate of both spontaneous and cisplatin-induced mutagenesis (12) have been observed in cell lines with increased pol β expression. This has lead to the suggestion that overexpression of pol β could be associated with cisplatin resistance and a mutator phenotype (12–14). If this hypothesis is correct it could be significant, since some spontaneously occurring tumors appear to overexpress pol β (15), and pol β gene amplification has been detected in tumors from patients that have failed cisplatin therapy (9). Although cell lines expressing pol β antisense RNA show decreased sensitivity to cisplatin (16), no increased sensitivity to cisplatin has been seen in pol β-deleted cells (17). Thus, the available data suggest that pol β overexpression might represent an important mechanism of platinum drug resistance and mutagenicity, but it is not yet clear whether pol β makes a significant contribution to error-prone translesion synthesis at normal levels of expression.

Although to date there is no direct evidence showing that pol β actually carries out error-prone DNA synthesis past bulky DNA adducts in vivo, one can envision at least two models for its involvement in translesion synthesis during in vivo replication. 1) Pol β might substitute for a replication complex that had stalled at Pt-DNA lesion sites during chromosomal replication. After error-prone translesion synthesis, pol β would dissociate. Then the replication complex could reassociate at the nascent primer terminus downstream from the lesion and complete DNA replication (18). 2) Alternatively, pol β might be involved in the error-prone filling of single-stranded gaps in the daughter strand opposite the lesion. These gaps could be formed as a part of lagging strand synthesis or as a result of activation of alternative origins of replication in damaged chromosomes after the progression of the active replication fork is blocked by a DNA lesion (19). Both the extent and fidelity of translesion synthesis by pol β...
could be affected by the DNA substrate (single-stranded versus gapped). It has been shown that DNA synthesis by pol β is distributive on DNA substrates in which primer is annealed to a single-stranded template, while pol β processes distributive synthesis on short gapped DNA templates with a 5'-phosphate at the end of the gap (16, 17, 20–22). With gaps of 2–6 nucleotides pol β exhibits increased catalytic efficiency (23–25) compared with primed single-stranded DNA. However, the overall base substitution fidelity of pol β appears to be very similar with 2–6 nucleotide gaps and single-stranded templates (23, 24).

The platinum complex (trans-R,R1,2-diaminocyclohexanoxalatoplatinum(II) (oxaloplatin) is currently in clinical trial for the treatment of tumors with intrinsic and acquired resistance to cisplatin. Both cisplatin and oxaloplatin form the same types adducts on the DNA, with the most abundant being Pt(GpG) intrastrand diadducts (26–29). However, the adducts formed by oxaloplatin contain (trans-R,R1,2-diaminocyclohexane (dach) carrier ligands, while the adducts formed by cisplatin contain cis-diamine carrier ligands. Previous data from our laboratory suggest that mammalian cells are able to replicate DNA in the presence of platinum-DNA adducts and that the extent of replicative bypass in vivo can be influenced by the carrier ligand of the adducts (1, 2, 4). We also have shown recently that pol β can discriminate between cisplatin and oxaloplatin adducts in vitro. The present study was designed to evaluate whether the efficiency and carrier ligand specificity of replicative bypass past Pt-DNA adducts by pol β could be determined by the mode of translesion synthesis (distributive synthesis on single-stranded DNA versus processive synthesis on gapped DNA) and whether the fidelity of replicative bypass depends on the carrier ligand of the Pt-DNA adducts and/or on the DNA template structure.

**EXPERIMENTAL PROCEDURES**

**Construction of Platinum Adduct-containing Templates—**

Primer-templates were constructed from synthetic oligonucleotides as described previously (8). Brieﬂy, cisplatin was obtained from Sigma; Pt-(dach) species information for oxaloplatin was provided by Dr. S. D. Wyrick (University of North Carolina). All platination reactions were carried out with aquated derivatives of the platinum complexes obtained by overnight stirring in the dark at room temperature. The reactions were carried out with aquated derivatives of the platinum complexes and oxaliplatin adducts (1, 2, 4). We also have shown recently that pol β can discriminate between cisplatin and oxaloplatin adducts in vitro. The present study was designed to evaluate whether the efficiency and carrier ligand specificity of replicative bypass past Pt-DNA adducts by pol β could be determined by the mode of translesion synthesis (distributive synthesis on single-stranded DNA versus processive synthesis on gapped DNA) and whether the fidelity of replicative bypass depends on the carrier ligand of the Pt-DNA adducts and/or on the DNA template structure.

**Steady-state Polymerization Kinetics—**

Steady-state kinetic parameters $K_m$ and $V_{max}$ for dCTP incorporation were measured in standing-start reactions as described previously (8) using the 44-mer templates hybridized to 24- or 25-mer primers alone or in combination with downstream 16-mer oligonucleotides (Fig. 1, B, D, E, and F). 150 fmol of DNA substrates were replicated at 37 °C in 10-μl reaction mixtures containing 5 fmol of pol β and variable concentrations of dCTP. Initial time course studies indicated that under standard conditions the reaction velocity is linear (i.e., conforms to steady-state kinetics) for up to 12 min for single-stranded templates and up to 6 min for gapped templates (data not shown). Less than 20% of the primers were extended under the steady-state conditions ensuring single hit conditions. As expected in steady state, $V_{max}$ values were proportional to enzyme concentration (data not shown).

The velocity of dCTP incorporation opposite the template 3'-G and 5'-G sites was determined using the equation $v = (I_{t,G} - I_{t,G}^0) / (1 + k_{cat,G} / |V_{max,G}|)$, with templates B and D (Fig. 1) for the 3'-G or $v = (I_{t,G} - I_{t,G}^0) / |V_{max,G}|$, with templates E and F for the 5'-G (where $t$ is the reaction time in min; $I_{t,G}$, $I_{t,G}^0$, and $I_{t,G}^0$ are the integrated gel band intensities at sites $-1, 0,$ and 1, respectively). The relationship between $v$ and dCTP concentration was determined by a Michaelis-Menten equation, as indicated by linearity in a Hanes-Woolf plot of [-dCTP]/v versus [dCTP]. $V_{max}$ (the maximum value of reaction velocity) and $K_m$ (dCTP concentration at which the reaction velocity is half-maximal) were determined from a Hanes-Woolf plot by linear least squares fit. The efficiency of nucleotide insertion by polymerase (p) was calculated as $V_{max}/K_m$. To facilitate comparison of different Pt adducts, the relative insertion efficiency $f_{rel}$ also is reported: $f_{rel} = V_{max,p} / V_{max,\text{control}}$.  

**Misincorporation Assays—**

To measure nucleotide misinsertion opposite Pt-GG adducts, DNA substrates shown in Fig. 1, B, D, E, and F were used. 150 fmol of these DNA substrates were incubated with 5 fmol of pol β and 5 mM of each dNTP individually for 15 and 30 min for control and damaged templates, respectively. Reactions were terminated as described previously (8). The kinetics of dTTP misincorporation opposite platinum and oxaloplatin GG sites using templates shown in Fig. 1, C, E, and F, were determined in reactions similar to ones described in the previous section. To detect extension products resulting from nucleotide misincorporation, it was necessary to increase incubation times from 1–2 to 15–30 min. The dTTP misinsertion efficiency ($V_{max}$) was determined as the ratio of dTTP to dCTP insertion efficiencies.
A: 22/44 DNA template
  22-mer
  GGTGTTGTTGGGC-GCTAAGGCTTCATTGAGATTGCATGGT-3'
  Stul
  44-mer
B: 24/44 DNA template
  24-mer
  GGAAGATACCTCTTCTACGACC-5'
  44-mer
C: 22/44/16 DNA template
  16-mer
  CCACCACCAACCCGACAG
  44-mer
D: 24/44/14 DNA template
  14-mer
  CCGAGATACCTCTTCTACGACC-5'
  44-mer
E: 25/44 DNA template
  25-mer
  GGTGTTGTTGGGC-GCTAAGGCTTCATTGAGATTGCATGGT-3'
  44-mer
F: 25/44 DNA template
  25-mer
  CCGAGATACCTCTTCTACGACC-5'
  44-mer

Fig. 1. Primed single-stranded and gapped DNA substrate configurations used in primer-extension and kinetic studies. The site-specifically modified DNA templates were constructed using combination of synthetic oligonucleotides as described under “Experimental Procedures.”

RESULTS

In a previous study (8) we established the carrier-ligand specificity of pol β catalyzed translesion synthesis using primer-templates where the primer was placed either 2 or 53 bases before the GG sites containing the platinum adduct. In those studies, oxaliplatin-GG adducts were bypassed to a greater extent than cisplatin-GG adducts, and the placement of the primer relative to the Pt-GG adduct had no effect on the carrier-ligand specificity of translesion synthesis. To study the effect of template structure on the extent and specificity of translesion synthesis by pol β in more detail, we have performed the primer extension assay using undamaged or site-specifically platinumated templates of different structure (Fig. 1, A–D). All these DNA substrates contained the same 44-mer template. In addition to a 22-mer primer used previously in primer extension assays (Fig. 1A), we have utilized a 24-mer primer (Fig. 1B) to examine the capacity of pol β to initiate DNA synthesis from the base preceding the lesion. The same experiments were also carried out using 6-base gapped DNA templates with the lesion either in the center of the gap (Fig. 1C) or at the beginning of the gap (Fig. 1D).

Fig. 2, A–H, shows the time course for elongation of 22-mer or 24-mer primers on 44-mer single-stranded and gapped DNA templates, either undamaged or containing a single cisplatin adduct. The positions of primers, full-length reaction product (44-mer), and target sequence are indicated. The enzyme to DNA substrate ratio was approximately 1:4 in these experiments, which corresponds to the minimum amount of pol β necessary to ensure measurable translesion synthesis on all templates. As expected for such reaction conditions, distinct bands corresponding to practically every nucleotide in the sequence were clearly visible when undamaged single-stranded templates were used (Fig. 2, A and E). Such a pattern is consistent with the distributive nature of pol β. Singhal and Wilson (30) have shown previously that pol β fills gaps of up to 6 nucleotides by a processive mechanism that requires a phosphate group at the 5′-end of the gap. In agreement with these findings we observed no strong pause sites within the 6-nucleotide gaps on undamaged DNA substrates (Fig. 2, C and G). At the pol β concentrations used in these experiments, some strand displacement was observed with gapped DNA templates. The extent of strand displacement depended on the DNA substrate utilized. For example, with the 22/44/16 DNA template (Fig. 1C) there was a major accumulation of products varying in length from 28 nucleotides (corresponding to the end of the gap) to 32 nucleotides (corresponding to strand displacement of four nucleotides). This was accompanied by significant time-dependent strand displacement to the end of the template (Fig. 2C). However, with the 24/44/14 DNA substrate (Fig. 1D) the major stop sites varied in length from 32 nucleotides (end of the gap) to 32 nucleotides (strand displacement of two nucleotides) (Fig. 2G). This was accompanied by limited strand displacement of the remaining duplex DNA, which did not increase with time. The difference in strand displacement between the 22/44/16 and 24/44/14 DNA substrates is most likely due to the stability of the duplex region immediately downstream of the gap. For the 24/44/14 DNA substrate, the first 5 base pairs of the duplex region downstream of the gap are all G-Cs, whereas the 22/44/16 DNA substrate contains an A-T base pair located 2 bases downstream from the gap.

Primer extension on all damaged templates was substantially reduced (Fig. 2, B, D, F, and H). When single-stranded templates were used, the decrease in the amount of full-length product was mainly due to a strong inhibition of dNTP incorporation opposite the Pt-GG adduct (24-mer band), Fig. 2, B and F, and 3′ to the lesion (22- and 23-mer bands), Fig. 2B). Weaker inhibition of dNTP incorporation opposite the 5′-G(Pt)-(25-mer band), Fig. 2, B and F) and of chain extension from 5′-G(Pt)- (26-mer band), Fig. 2, B and F) was also observed. Primer extension on damaged gapped DNA templates produced multiple stop sites, although the relative distribution of the products of replication was somewhat different from that seen with single-stranded templates. Stop sites 3′ from the lesion weakened. This was most apparent for the DNA substrates with the Pt adduct in the center of the gap (compare the 23- and 24-mer bands in gel 2D (gapped DNA) with gel 2F (single-stranded DNA)). In addition, the stop site across from the 3′-G(Pt) became more prominent and additional pause sites 1 and 2 nucleotides beyond the adduct appeared. This was most apparent for the DNA substrates with the Pt adduct at the beginning of the gap (compare the 27- and 28-mer bands in gel 2H (gapped DNA) and gel 2F (single-stranded DNA)). Some strand displacement was seen with both gapped DNA templates with platinum adducts. However, in contrast to the pattern of strand displacement for undamaged templates, less strand displacement was seen for the 22/44/16 DNA template (Fig. 2D) than for 24/44/14 DNA template (Fig. 2F).

The extent of translesion synthesis past the cisplatin-GG and oxaliplatin-GG adducts was calculated as described under
“Experimental Procedures” and is summarized in Fig. 2, I and J (the stop sites were identical for cisplatin- and oxaliplatin-GG adducts, so the gels for the experiment with oxaliplatin adduct are not shown). Levels of translesion synthesis past platinum adducts (sum of products longer than 26 nucleotides (Fig. 2, B, D, F, and H) as a percent of total primer termini) appeared to be similar for both the 22/44 and 24/44 single-stranded DNA templates (Fig. 2, I and J, closed symbols). The extent of translesion synthesis past Pt-DNA adducts was increased 1.5–3-fold with gapped DNA templates (Fig. 2, I and J, open symbols) relative to single-stranded primed DNA templates. Furthermore, a higher extent of translesion replication was observed for DNA templates with the Pt adduct in the center of the gap (Fig. 2, I and J, open circles) compared with DNA templates with the Pt adduct at the beginning of the gap (Fig. 2, I and J, open triangles).

In agreement with our previous data on pol β-catalyzed primer extension at low enzyme concentrations (8), cisplatin adducts were about 2.5-fold more effective than oxaliplatin adducts at blocking translesion synthesis on both 22/44 and 24/44 single-stranded primed templates (compare Fig. 2, I and J, closed symbols). However, with both gapped DNA substrates, there was relatively little difference in the extent of translesion synthesis between templates with cisplatin-GG and oxaliplatin-GG adducts (Fig. 2, I and J, open symbols).

We have also shown previously that when the enzyme is present in excess to primed single-stranded DNA templates, pol β does not appear to discriminate between cisplatin- and oxaliplatin-DNA adducts. Thus, we examined the effect of polymerase concentration on replicative bypass using the DNA substrates described above (Fig. 3). At high enzyme concentrations (2.5–25-fold excess over primer-template), pol β did not differentiate between cisplatin and oxaliplatin adducts for any DNA substrate tested (data not shown); therefore data are presented for cisplatin-damaged templates only. Fig. 3 shows the enzyme concentration-dependence and time course for the gels for the experiment with oxaliplatin adduct (the stop sites were identical for cisplatin- and oxaliplatin-GG adducts; therefore data are presented for cisplatin-damaged templates only. Fig. 3 shows the enzyme concentration-dependence and time course for translesion synthesis past cisplatin-GG adducts using all four DNA constructs. The extra band seen at 45 nucleotides is characteristic of primer extension reactions with high concentrations of pol β and is most likely due to the intrinsic nucleotidyl transferase activity of pol β (8). With both 22/44 and 24/44 primed single-stranded templates at pol β to template ratios of 25:1, strong stop sites 1 and 2 nucleotides past the Pt-GG adducts were observed in addition to the stop sites 3’ to the Pt-GG adduct and across the lesion (Fig. 3, A and C). For both gapped templates, the stop site immediately preceding the Pt-GG adduct was decreased in intensity and the stop site immediately following the Pt-GG adduct was increased in intensity compared with the single-stranded templates (compare Fig. 3, B and D, with 3, A and C). Finally, the stop sites 1 and 2 nucleotides past the Pt-GG adducts were considerably weaker and strand displacement was significantly greater for the template with the Pt-GG adduct at the beginning of the gap compared with the template with the Pt-GG adduct in the
center of the gap. For example, with the DNA substrate containing the Pt adduct at the beginning of the gap, no stop site was observed at the end of the gap and strand displacement was essentially complete (Fig. 3D). In contrast, with the DNA substrate containing the Pt adduct in the center of the gap, a strong stop site at the end of the gap and significantly less strand displacement were observed (Fig. 3B).

The extent of translesion synthesis past cisplatin adducts with high levels of pol β appeared to be identical for 22/44 and 24/44 primed single-stranded DNA substrates (Fig. 3, A and C; Fig. 3, E and F, closed symbols). The effect of adding the 14- or 16-mer 5'-phosphorylated downstream oligonucleotides to create 6-base pair gaps (Fig. 3) was very similar to the effects observed at lower pol β concentrations (Fig. 2). The extent of translesion synthesis was increased with both gapped templates compared with single-stranded templates (Fig. 3, E and F, open symbols versus closed symbols). The most significant effect of pol β concentration was on the relative extent of translesion synthesis with the two gapped templates. At enzyme to template ratios of up to 12:1, gapped DNA with the lesion in the center of the gap was a better substrate for translesion synthesis than gapped DNA with the lesion at the beginning of the gap (Fig. 3E). However, at enzyme to template ratios of greater than 12:1, the total amount of translesion synthesis past Pt-GG sites was equal for these two DNA substrates at all reaction times tested (Fig. 3F).

Steady-state Kinetics of dCTP Insertion Opposite GG Sites on Damaged and Undamaged Templates—As shown above, oxaliplatin-GG adducts were bypassed more readily than cisplatin-GG adducts when the primer extension assay was performed with single-stranded DNA substrates under conditions of low enzyme concentration (Fig. 2, I and J, closed symbols) and/or limiting dNTP concentrations (data not shown). However, during processive gap-filling synthesis, replication past cisplatin and oxaliplatin adducts did not differ significantly (Fig. 2, I and J, open symbols). Goodman and colleagues (31) have shown that the steady-state kinetic assay is more sensitive than the primer extension assay for determining whether a polymerase is able to discriminate between adducts and can provide information about the mechanism of discrimination between adducts. This assay is performed under limiting enzyme concentrations and is more representative of true intracellular conditions than the primer extension assay. To characterize the extent of lesion bypass more quantitatively, kinetic experiments were performed on undamaged templates and templates with cisplatin and oxaliplatin adducts. In the previous primer-extension experiments, the strongest inhibitory effect of platinum adducts on DNA replication by pol β at low enzyme concentrations was reduced incorporation across from the 3'- and 5'-platinated guanines. Therefore, steady-state kinetic assays of dCTP incorporation opposite these two DNA substrates were performed. Our study was done using standing start reaction conditions where nucleotide insertion is measured at the first template site adjacent to the original primer 3' terminus. Although the absolute values of kinetic parameters, $K_{\text{cat}}$ and $K_m$, derived from the standing start assay have limited mechanistic significance, the ratios of $V_{\text{max}}/K_m$ for damaged versus undamaged templates are reliable measurements of the effect of different lesions on polymerase activity (32). All experiments were carried out in the presence of 30-fold excess of template over enzyme. Prior to kinetic studies, experiments were performed to determine the enzyme concentrations, dNTP concentrations, and times for which product accumulation was linear as a function of time.

The kinetic parameters for dCTP insertion opposite the template 3'- and 5'-Gs using control and damaged primed single-stranded and gapped DNA substrates (Fig. 1, B, D, E, and F) are presented in Table 1. As anticipated, the catalytic efficiency ($V_{\text{max}}/K_m$) for dCTP incorporation opposite the 3'- and 5'-Gs was enhanced substantially with 5-nucleotide and 6-nucleotide gapped DNA compared with primed single-stranded DNA substrates, mainly due to increases in $V_{\text{max}}$. The observed 4-fold increase in catalytic efficiency on gapped DNA relative to single-stranded DNA is comparable with that reported previously for 6-nucleotide gaps (24). Chagovetz et al. (24) reported that the increase in catalytic efficiency with 6-nucleotide gapped DNA was predominantly $K_m$-mediated. The apparent discrepancy between this report and our data may relate to differences in the assays used to measure kinetic parameters. Chagovetz et al. (24) used the running start kinetic assay. Since the kinetic parameters in our experiments were determined by the standing start assay, the $V_{\text{max}}$-governed discrimination might not
TABLE I

| Target (template) | Pt adduct | $V_{max}$ | $K_m$ | $V_{max}/K_m$ | $f_{rel}$ |
|------------------|-----------|-----------|-------|---------------|----------|
| 3'-G (primed single-stranded DNA) | Control | 0.022 ± 0.002 | 2.6 ± 0.3 | 8300 ± 470 | 0.011 |
| | Cisplatin | 0.020 ± 0.002 | 234 ± 22 | 90 ± 12 | 0.011 |
| | Oxaliplatin | 0.019 ± 0.001 | 110 ± 14 | 181 ± 25 | 0.022 |
| 5'-G (primed single-stranded DNA) | Control | 0.021 ± 0.002 | 3.2 ± 0.7 | 6460 ± 174 | 0.027 |
| | Cisplatin | 0.014 ± 0.001 | 95 ± 8 | 141 ± 22 | 0.027 |
| | Oxaliplatin | 0.016 ± 0.001 | 96 ± 6 | 165 ± 24 | 0.032 |
| 3'-G (6-nt gap) | Control | 0.125 ± 0.039 | 3.9 ± 1.7 | 32,000 ± 3000 | 0.068 |
| | Cisplatin | 0.083 ± 0.004 | 36.8 ± 2.3 | 2200 ± 200 | 0.075 |
| | Oxaliplatin | 0.086 ± 0.003 | 39.6 ± 3.2 | 2400 ± 270 | 0.075 |
| 5'-G (5-nt gap) | Control | 0.090 ± 0.014 | 3.3 ± 0.7 | 27,000 ± 600 | 0.048 |
| | Cisplatin | 0.092 ± 0.015 | 77 ± 6 | 1300 ± 290 | 0.048 |
| | Oxaliplatin | 0.100 ± 0.011 | 80 ± 7 | 1450 ± 250 | 0.054 |

necessarily represent the differences in the rate of polymerization from one site to the next, but could also be due to the differences in the rate of enzyme-DNA dissociation or association (32).

Pt-GG adducts had little effect on the $V_{max}$ for dCTP insertion opposite either the 3'- or 5'-Gs (Table I). On the other hand, Pt-GG adducts increased the $K_m$ values for dCTP insertion substantially (Table I). The increase in the $K_m$ values was greater with single-stranded DNA templates (30–90-fold) than with gapped DNA substrates (10–25-fold). Thus, the use of gapped DNA led to a considerable increase (2–3.5-fold) in the efficiency of nucleotide insertion across both 3'-G(Pt) and 5'-G(Pt) relative to single-stranded DNA. With a single-stranded DNA template, the $K_m$ for insertion of dCTP opposite the 3'-G of an oxaliplatin-GG adduct was about one-half that for insertion of dCTP opposite the 3'-G of a cisplatin-DNA adduct, while the $V_{max}$ values for both reactions were very similar (Table I). This resulted in overall 2-fold increase in insertion efficiency across the 3'-G of an oxaliplatin adduct. However, no significant differences were evident between templates with cisplatin and oxaliplatin adducts in the relative insertion efficiency of dCTP opposite the 5'-G(Pt) on single-stranded DNA or opposite either the 3'-G(Pt) and 5'-G(Pt) on gapped DNA (Table I).

Nucleotide Misincorporation Opposite GGs on Undamaged and Platinated Templates—pol β is known to lack associated proofreading activity and to exhibit high fidelity during DNA replication in vitro (33, 34). It is reasonable to expect that the presence of platinum adducts on DNA might influence polymerase fidelity and that the conformation of the adduct might be important in determining the miscoding potential of the lesion. Therefore, a kinetic fidelity assay to measure single nucleotide incorporation opposite the template 3'-G and 5'-G was performed on damaged and control single-stranded and gapped DNA substrates. Initially, extension studies by pol β were performed in the presence of 5 mM of either dTTP, dGTP, and dATP to obtain qualitative information about misincorporation across from the platinum adducts (data not shown). Primer extension assays were performed for 15 min on control templates and for 30 min on damaged templates with a 30-fold excess of DNA substrate over enzyme using primers which ended just prior the template 3'-G and 5'-G sites. Both single-stranded and gapped templates were utilized. Some misincorporation of T was detected for both control and damaged templates, except for the incorporation across from the 5'-G on single-stranded templates. However, none of the other nucleotides was inserted opposite the 3'-G and 5'-G sites by pol β under the conditions of these assays with any of the DNA templates used.

A steady-state kinetic analysis of dTTP incorporation was then performed to characterize the misincorporation efficiency more quantitatively (Table II). Since no detectable dTTP misincorporation across from the 5'-G on single-stranded templates was found in the primer extension studies, kinetic experiments were not performed with the 25/44 DNA substrate. Therefore, the frequency of dTTP misincorporation on single-stranded versus gapped DNA could only be compared for the 3'-G. The efficiency ($V_{max}/K_m$) of dTTP incorporation opposite the undamaged 3'-G residue for 6-nucleotide gapped DNA substrate was about 10 times higher compared with the single-stranded DNA, mainly due to a decrease in apparent $K_m$ values. This enhanced dTTP incorporation efficiency resulted in about 2.4-fold enhanced misinsertion frequency ($f_{rel}$) compared with single-stranded DNA. The presence of a platinum lesion on the DNA, mainly due to a decrease in apparent $K_m$ values, increased the misinsertion frequency with all DNA templates except for the incorporation across from the 5'-G on single-stranded templates. However, none of the other nucleotides was inserted opposite the 3'-G and 5'-G sites by pol β under the conditions of these assays with any of the DNA templates used.

For both single-stranded and gapped DNA substrates, the efficiency of dTTP incorporation ($V_{max}/K_m$) and frequency of misinsertion ($f_{rel}$) opposite the 3'-G (Pt) were both approximately 1.7-fold higher for oxaliplatin adducts than for cisplatin adducts (Table II). The greater efficiency of dTTP misincorporation across from the 3'-G(Pt) on oxaliplatin adducts compared with cisplatin adducts on single-stranded templates was determined by both lower $K_m$ and higher $V_{max}$ values, while for gapped DNA substrates this difference was primarily due to an increase in $V_{max}$. Misincorporation opposite the 5'-G of a cisplatin adduct on the gapped substrate was too low for reliable determination of kinetic parameters, and dTTP misincorporation opposite the 5'-G(Pt) on single-stranded templates was too low to be detected for both cisplatin and oxaliplatin adducts.
Translesion Synthesis past Pt-DNA Adducts by Pol β

TABLE II

| Target (template) | Pt adduct | $V_{\text{max}}$ | $K_m$ | $V_{\text{max}}/K_m$ | $f_{\text{rel}}$ |
|------------------|-----------|------------------|-------|---------------------|----------------|
| 3'-G (primed single-stranded DNA) | Control | 0.0027 ± 0.0005 | 640 ± 150 | 4.52 ± 0.39 | 5.4 × 10^{-4} |
| | Cisplatin | 0.0058 ± 0.0009 | 820 ± 95 | 0.76 ± 0.14 | 84.4 × 10^{-4} |
| | Oxaliplatin | 0.00104 ± 0.00004 | 440 ± 57 | 2.52 ± 0.34 | 139.2 × 10^{-4} |
| 5'-G (primed single-stranded DNA) | Control | ND | ND | ND | ND |
| | Cisplatin | ND | ND | ND | ND |
| | Oxaliplatin | ND | ND | ND | ND |
| 3'-G (6-nt gap) | Control | 0.0020 ± 0.0013 | 50.3 ± 9.1 | 41.6 ± 4.1 | 13 × 10^{-4} |
| | Cisplatin | 0.0026 ± 0.0003 | 1700 ± 200 | 1.5 ± 0.1 | 6.8 × 10^{-4} |
| | Oxaliplatin | 0.0054 ± 0.0013 | 2040 ± 500 | 2.9 ± 0.5 | 12.1 × 10^{-4} |
| 5'-G (5-nt gap) | Control | 0.0015 ± 0.0008 | 260 ± 80 | 7.85 ± 1.83 | 2.9 × 10^{-4} |
| | Cisplatin | ND | ND | ND | ND |
| | Oxaliplatin | 0.0011 ± 0.0006 | 880 ± 80 | 1.30 ± 0.14 | 9 × 10^{-4} |

* ND, not determined. No detectable dTTP misincorporation was observed opposite the 5'-G on primed single-strand DNA. Bands produced as a result of dTTP misincorporation across from the 3'-G of the cisplatin adduct on the gapped DNA template were detectable but were too weak for reliable measurement of kinetic parameters.

DISCUSSION

It has been recently postulated that pol β overexpression facilitates translesion synthesis of cisplatin adducts, leading to increased drug tolerance and induced mutagenesis (12, 13). It is not clear whether pol β at normal levels catalyzes sufficient amounts of translesion synthesis to influence cisplatin cytotoxicity, since pol β-deleted cells do not exhibit increased sensitivity to this drug treatment (17). However, mutation frequency in response to cisplatin treatment was not measured for pol β-deleted cells. Therefore, the available data do not rule out the possibility that pol β may play a role in error-prone translesion synthesis even when expressed at normal levels.

The experiments presented here confirm the previously reported data (5, 6, 8) showing that pol β can catalyze extensive bypass of platinum-DNA adducts in a single-stranded region of DNA. These data may be relevant to pol β’s postulated participation in leading strand and most lagging strand translesion synthesis at the replication fork. In the present study we also found that translesion synthesis by pol β is even greater during short gap-filling synthesis (Figs. 2 and 3), which might occur during some lagging strand translesion synthesis (19). In agreement with the primer-extension data, steady-state kinetic analysis also showed that the catalytic efficiency ($V_{\text{max}}/K_m$) of translesion synthesis by pol β was greater with gapped DNA than with single-stranded DNA templates. Thus, when primed single-stranded DNA was used, the efficiency of dCTP incorporation across from both the 3' and 5'-Gs of Pt-GG adducts was decreased by a factor of 40–100 compared with incorporation across from undamaged guanines, while for gapped DNA only a 13–20-fold decrease was observed. The decrease in dCTP insertion opposite platinated guanines resulted from a substantial increase in the $K_m$ values for dCTP with all damaged DNA substrates (Table I). At the same time, the Pt adducts did not appear to affect the rate of phosphodiester bond formation, since the apparent $V_{\text{max}}$ values were similar for all templates.

For the cisplatin-GG adduct, the efficiency ($V_{\text{max}}/K_m$) of dCTP incorporation across from the 5'-G(Pt) was two times higher than that across from the 3'-G(Pt) for primed single-stranded substrates. These data contradict the prediction made by Bradley et al. (35) that polymerases should have more difficulty traversing the 5’-nucleotide than the 3’-nucleotide. The crystal structure of the cisplatin-GG adduct shows a greater distortion of the 3'-G-C base pair than the 5'-G-C base pair (36), while the NMR solution structure shows a greater distortion in the vicinity of the 5'-G-C base pair (37). Our data suggest that, when bound to the active site of pol β, the cisplatin-GG adduct may adapt a conformation which is closer to that seen in crystals than to that seen in solution.

With the primer extension assay, primed single-stranded DNA templates, and physiological conditions (low enzyme concentrations (Fig. 2, B and F) and/or low dNTP concentrations (data not shown)), pol β catalyzes 2.5-fold more translesion synthesis than oxaliplatin adducts than past cisplatin adducts (Ref. 8, Fig. 2). These findings were confirmed by steady-state kinetic experiments that showed a 2-fold higher efficiency of dCTP incorporation opposite the 3'-G of oxaliplatin-GG adducts than opposite the 3'-G of cisplatin-GG adducts. This was due almost entirely to a 2-fold decrease in the $K_m$ for dCTP incorporation opposite the 3'-G for the oxaliplatin adducts compared with cisplatin adducts. In contrast, the efficiency ($V_{\text{max}}/K_m$) of nucleotide insertion across from the 5'-G(Pt) was similar for both cisplatin- and oxaliplatin-GG adducts. Therefore, the increased translesion synthesis past oxaliplatin adducts seen in the primer extension experiments is determined primarily by enhanced nucleotide incorporation across from the 3'-G(Pt). The structure of the oxaliplatin-GG adduct has not been studied in detail, but our kinetic data with pol β suggest that the oxaliplatin- and cisplatin-GG adducts will show the greatest conformational differences in the vicinity of the 3'-G(Pt). Our previous data show that oxaliplatin-GG adducts are bypassed more readily than cisplatin-GG adducts by human pol γ and yeast pol ζ, as well as by human pol β (8). Those data suggest that the differences in bypass of oxaliplatin-GG and cisplatin-GG adducts are most likely due to the conformational differences of the adducts themselves rather than to unique interactions of the adducts with the active site of an individual DNA polymerase.

In primer extension experiments using an excess of enzyme over single-stranded DNA, pol β did not appear to differentiate between cisplatin and oxaliplatin adducts (Ref. 8 and Fig. 3). Similar levels of translesion synthesis past cisplatin and oxaliplatin adducts over a wide range of enzyme concentrations were also found with the primer extension assay during pro-
cessive gap-filling DNA synthesis (Fig. 3). The primer extension data with gapped DNA templates were supported by steady-state kinetic experiments which showed that pol β did not discriminate between cisplatin and oxaliplatin adducts in the efficiency of dCTP incorporation across from either the 3'- or the 5'-G(Pt) of gapped DNA templates (Table I). The fact that both increased enzyme concentration and the switch to a more processive mode of action diminish the differences between oxaliplatin and cisplatin adducts suggests that the ability of pol β to discriminate between cisplatin and oxaliplatin adducts is strongly influenced by the rate of polymerase dissociation from the damaged template.

For the gapped DNA substrates, the distance of the lesion from both the 3'-primer terminus and the 5' terminus of the downstream oligonucleotide seemed to have a considerable effect on the extent and pattern of translesion synthesis. Our data suggest that association between pol β and gapped DNA may be more difficult when the primer 3'-end is immediately adjacent to the lesion than when the primer is separated from the lesion by two nucleotides. Once the polymerase successfully associates with either of the gapped templates, replicative bypass proceeds processively due to binding of the 8-kDa domain of pol β to the 5'-phosphate moiety at the end of the gap (30). This results in an increased level of replicative bypass of Pt-DNA adducts (Figs. 2 and 3) and an increased efficiency of nucleotide incorporation (V_{max}/K_{m}) opposed platinated guanines (Table I) on gapped DNA compared with single-stranded DNA. The position of the lesion relative to the end of the gap also influenced the extent of strand displacement. When the Pt adduct was located at the beginning of the gap, strand displacement proceeded with the same effectiveness as with undamaged DNA. However, when the Pt adduct was located in the center of the gap, completion of gap filling and subsequent strand displacement was inhibited (Fig. 3B).

Cisplatin is known to be a mutagen in mammalian cells (38). However, the relative mutagenicity of cisplatin and oxaliplatin in mammalian cells has not been reported. Cisplatin-induced mutations in human cells have been detected at both the 5'- and 3'-Gs of Pt-GG adducts, and both G-C to T-A transversions and G-C to A-T transitions have been observed at high frequency (39, 40). Hoffmann et al. (6) have demonstrated that replication past cisplatin-GG adducts by calf thymus DNA polymerase β in vitro is mutagenic. The present study compared the fidelity of pol β during translesion synthesis past cisplatin and oxaliplatin adducts on DNA substrates of different structure. Under our experimental conditions, dTTP was the only dNTP that was misinserted opposite 3'-G(Pt) with both single-stranded and gapped DNA (data not shown). This observation appears to conflict with previous reports that pol β catalyzes G-T and G-A mispairs with similar efficiency (24). However, previous studies have also shown that the fidelity of pol β is highly dependent on sequence context (23). Thus, the differences between the current and previous studies may be related to differences in sequence context and/or the assays used. With undamaged DNA the misincorporation frequency for dTTP opposite Gs was 2.4-fold greater for gapped DNA than for single-stranded DNA (Table II). A similar increase in misincorporation frequency for G-T mispairing during 6 nucleotide gap filling synthesis was reported by Chagovetz et al. (24). Moreover, the overall frequencies of dTTP misinsertion opposite guanine on undamaged single-stranded DNA and 6-nucleotide gapped DNA are in agreement with previous reports (24, 41). In contrast to misincorporation opposite undamaged 3'-G, the fidelity of dNTP insertion opposite 3'-G(Pt) was significantly (about 12-fold) greater with gapped DNA than with single-stranded DNA with both cisplatin- and oxaliplatin-GG adducts (Table II).

The dTTP misincorporation frequency opposite the 3'-G(Pt) with both single-stranded and gapped DNA was 1.7-fold greater for oxaliplatin adducts than for cisplatin adducts (Table II), which reinforces the suggestion that the conformation of cisplatin- and oxaliplatin-GG adducts differs significantly in the vicinity of the 3'-G(Pt). However, it is important to note that dTTP misincorporation does not appear to represent the major mutagenic effect of platinum adducts on pol β. Hoffmann et al. (6) have recently shown that the mutational hot spots generated by pol β in vitro are situated 5' to the lesion and that the frequency of frameshift mutations in that region is much greater than the frequency of base substitution mutations. Therefore, these data do not necessarily predict the relative mutagenicity of cisplatin and oxaliplatin adducts in pol β over-expressing cells.

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