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Replication Past the *N*⁵-Methyl-Formamidopyrimidine Lesion of Deoxyguanosine by DNA Polymerases and an Improved Procedure for Sequence Analysis of In Vitro Bypass Products by Mass Spectrometry

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Abstract

Oligonucleotides containing a site-specific *N*⁶-(2-deoxy-D-erythro-pentofuranosyl)-2,6-diamino-3,4-dihydro-4-oxo-5-*N*-methylformamidopyrimidine (MeFapy-dGuo) lesion were synthesized and their *in vitro* replication by *Escherichia coli* DNA polymerase I Klenow fragment (exo[−]) and *Sulfolobus solfataricus* P2 DNA polymerase IV (Dpo4) resulted in the misincorporation of Ade, Gua and Thy opposite the MeFapy-dGuo lesion in addition to the correct insertion of Cyt. However, sequencing of the full-length extension product revealed that the initial insertion of Cyt opposite the lesion was extended most efficiently. Two sequences were examined and the misincorporation was sequence dependent. Improvements in the method for the mass spectrometric sequencing of the extension products were developed; a 5'-biotinylated primer strand was used that contained a dUrd near the template-primer junction. The extended primer was immobilized with streptavidin-coated beads allowing it to be washed free of polymerase, the template strand, and other reagents. The extended primer was cleaved from the solid support with uridine DNA deglycosylase and piperidine treatment, and the extension products were sequenced by LC-ESI-MS-MS. The purification steps afforded by the biotinylated primer resulted in improved sensitivity for the MS analysis. Translesion synthesis of a template with a local 5'-T-(MeFapy-dGuo)-G-3' sequence resulted in only error-free bypass and extension, whereas a template with a local 5'-T-(MeFapy-dGuo)-T-3' sequence also resulted in an interesting deletion product and the mis-incorporation of Ade opposite the MeFapy-dGuo lesion.

Introduction

The N7-position of guanine is generally regarded as the most nucleophilic site in DNA and cationic N7-dGuo adducts are formed as the predominant species from the reaction of DNA with many alkyl halides, nitrogen and sulfur mustards, and epoxides (1). The cationic N7-dGuo species can undergo depurination to produce the well-studied abasic site (2,3). A competing reaction to depurination is the ring-opening of the imidazolium ion through the addition of hydroxide ion to the C8 resulting in a formamidopyrimidine (Fapy) in which the formamide

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nitrogen (N^5) is substituted (Scheme 1). The parent Fapy-dGuo lesion in which the N^5 -position is unsubstituted ($R=H$) arises from oxidative damage to DNA (1,4). Fapy-dGuo and Fapy-dAdo lesions from the oxidative pathways have been observed in biological samples and are excised from genomic DNA by base-excision repair proteins (5–14).

Methylation of DNA occurs as a result of exposure to xenobiotics such as *N*-methylnitrosoamines; DNA can also be methylated through the non-enzymatic reaction with *S*-adenosyl-L-methionine (15). The major product from the reaction of DNA with methylating agents is N7-methyl-dGuo; however, N7-dGuo adducts are generally regarded as non-miscoding since the Watson-Crick hydrogen bonding face is not altered, and the $G \rightarrow A$ transitions that results from exposure to methylating agents have been ascribed to O^6 -methyl-dGuo (16–18). Methylation damage at the O^6 -position of dGuo, O^4 -position of dThd, and the phosphate oxygen atoms is repaired by alkylguanine transferases, which are inactivated by transfer of the methyl group from DNA to an active site cysteine (19). Interestingly, it was observed that the major persistent DNA adduct in the bladders and livers of rats following exposure to *N*-methylnitrosourea, *N,N*-dimethylnitrosamine or 1,2-dimethylhydrazine was the MeFapy-dGuo adduct (20,21). This observation suggests that MeFapy-dGuo could contribute to the mutagenicity of methylating agents.

We report here *in vitro* replication studies of two oligonucleotides containing the MeFAPy-dGuo lesion at a define site. Single-nucleotide incorporation studies with exonuclease-deficient *Escherichia coli* DNA polymerases I Klenow fragment (Kf^-) and *Sulfolobus solfataricus* P2 DNA polymerase IV (Dpo4) suggest that MeFapy-dGuo has miscoding potential; however, further extension of the product from the correct insertion of dCTP opposite the template MeFapy-dGuo is much more efficient than from beyond MeFapy-dGuo paired with other bases, thereby reducing the proportion of error-prone translesion synthesis. We utilized an LC-ESI-MS-MS method previously developed in our lab to sequence the extension products and the sensitivity of this method was improved by using primers containing a 5'-biotin group for purification of the extension product before MS analysis.

Experimental Procedures

Oligonucleotide Synthesis

The oligodeoxynucleotides were synthesized on a Perseptive Biosystems Model 8909 DNA synthesizer on a 1 μ mol scale using their Expedite reagents with the standard synthetic protocol for the coupling of the unmodified bases. The coupling of the MeFapy-dGuo phosphoroamidite was performed off-line manually for 30 min as previously described (22). The DMTr group of the MeFapy-dGuo was removed automatically with using a “short” deprotection cycle (160 μ L of Cl_3CCO_2H for 20 s) to minimize rearrangement to the pyranose form as we previously reported (23). The remainder of the synthesis was performed on-line using standard protocols. The modified oligodeoxynucleotides were cleaved from the solid support and the exocyclic amino groups were deprotected in a single step using 0.1 M NaOH at room temperature overnight. Gel purification of the oligonucleotides was conducted on a denaturing gel containing 8.0 M urea and 16% acrylamide (w/v) (from a 19:1 acrylamide/bisacrylamide solution (w/w), AccuGel, National Diagnostics, Atlanta, GA) with 80 mM Tris borate buffer (pH 7.8) containing 1 mM EDTA. Modified oligonucleotides were characterized by MALDI-TOF MS.

HPLC purification

Oligonucleotides were purified on a YMC ODS-AQ column (250 $\times$ 4.6 mm, flow rate 1.5 mL/min or 250 $\times$ 10 mm, flow rate 5 mL/min) or Phenomenex Gemini-C18 column (250 $\times$ 4.6 mm, flow rate 1.5 mL/min or 250 $\times$ 10 mm, flow rate 5 mL/min) with UV detection at 254 nm.

HPLC gradients consisted of 100 mM aqueous ammonium formate and CH₃CN for oligonucleotide purification. Gradient: initial conditions were 1% CH₃CN; a linear gradient to 8% CH₃CN over 5 min; a linear gradient to 20% CH₃CN over 15 min; a linear gradient to 80% CH₃CN over 2 min; isocratic at 80% CH₃CN for 1 min; then a linear gradient to the initial conditions over 2 min.

5'-TCAT-(MeFapy-dGuo)-GAATCCTTACGAGCATCGCCCC-3' (1)

Purified by gel electrophoresis. MALDI-TOF MS (HPA) *m/z* calcd for (M-H), 8495.1; found 8496.4.

5'-TCGT-(MeFapy-dGuo)-TCAATCCTTACGAGCATCGCCCC-3' (2)

Purified by gel electrophoresis. MALDI-TOF MS (HPA) *m/z* calcd for (M-H), 8777.4; found 8775.3.

Oligonucleotide labeling and annealing

The labeling and annealing of the oligonucleotides was performed as previously described (24).

Single-nucleotide Incorporation Assays

These assays were performed as previously described with the following modifications (24). The reactions with Kf[−] (25) and Dpo4 (26) were initiated by the addition of the dNTP with final concentrations of 25, 50, and 100 μM. The final concentrations of DNA, Kf[−], and Dpo4 were 100, 24, and 80 nM, respectively. The Kf[−] reactions were run at room temperature for 10 min, and the Dpo4 reactions were run at 37 °C for 30 min. The reactions with the primer and unmodified template were run under the same reaction conditions but with one-half the enzyme concentration. Reactions were quenched with 70 μL of 20 mM EDTA in 95% formamide (v/v) containing xylene cyanol and bromophenol blue dyes, and heated at 95 °C for 10 min (24). Aliquots (6 μL) were separated by electrophoresis on a denaturing gel.

Steady-state Kinetics

These assays were performed according to a published procedure with the following modifications (24). All reactions (10 μL final volume, 100 nM DNA, and 24 nM Kf[−] or 80 nM Dpo4) were run at nine dNTP concentrations and quenched with 70 μL of 20 mM EDTA in 95% formamide (v/v) containing xylene cyanol and bromophenol blue dyes and heated at 95 °C for 10 min (24). Aliquots (6 μL) were separated by electrophoresis on denaturing gels. Each analysis was performed in duplicate.

Full-length Polymerase Extension Assays

These assays were performed as previously described with the following modifications (24). The final dNTP concentrations for the reactions with Kf[−] and Dpo4 were 25, 50, and 100 μM each. The final concentrations of DNA, Kf[−], and Dpo4 were 100 nM, 24 nM, and 80 nM, respectively. The Kf[−] reactions were run at room temperature for 10 min, the Dpo4 reactions were run at 37 °C for 30 min. The reactions with the primer and unmodified template were run using the same reaction conditions but with one-half the enzymes concentration. Reactions were quenched by the addition of 70 μL of 20 mM EDTA in 95% formamide (v/v) containing xylene cyanol and bromophenol blue dyes and heated at 95 °C for 10 min. Aliquots (6 μL) were separated by electrophoresis on denaturing gels (24).

LC-ESI-MS-MS Sequencing of the Extension Reactions

The Kf[−] and Dpo4 extension reactions were performed for 6 h in Tris-HCl buffer (50 mM, pH 7.8) containing DNA duplex (1.55 nmol) with the biotinylated primer, dithiothreitol (DTT, 1 mM), bovine serum albumin (BSA, 50 μg/mL), NaCl (50 mM), and MgCl₂ (5 mM); the total volume of the reaction was 200 μL. The reactions were performed with all four dNTP's (1 mM each) and polymerase concentrations of 100 nM of Kf[−] or Dpo4. After the extension reaction was complete, streptavidin-coated beads (0.5 mL of the streptavidin-coated bead suspension was centrifuged and subsequently washed with 3 × 500 μL 100 mM of sodium phosphate buffer, pH 7.0) and 800 μL of sodium phosphate buffer (pH 7.0) were added to the reaction and the resulting suspension was placed in a rotating shaker for 2 h. The liquid was removed and the streptavidin-coated beads were washed with H₂O (3 × 300 μL). A solution (500 μL) containing uracil DNA glycosylase (UDG, 20 units), EDTA (1 mM), and DTT (1 mM) in Tris-HCl buffer (50 mM, pH 7.8) was added to the streptavidin-coated beads and mixture shaken for 4 h at 37 °C. The liquid was removed and streptavidin-coated beads were washed with H₂O (3 × 300 μL). A solution of piperidine (final concentration of 0.25 M in 400 μL) was added to the streptavidin-coated beads and the mixture was heated at 95 °C for 1 h. The liquid was decanted and the beads were washed with H₂O (3 × 200 μL). The piperidine cleavage fraction was combined with the H₂O washes, lyophilized, and the residue was dissolved in H₂O (70 μL). An aliquot (20 μL) was removed and the 5'-pCTTACGAGCCCCC-3' oligonucleotide standard (0.2 nmols) was added; a 10-μL aliquot was used for the analysis. MS analysis was performed in the Vanderbilt University facility on a Waters Acquity UPLC system (Waters, Milford, MA) connected to a Finnigan LTQ mass spectrometer (ThermoElectron) equipped with an Ion Max API source and a standard electrospray probe using an Acquity UPLC BEH C18 column (1 μm, 1.0 mm × 100 mm). LC conditions were as follows: buffer A contained 10 mM NH₄CH₃CO₂ plus 2% CH₃CN (v/v) and buffer B contained 10 mM NH₄CH₃CO₂ plus 95% CH₃CN (v/v). The following gradient program was used with a flow rate of 150 μL/min: initially 0% B; 3 min linear gradient to 3% B; 1.5 min linear gradient to 20% B; 0.5 min linear gradient 100% B; isocratic at 100% B for 0.5 min; 1 min linear gradient to 0% B; isocratic at 0% B for 3 min. The temperature of the column was maintained at 50 °C and the samples (10 μL) were infused with an auto-sampler. The electrospray conditions were as follows: source voltage 4 kV, source current 100 μA, N₂ was used as the auxiliary gas and the flow-rate setting was 20, sweep gas flow-rate setting 5, sheath gas flow setting 34, capillary voltage −49 V, capillary temperature 350 °C, and tube lens voltage −90 V. No CID offset was employed. MS/MS conditions were as follows: normalized collision energy 35%, activation *Q* 0.250, and activation time 30 ms. The isolation width in MS/MS was 2. The automatic gain control (AGC) settings in full MS and MSⁿ were 10000. The maximum injection time in full MS and MSⁿ were 10 ms and 40 ms, respectively. The MS data were acquired in negative mode. Helium was used as the collision damping gas in the ion trap and was set at a pressure of 1 mTorr. The number of μscan used for data acquisition in full MS and MSⁿ modes was 2 and 1, respectively. Product ion spectra were acquired over the range *m/z* 345–2000. The ions were selected for CID analysis and the elucidation of the CID fragmentations of the candidate oligonucleotide sequence was done with the aid of the Mongo Oligo Mass Calculator (v. 2.6) from the University of Utah (<http://library.med.utah.edu/masspec/>). After the oligonucleotide sequence was identified, the proposed sequence was purchased from Midland Certified Reagents (Midland, TX) and subjected to the same LC-ESI-MS-MS analysis in order to compare the CID spectra.

Construction of the Calibration Curves

Standard calibration curves were constructed using 5 – 7 different concentrations (from 0.02 to 0.4 nmol) of the corresponding oligonucleotide (analyte) and a constant amount of the 5'-pCTTACGAGCCCCC-3' standard (0.10 nmol). The amount of the standard corresponded to ~50% yield for the extension reaction. The analytes and the standard were purchased from

Midland Certified Reagents (Midland, TX). The calibration curves were constructed in full scan mode using the sum of the extracted [M-2H] and [M-3H] ions for the analyte oligonucleotides and the sum of the [M-3H] and [M-4H] ions for the standard. The correlation coefficient (r^2) were typically > 0.98 . The yield of formation of the corresponding analyte was calculated based on ratio of the amount (nmol) of the full-extension product and the amount (nmol) of the biotinylated primer used for the full-length extension reaction. Each peak consisted of at least 15 scans.

The 5'-pCTTACGAGCCCCC-3' standard (0.10 nmol) was added to known amounts of the 5'-p-TGACACGA-3' (0.28 nmol), 5'-p-TGACACGAG-3' (0.68 nmol), 5'-p-TGAAAGA-3' (0.40 nmol), 5'-p-TGACCGA-3' (0.20 nmol), and 5'-p-TGACCGAC-3' (0.14 nmol) analytes. The mixture volume was brought to 20 μ L and 10 μ L was injected into the UPLC. The amounts of the analytes were calculated based on the ratio of the area of analyte to standard (13mer) and the corresponding calibration curve.

Results

Oligonucleotides Synthesis

We have recently described the synthesis of a phosphoramidite reagent of MeFapy-dGuo and its incorporation into oligonucleotides by solid-phase techniques (23). The ribose unit of Fapy derivatives can rearrange from the furanose to the pyranose form when the 5'-hydroxyl group is free (27,28). We found that this isomerization could be minimized by altering the deprotection cycle for removal of the 5'-DMTr group from the MeFapy-dGuo nucleotide. A "short" deprotection cycle favored the furanose form while a "long" deprotection cycle favored the pyranose form. The MeFapy-dGuo was incorporated at position 5 of oligonucleotides **1** (28mer) and **2** (29mer) using the short deprotection cycle (see Figure 1 for sequences). The position of the lesion was chosen to facilitate MS sequencing of the extension products. The modified oligonucleotides were initially purified by HPLC to remove minor amounts of the pyranose form and then further purified by preparative PAGE; the purity was estimated to be $>99\%$ by capillary gel electrophoresis. Oligonucleotides **1** and **2** were characterized by MALDI-TOF mass spectrometry and the presence of the MeFapy-dGuo was confirmed by enzymatic digestion (23).

Polymerase Bypass of the MeFapy-dGuo Adduct

The *in vitro* replication bypass and extension of the MeFapy-dGuo adduct was examined with the model DNA polymerases Kf⁻ and Dpo4. The 28mer oligonucleotide **1** containing the MeFapy-dGuo lesion in a 5'-T-(MeFapy-dGuo)-G-3' local sequence was annealed to a complementary 23mer (-1) primer strand, which was 5'-labeled with a ³²P-phosphate. The steady-state insertion rates were determined for the individual dNTPs (Table 1) (29).

The MeFapy-dGuo adduct was found to be miscoding; in addition to the correct insertion of dCTP opposite the lesion, Kf⁻ and Dpo4 misincorporated dATP, dGTP, and TTP (see Figures S8 and S9 of the supporting information for the gel analyses). Incorporation of dCTP was surprisingly efficient. The k_{cat}/K_m values for dCTP insertion opposite the MeFapy-dGuo lesion with Kf⁻ and Dpo4 were only 8.3 and 1.4-fold lower than insertion opposite an unmodified dGuo (Table 1 and Table 2, respectively). Misincorporation frequencies (f) for the other dNTPs ranged from 4–30 percent for Kf⁻; Dpo4 was more discriminating with misincorporation frequencies of $<2\%$. Interestingly, Kf⁻ and Dpo4 inserted only dCTP and dATP opposite the MeFapy-dGuo adduct of the 5'-T-(MeFapy-dGuo)-T-3' local sequence (**2**) with dCTP insertion being 13.7- and 23-fold more efficient (Table 2, see Figures S10 and S11 of the supporting information for gel analyses). Insertion of dATP opposite the lesion in the 5'-T-(MeFapy-dGuo)-G-3' sequence (**1**) was the least efficient.

The ability of the polymerases to extend past the MeFapy-dGuo adduct was also examined in the presence of all four dNTPs. Starting from the -1 primer, full-length products were observed for both polymerases (Figure 1). Kf⁻ showed a significant pause after insertion opposite the MeFapy-dGuo lesion as well as unextended primer for both sequences. Dpo4 extended past the lesion more efficiently; intermediate length extension product could be observed in minor amounts, reflective of the distributive nature of Dpo4. The 5'-T-(MeFapy-dGuo)-T-3' sequence (2) appears to be for efficiently extended, which may reflective of lower misincorporation frequencies for the "incorrect" bases.

Sequencing of the Extension Products

To better understand the mechanism of lesion bypass, we utilized an LC-ESI-MS-MS method previously developed in our laboratories to analyze the sequence of the extension products (24,26,30–38). A dUrd was incorporated near the template-primer junction and the extension reaction was carried out in the presence of all four dNTPs. The reaction mixture was then treated with UDG followed by piperidine, providing a 3'-fragment of the extension product for mass spectrometric analysis. Details of this method have been previously described (24,26). The nucleotide composition of each extension product can be calculated from its mass; CID fragmentation provided sequence information (39,40). We were initially unable to detect a product from the extension reaction of the 5'-T-(MeFapy-dGuo)-G-3' template (1) with Kf⁻. We suspected that byproducts from the protocol interfered with the detection of the extension products because there is no purification step prior to MS analysis. A protocol to clean-up the extension reaction was developed that utilized a primer containing a biotin group on the 5'-end (Scheme 2). Streptavidin-coated polystyrene beads were added after the extension was complete. The beads were washed and then UDG was added. After a second wash step, piperidine was added to cleave the desired 3'-end of the extension product from the bound oligonucleotide. The eluate was collected and dried to remove excess piperidine before LC-ESI-MS-MS analysis. When a 5'-biotinylated primer was utilized, poor recovery of the extension product was initially observed. We suspected that binding of the biotin to the streptavidin-coated beads physically inhibited efficient deglycosylation by UDG. The incorporation of ten dThds between the biotin-containing moiety and the primer sequence dramatically improved the recovery of the product. An example of the analysis is shown below for the Kf⁻ extension reaction of the 5'-T-(MeFapy-dGuo)-G-3' 1 (Figure 2).

Extension of the 5'-T-(MeFapy-dGuo)-G-3' template 1 by Kf⁻ resulted in a single extension product. The TIC spectrum showed peaks at m/z 718.9 and 1078.7, which were assigned as the [M-3H] and [M-2H] ions of the same oligonucleotide product by the observation of the appropriate mass difference of the isotopic cluster (~0.3 and 0.5 Da, respectively). The peaks at m/z 982.7 and 1310.7 are the [M-4H] and [M-3H] ions of the 5'-pCTTACGAGCCCCC-3' oligonucleotide, which was added to the sample as an internal standard in order to quantitate the extension products (*vide infra*). Three possible nucleotide compositions were calculated for an oligonucleotide of mass 2159.4 ± 2 Da (<http://library.med.utah.edu/masspec/>). Given the sequence of the starting template, the most likely composition of the product contained two dCyds, two dThds, two dAdos, one dGuo and a phosphate (pC₂T₂A₂G, m/z 2159.4), which is the expected composition for error-free bypass and extension of the template. Analysis of the CID spectrum (Figure 2C) of the m/z 1078.7 ion confirmed that the species is the error-free product, 5'-pTCCATGA-3'. The observed and theoretical CID fragment ions for this product are listed in Table 3. This product was further confirmed by comparison of the CID spectrum to that of an authentic oligonucleotide (Figure 2D). The identity of the component corresponding to t_r 4.40 min is unknown, but its mass spectrum indicates that it is not an oligonucleotide. Extension products from the *in vitro* bypass of the MeFapy-dGuo adduct by Dpo4 were also subjected to sequence analysis. As with Kf⁻, a single product arising from error-free bypass and extension was observed.

Bypass and extension of the alternative 5'-T-(MeFapy-dGuo)-T-3' local sequence (**2**) was found to be more error-prone. The predominant extension product of the 5'-T-(MeFapy-dGuo)-T-3' template was still the error-free product for both polymerases examined. A second error-free extension product was observed for the Kf^- reaction and was derived from a blunt end addition of dGTP to the expected error-free product. For both polymerases, a minor product derived from the mis-insertion of dATP opposite the adduct followed by error-free extension was identified. A summary of the extension products is given in Table 5. Of interest, a product derived from insertion of dCTP opposite the MeFapy-dGuo lesion, followed by deletion of the 3'-dThd (5'-pTGAC-CGA-3') was observed (Figure 3). A related product from the Kf^- polymerization reaction derived from the one-base deletion of the 3'-dThd but with a blunt end addition of dCTP (5'-pTGAC-CGAC-3') was also identified. We note that a w_1 -fragment, which would definitively identify the 3'-terminal nucleotide, was not observed for this product; therefore, the two nucleotides of the 3'-end could be reversed.

Quantitation of Extension Products

We previously reported relative yields of the extension products by comparing peak intensities of the TIC spectra (24,26,30–38). After the extension products were identified, the polymerization reaction was repeated and a 13mer oligonucleotide standard (5'-pCTTACGAGCCCCC-3') was added prior to MS analysis in order to obtain an estimate of the efficiency of translesion synthesis and distribution of products. Calibration curves between the standard and each of the extension products were individually developed using a constant concentration of the standard that corresponded to ~50% yield of the extension reaction and 5–7 different concentrations of the product (see Figures S14, S20, S24, S28, S31, and S35 in the Supporting Information). The amount of each oligonucleotide was based on the total ion current corresponding to a particular oligonucleotide, which were the $[M-2H]^- + [M-3H]^-$ ions for the extension products and $[M-3H]^- + [M-4H]^-$ ions for the 13mer standard.

Replication of the 5'-T-(MeFapy-dGuo)-G-3' oligonucleotide (**1**) by Kf^- and Dpo4 afforded only the error-free product 5'-pTCCATGA-3'. The yield of this product was estimated to be 31 and 74 % for Kf^- and Dpo4, respectively. Analysis of the full-length extension reaction from the Kf^- polymerization revealed a significant build-up of products corresponding to the insertion of one-nucleotide and unextended primer, which accounts for the lower yield (Figure 1).

Three different product types were afforded from the replication of the 5'-T-(MeFapy-dGuo)-T-3' oligonucleotide (**2**). The major extension product(s) was derived from error-free translesion synthesis, which also included the blunt end addition of dGuo (5'-pTGACACGAG-3'), and were formed in 36% and 51% yield from Kf^- and Dpo4, respectively. Products that resulted from a one-base deletion, 5'-pTGAC-CGA-3' and 5'-pTGAC-CGAC-3', were observed at levels of 17% with Kf^- and 11% with Dpo4. The final product from the extension of **2**, derived from the misinsertion of dAdo opposite the MeFapy-dGuo lesion (5'-pTGAAACGA-3'), was observed to the extent of 2–3%.

Replication of the 5'-T-(MeFapy-dGuo)-G-3' oligonucleotide (**1**) resulted in a single extension product and the quantitation is likely to be accurate. The extension of the 5'-T-(MeFapy-dGuo)-T-3' oligonucleotide (**2**) resulted in five products for Kf^- and three for Dpo4. Separation of the multiple products and standard was not achieved. Co-eluting analyte mixtures can change the properties of the droplet that is formed during the electrospray ionization process, which in turn affects the amount of ions in the gas phase; this phenomena is referred to a ion suppression or ion enhancement (41,42). Since the calibration curves were constructed with a single analyte and standard, ion suppression/enhancement can result in quantitation errors when multiple analytes co-elute. To address the potential influence of ion suppression/enhancement on the quantitation of the extension products, a sample was prepared that contained known amounts

of the five oligonucleotides products from the extension of the 5'-T-(MeFapy-dGuo)-T-3' template (**2**) and the 13mer standard and subjected to LC-ESI-MS analysis. The extracted ion profile for the sum of the [M-2H] and [M-3H] ions of the extension product oligonucleotides and the sum of the [M-3H] and [M-4H] ions of the standard is shown in Figure 4. We observed a 20% deviation between the calculated and known concentration for only one oligonucleotide (5'-pTGAAACGA-3'), while the deviation was <10% for the other four extension products.

Discussion

The MeFapy-dGuo and Fapy-dGuo lesions have been the subjects of previous replication studies. MeFapy-dGuo has been reported to be a significant block to replication by Kf and T4 DNA polymerase (43–47). Ide and coworkers utilized an oligonucleotide containing a site-specific MeFapy-dGuo lesion and observed that exonuclease proficient Kf (Kf⁺) preferentially inserted dCTP opposite the MeFapy-dGuo lesion with a V_{max}/K_m value 10^4 greater than the other three dNTPs and about 67-fold lower than dCTP insertion opposite an unmodified template (47); the local sequence context for these studies was a 5'-CT-(MeFapy-dGuo)-TC-3', which is similar to oligonucleotide **2** of our work. Additionally, the extension of the MeFapy-Gua•Cyt template-primer terminus was much *more* favorable than when the primer was mismatched. It was concluded that the extension step was the major kinetic barrier to the lesion bypass of MeFapy-dGuo by Kf. In the case of **2**, we observed that Kf⁺ inserted dCTP 13.7-times more efficiently than dATP. The exonuclease activity of the polymerase may account for the higher degree of discrimination for the insertion opposite the lesion in the study by Ide and coworker. Full sequencing of the extension products demonstrated that further extension of the primer with Cyt opposite the MeFapy-Gua lesion was most favorable and was the only product observed for 5'-T-(MeFapy-dGuo)-G-3' template (**1**). Overall, our results compare favorably with the previous replication studies of MeFapy-dGuo.

In vitro by-pass and extension of the MeFapy-dGuo lesion in a 5'-T-(MeFapy-dGuo)-G-3' sequence (**1**) by Kf⁺ and the model Y-family polymerase Dpo4 resulted in error-free products paralleling the results of Ide and coworkers with Kf⁺. The bypass and extension of the MeFapy-dGuo lesion by Kf⁺ proceeded in low yield. Gel analysis of the extension reactions revealed a significant amount of unextended primer and a product that was extended by one nucleotide (Figure 1). The latter can be attributed to initial mis-insertion opposite the MeFapy-dGuo lesion because only insertion of dCTP opposite the MeFapy-dGuo leads to further extension. We attribute the accumulation of unextended primer to the presence of the α -anomer of the MeFapy-dGuo lesion. Carell and coworkers have prepared oligonucleotides containing a configurationally stable, carbocyclic analogue of the Fapy-dGuo lesion and found that the α -anomer is a strong block to yeast pol η and *Bacillus stearothermophilus* pol I (48,49). Error-free bypass and extension by Dpo4 proceeded in higher yield. The efficiency of dCTP insertion opposite the MeFapy-dGuo lesion by Dpo4 was only ~2-fold lower than opposite an unmodified template dGuo, and Dpo4 better discriminated in favor of dCTP insertion over mis-insertion of the other dNTPs. The efficiency for the replication past the MeFapy-dGuo lesion by Dpo4 suggests a possible role for DinB DNA polymerases in the bypass of MeFapy-dGuo lesion.

Previous *in vitro* extension studies with the Fapy-dGuo lesion reported that insertion of dCTP opposite the lesion is favored by Kf⁺ (50). Fapy formation from either initial oxidation or N7-alkylation of dGuo does not affect the Watson-Crick hydrogen-bonding face of the base; thus, preferential insertion of dCTP opposite a FAPy lesion is easily understood (47). Of the other dNTPs, insertion of dATP opposite Fapy-dGuo was most favored (50). Wiederholt and Greenberg have proposed two models for the instructive insertion of dATP opposite Fapy-dGuo (Figure 5) both involving the formyl N⁵-H acting as a hydrogen-bond donor with N1 of dATP (50). The N⁵-H is replaced with an N⁵-CH₃ in MeFapy-dGuo and eliminates this

hydrogen-bonding interaction. Consistent with this model, the insertion efficiency of dATP opposite MeFapy-dGuo is <5% that of dCTP in the 5'-T-(MeFapy-dGuo)-G-3' sequence (**1**) and similar to insertion of dATP opposite an unmodified dG template (Table 1 and Figure S8). The insertion of dATP opposite the MeFapy-dGuo lesion is less efficient in a 5'-T-(MeFapy-dGuo)-T-3' sequence (**2**) when the polymerase was Kf⁻ and the same for Dpo4; however, the extension from the dAdo primer terminus opposite the MeFapy-dGuo is competitive in this sequence. It is possible that the dATP is templating off the 5'-dThd that flanks the MeFapy-dGuo in both oligonucleotide sequences.

Site-specific mutagenesis studies of the Fapy-dGuo reported G→T transversions when replicated in *E. coli* or mammalian cells (51,52). The mutational frequency in *E. coli* was <2%. It was found that MeFapy-dGuo is about 3.5-fold more mutagenic in a local 5'-T-(Fapy-dGuo)-T-3' sequence than a 5'-T-(Fapy-dGuo)-A-3' sequence (29% vs. 8%) when replicated in COS-7 cells (51,52). The later finding inspired us to examine the *in vitro* replication of MeFapy-dGuo in oligonucleotide **2** containing a local 5'-T-(MeFapy-dGuo)-T-3'. Bypass of the 5'-T-(Fapy-dGuo)-T-3' template (**2**) resulted in largely error-free free products similar to the 5'-T-(Fapy-dGuo)-G-3' sequence (**1**). We observed a low level of an error-prone bypass product derived from mis-insertion of dATP followed by error-free extension. An interesting product that results from bypass of the 5'-T-(Fapy-dGuo)-T-3' template (**2**) is the correct insertion of dCTP opposite the MeFapy-dGuo lesion followed by deletion of the 5'-dThd. The analogous deletion product was not observed for the bypass of the 5'-T-(Fapy-dGuo)-G-3' oligonucleotide (**1**) (Table 5). A mechanism for the deletion that takes into account the sequence effect is not obvious to us.

The products from translesion synthesis past the MeFapy-dGuo lesion and their distribution were determined by mass spectrometry. The most accurate way to quantitate products by mass spectrometry is by the stable isotope dilution method in which the internal standard is an isotopically labeled version (isotopomer) of the analyte of interest. Uniformly labeled nucleosides are currently available in small quantities and are expensive; therefore, the use of isotopically labeled oligonucleotide standards may be impractical, particularly when multiple extension products are formed. We utilized a 13mer oligonucleotide as a standard to quantitate the extension products. In the one case examined, we estimate that ion suppression/enhancement typically caused ~10 % error in the quantitation but can be as high as 20%.

Conclusion

Oligonucleotides containing MeFapy-dGuo lesions were synthesized using a protected phosphoramidite and used for *in vitro* polymerase extension reactions with Kf⁻ and the model Y-family polymerase Dpo4. These studies demonstrated that the MeFapy-dGuo lesion was miscoding in that insertion of all four dNTPs was observed at various efficiencies depending on the DNA polymerase. However, sequencing of the full-length extension products revealed that further extension of a template-primer terminus consisting of MeFapy-dGuo•dCyd was most favorable, in accord with previous results of Ide and coworkers (47). The results of the bypass studies were dependent on local sequence. Full-length extension products were sequenced by an improved LC-ESI-MS-MS protocol involving a biotinylated primer that resulted in improved sensitivity; in addition, a 13mer oligonucleotide was added as an internal standard that allowed us to quantify the yield of the extension products. Only error-free bypass and extension was observed for oligonucleotide **1** with a local 5'-T-(MeFapy-dGuo)-G-3', whereas oligonucleotide **2** in a local 5'-T-(MeFapy-dGuo)-T-3' sequence also resulted in an interesting deletion product and the mis-incorporation of A opposite the MeFapy-dGuo lesion. Bypass and extension of the MeFapy-dGuo was more efficient with Dpo4 and suggests a possible role for DinB polymerases in Fapy replication.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

Fapy, formamidopyrimidine
 Fapy-dGuo, *N*⁶-(2-deoxy-D-*erythro*-pentofuranosyl)-2,6-diamino-3,4-dihydro-4-oxo-5-formamidopyrimidine
 MeFapy-dGuo, *N*⁶-(2-deoxy-D-*erythro*-pentofuranosyl)-2,6-diamino-3,4-dihydro-4-oxo-5-*N*-methylformamido-pyrimidine
 Kf, *Escherichia coli* polymerase I, Klenow fragment ("-" denotes exonuclease deficient)
 Dpo4, *Sulfolobus solfataricus* P2 DNA polymerase IV
 UDG, uracil DNA glycosylase
 ES, electrospray
 DMTr, 4,4'-dimethoxytrityl
 TIC, total ion current
 CID, collision-induced dissociation
 BSA, bovine serum albumin
 DTT, dithiothreitol
 MALDI-TOF, matrix-assisted laser desorption/time-of-flight (MS)

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3'-CCCCCGCTACGAGCATTCCTAAG-(MeFapy-dGuo)-TACT-5' (1)
 5'-GGGGGCGATGCTCGTAAGGATTTC

3'-CCCCCGCTACGAGCATTCCTAACT-(MeFapy-dGuo)-TGCT-5' (2)
 5'-GGGGGCGATGCTCGTAAGGATTGA

Figure 1.
 Bypass and extension of the MeFapy-dGuo lesion by Kf⁻ and Dpo4 with increasing concentration of dNTPs (μM).

Figure 2. UPLC-ESI-MS-MS analysis of the extension of the 5'-T-(MeFapy-dGuo)-G-3' template **1** by Kf^- . **A.** UPLC trace of the extension reaction vs. total ion current. **B.** Full-scan product ion spectrum of extension reaction products with an internal standard. **C.** CID product ion spectrum of the extension product ($m/z=1078.7$ Da.) **D.** CID spectrum of an authentic standard of the extension product.

Figure 3. UPLC-ESI-MS-MS analysis of the extension of the 5'-T-(MeFapy-dGuo)-T-3' template **2** by Dpo4. **A.** Extracted ion profile of the sum of the extracted [M-2H] and [M-3H] ions extension products and [M-3H] and [M-4H] of the standard. **B.** Full-scan mass spectrum of extension reaction products at retention time 4.75 min with an internal standard. **C.** CID spectrum of the extension product 5'-pTGACCGA-3' ($m/z = 1091.5$). **D.** CID spectrum of an authentic standard of the extension product.

Figure 4.

Averaged full scan spectrum from $t = 3.90 - 4.20$ min (**A**) and the extracted ion profile (**B**) of a known mixture of the five extension products (sum of the $[M-2H]$ and $[M-3H]$ ions) from of the 5'-T-(MeFapy-dGuo)-T-3' template (**2**) with Kf^- and the 13mer standard (sum of the $[M-3H]$ and $[M-4H]$ ions).

Figure 5.
Models for the instructive insertion of dCTP and dATP opposite Fapy-dGuo lesions.

Scheme 1.

Scheme 2.

Table 1

Steady-state kinetic parameters for single-nucleotide incorporation opposite the MeFapy-dGuo adduct in a local 5'-T-(MeFapy-dGuo)-G-3' sequence (1) by Kf⁻ and Dpo4.

Polymerase	Template	dNTP	K_m (μM)	k_{cat} ($s^{-1} \cdot 10^{-3}$)	k_{cat}/K_m ($\mu M^{-1} \cdot s^{-1} \cdot 10^{-3}$)	f^a
Kf ⁻	MeFapy-dGuo	C	0.066 ± 0.02	2.0 ± 0.1	30	1
		T	0.33 ± 0.09	2.8 ± 0.3	8.5	0.28
		G	0.19 ± 0.02	1.7 ± 0.1	8.9	0.29
	dG	A	1.20 ± 0.7	1.6 ± 0.2	1.3	0.04
		C	0.013 ± 0.001	3.3 ± 0.1	250	1
		T	3.4 ± 0.2	1.2 ± 0.3	0.4	0.001
Dpo4	MeFapy-dGuo	G	2.1 ± 0.02	5.2 ± 0.3	2.5	0.01
		A	2.8 ± 0.9	5.5 ± 0.5	2.0	0.008
		C	0.098 ± 0.02	0.27 ± 0.01	2.8	1
		T	6 ± 1	0.13 ± 0.1	0.02	0.01
		G	13 ± 4	0.60 ± 0.01	0.05	0.02
		A	7 ± 2	0.20 ± 0.08	0.03	0.01
		C	0.077 ± 0.017	0.30 ± 0.02	3.9	1
		T	29 ± 8	0.29 ± 0.03	0.01	0.003
		G	49 ± 14	0.43 ± 0.04	0.01	0.003
		A	22 ± 6	0.25 ± 0.01	0.01	0.003

f^a = misincorporation frequency = $(k_{cat}/K_m)_{incorrect} dNTP / (k_{cat}/K_m)_{correct} dNTP$ (dCTP)

Steady-state kinetic parameters for single-nucleotide incorporation opposite the MeFapy-dGuo adduct in a local 5'-T-(MeFapy-dGuo)-T-3' sequence (2) by Kf⁻ and Dpo4.

Polymerase	Template	dNTP	K _m (μM)	k _{cat} (s ⁻¹ •10 ⁻³)	k _{cat} /K _m (μM ⁻¹ •s ⁻¹ •10 ⁻³)	f _e
Kf ⁻	MeFapy-dGuo	C	0.16 ± 0.1	0.66 ± 0.07	4.1	1
	dG	A	5.9 ± 0.1	1.8 ± 0.3	0.3	0.07
		C	0.026 ± 0.001	2.1 ± 0.03	66	1
Dpo4	MeFapy-dGuo	A	3.8 ± 0.2	1.0 ± 0.1	0.3	0.005
		C	0.71 ± 0.09	0.53 ± 0.06	0.7	1
		A	8.8 ± 2.7	0.24 ± 0.02	0.03	0.04
	dG	C	0.20 ± 0.06	0.24 ± 0.06	1.2	1
		A	30 ± 9	0.29 ± 0.02	0.01	0.01

$$a_f = \text{misincorporation frequency} = (k_{cat}/K_m)_{\text{incorrect dNTP}} / (k_{cat}/K_m)_{\text{correct dNTP (dCTP)}}$$

Table 3Observed and theoretical CID ions of the 5'-pTCCATGA-3' extension product ($m/z = 1078.7$).

CID ion assignment	observed	theoretical
5'-pT (a_2 -B)	481.1	481.0
5'-pTC (a_3 -B)	770.2	770.9
5'-pTCC (a_4 -B)	1059.2	1059.1
5'-pTCCAT (a_6 -B)	1676.1	1676.2
(a_6 -B, -2)	837.7	837.6
pCCATGA-3' (w_6)	926.7	926.7
pCATGA-3' (w_5)	1565.2	1565.3
pATGA-3' (w_4)	1276.2	1276.2
pTGA-3' (w_3)	963.2	963.2
pGA-3' (w_2)	659.2	659.1

Table 4Observed and theoretical CID ions of the 5'-pTGAC-CGA-3' extension product ($m/z = 1091.5$).

CID ion assignment	observed	theoretical
5'-pT (a_2 -B)	480.8	481.04
5'-pTG (a_3 -B)	810.0	810.09
5'-pTGA (a_4 -B)	1122.8	1123.15
5'-pTGAC (a_5 -B)	1411.6	1412.20
5'-pTGACC (a_6 -B)	1702.6	1701.24
(a_6 -B, -2)	849.9	850.12
pACCGA-3' (w_5)	1550.0	1550.26
pCCGA-3' (w_4)	1236.9	1237.20
pCGA-3' (w_3)	948.0	948.16
pGA-3' (w_2)	659.0	659.11

Table 5

Summary of the bypass and extension of the 5'-T-(MeFapy-dGuo)-G-3' (**1**) and 5'-T-(MeFapy-dGuo)-T-3' (**2**) templates as determined by LC-ESI-MS-MS sequencing.

Polymerase	Extension products from the 5'-T-(MeFapy-dGuo)-G-3' template 1 (yield)	Extension products from the 5'-T-(MeFapy-dGuo)-T-3' template 2 (yield)	Comment
Kf ⁺	5'-pTCCATGA-3' (31 %)	5'-pTGACACGA-3' (26 %)	Error-free
		5'-pTGACACGAG-3' (12 %)	Error-free
		5'-pTGAAACGA-3' (3 %)	Error-prone
		5'-pTGACCGA-3' (14 %)	One-base deletion
		5'-pTGACCGAC-3' (3 %)	One-base deletion
Dpo4	5'-pTCCATGA-3' (74 %)	5'-pTGACACGA-3' (51 %)	Error-free
		5'-pTGAAACGA-3' (2%)	Error-prone
		5'-pTGACCGA-3' (11%)	One-base deletion