Synthesis and Characterization of Oligonucleotides Containing a Nitrogen Mustard Formamidopyrimidine Monoadduct of Deoxyguanosine

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Supporting Information

ABSTRACT: N3-Substituted formamidopyrimidine adducts have been observed from the reaction of dGuo or DNA with aziridine containing electrophiles, including nitrogen mustards. However, the role of substituted Fapy-dGuo adducts in the biological response to nitrogen mustards and related species has not been extensively explored. We have developed chemistry for the site-specific synthesis of oligonucleotides containing an N3-nitrogen mustard Fapy-dGuo using the phosphoramidite approach. The lesion was found to be a good substrate for Escherichia coli endonuclease IV and formamidopyrimidine glycosylase.

INTRODUCTION

Nitrogen mustards (bis(2-chloroethyl)amine derivatives; Figure 1) are a family of bifunctional DNA alkylating agents that were first introduced into a clinical setting in 1942 for the treatment of non-Hodgkin lymphoma.1,2 Nitrogen mustards (NM) are still used in combination with other agents for the treatment of a variety of cancers.3 Among the predominant site of reaction of nitrogen mustards with DNA is at N7-dGuo. Cationic N7-dGuo adducts are generally considered to be benign,3,4 but, like DNA alkylating agents, an array of minor products are also formed.5–7 The cytotoxicity of simple monofunctional alkylating agents has been ascribed to minor O6-dGuo, O7-dThd, and N3-dAdo adducts.8–9

The mechanism of action of nitrogen mustards involves an initial intramolecular S,N2 reaction to form an aziridinium ion, which is the DNA modifying agent.10,11 The nucleophilicity of the N atom can be attenuated by the third substituent, designated as R in Figure 1. Although the N7-dGuo adduct is generally the major adduct (N7-NM-dGuo, Figure 1), with the second chloride (X = Cl) partially or fully hydrolyzed to the corresponding alcohol (X = OH),12 the bifunctional nature of nitrogen mustards allows them to react with a second nucleophilic site in DNA to afford intra- and interstrand cross-links as well as with nucleophilic sites of proteins to form DNA–protein cross-links.13–15 This second adduction reaction presumably involves aziridination of the initial N7- adduct. Cross-links between the N7 positions of dGuo (N7-N7-NM-dGuo), between the N3-positions of dAdo, and between N7-dGuo and N3-dAdo have been identified, among others.16–19 Many crucial cellular processes (i.e., replication and repair) require the transient separation of the DNA strands. DNA interstrand cross-links prevent this strand separation and are therefore predicted to be highly cytotoxic to cells.20,21 Indeed, although DNA interstrand cross-links typically account for only 1–5% to the total adduct burden, they are believed to be the key lesion in the mechanism of action of nitrogen mustards and related agents. Interestingly, nitrogen mustards prefer to form interstrand cross-links in a 5′-GNC-3′ rather than in a 5′-GC-3′ sequence context.21–24

The products from the reaction of nitrogen mustards with Guo, dGuo, and DNA have been well-studied. Chetsanga25 and Hemminki26–28 have independently characterized the imidazole ring-opening of the N7-dGuo adduct of a nitrogen mustard to afford the corresponding N3-substituted formamidopyrimidine (Fapy) lesion (Figure 1). N3-(2-Aminoethyl)-Fapy-dGuo (AE-Fapy-dGuo) has been observed to be the major product from the reaction of aziridine with dGuo and DNA.29,30 A Fapy-dGuo adduct has also been characterized from the reaction of DNA with acid-activated mitomycin, an aziridine-containing natural product.31 However, little attention has been paid to nitrogen mustard Fapy-dGuo adducts (NM-Fapy-dGuo). Previous studies have shown that Fapy-dGuo adducts have mutagenic potential32 and are substrates for the base excision repair pathway.33–34 Oligonucleotides containing site-specific NM-Fapy-dGuo lesions will allow for future replication and repair studies both in vitro and in cells. We have reported the synthesis of oligonucleotides containing the N3-methyl-Fapy-dGuo lesion (MeFapy-dGuo) by solid-phase methods.35–40 The extension of this chemistry to the synthesis of oligonucleotides containing the NM-Fapy-dGuo lesion (R = Et) is described herein. We find that the NM-Fapy-dGuo lesion is a substrate for Escherichia coli endonuclease IV (Endo IV) and formamidopyrimidine glycosylase (FPG) in vitro.

Supporting Information

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Experimental Procedures

\(\text{N}^2-\{\text{[Dimethylamino]methylene}\} \cdot \text{O}^6-\{2-(\text{trimethylsilyl})\} \cdot \text{ethyl}\} \cdot \text{2'-deoxyguanosine} (2)\). A solution of 1 \(426 \text{ mg}, 1.16 \text{ mmol}\) and \(\text{NNN}-\text{dimehtylformamide dimethyl acetal} (1.22 \text{ mL}, 9.3 \text{ mmol})\) in dry methanol (20 \text{ mL}) was heated at 60 °C for 6 h. The methanol was then removed in vacuo on a rotary evaporator. Purification of the resulting solid by flash chromatography on silica afforded 2 \(441 \text{ mg}, 90\%\). The eluent was initially 96:4 \text{CH}_3\text{Cl}_2/\text{CH}_3\text{OH}, and the \text{CH}_3\text{OH} content was increased by 1\% every column volume \(\sim 200 \text{ mL}\) to a final ratio of 92:8. 1H NMR (DMSO-\(d_6\)): \(\delta \ 8.57 (s, 1 \text{H}, \text{N}=\text{CH})\), 8.25 \((s, 1 \text{H}, \text{H}-8)\), 6.30 \((t, 1 \text{H}, J = 6.00 \text{ Hz}, \text{H}-1')\), 5.29 \((s, 1 \text{H}, 3'-\text{OH})\), 5.14 \((s, 1 \text{H}, \text{OH}^5-S)\), 4.59 \((t, 2 \text{H}, J = 6.00 \text{ Hz}, \text{OCH}_2\text{CH}_3\text{Si})\), 4.39 \((m, 1 \text{H}, \text{H}^3')\), 3.85 \((m, 1 \text{H}, \text{H}^4')\), 3.62–3.58 \((m, 2 \text{H}, \text{H}^5', \text{H}^6')\), 3.17 \((s, 3 \text{H}, \text{N}=\text{CH}-)\), 3.08 \((s, 3 \text{H}, \text{CH}_3\text{-N}=\text{CH}^-)\), 2.65 \((m, 1 \text{H}, J=2.2', 2.2' = 6.00 \text{ Hz}, \text{H}-1')\), 2.27 \((m, 1 \text{H}, \text{H}-2')\), 2.10 \((t, 2 \text{H}, J = 6.00 \text{ Hz}, \text{OCH}_2\text{CH}_3\text{Si})\), 0.15 \((s, 9 \text{H}, \text{Me}_3\text{-Si})\). HRMS \((\text{FAB}+)\) \(m/z\) calc for \(\text{C}_{22}\text{H}_{27}\text{N}_5\text{O}_5\text{Si} [M + H]^+\): 423.2180; found, 423.2171.

\(\text{N}^2-\{\text{Dimethylamino}methylene\} \cdot \text{O}^5-\{\text{bis}[4-(\text{methoxyphenyl})\text{phenoxyethyl})\} \cdot \text{O}^2-\{\text{trimethylsilyl}ethyl\} \cdot \text{2'-deoxyguanosine} (3)\). Compound 2 \(420 \text{ mg}, 1.00 \text{ mmol}\) was dissolved in anhydrous pyridine, evaporated on a rotary evaporator, dried overnight under high vacuum, and then dissolved in dry pyridine \(15 \text{ mL}\). Dimethoxytrimethane chloride \(372 \text{ mg}, 1.11 \text{ mmol}\) was added in three equal portions, and the mixture stirred at room temperature overnight. The pyridine was removed in vacuo on a rotary evaporator. Purification of the residue by flash chromatography on silica afforded 3 \(677 \text{ mg}, 85\%\). The eluent was initially 94:5:1 \text{CH}_3\text{Cl}_2/\text{CH}_3\text{OH}/\text{pyridine}, and the methanol content was increased by 1\% every column volume \(\sim 200 \text{ mL}\) to a final ratio of 91:7.1. 1H NMR (DMSO-\(d_6\)): \(\delta \ 9.02 (s, 1 \text{H}, \text{N}=\text{CH})\), 8.42 \((s, 1 \text{H}, \text{H}-8)\), 7.30–7.13 \((m, 9 \text{H}, \text{ArH})\), 6.80–6.70 \((m, 4 \text{H}, \text{ArH})\), 6.43 \((t, 1 \text{H}, J = 6.00 \text{ Hz}, \text{H}-1')\), 5.54 \((s, 1 \text{H}, 3'-\text{OH})\), 4.74 \((t, 2 \text{H}, J = 6.00 \text{ Hz}, \text{OCH}_2\text{CH}_3\text{Si})\), 4.45 \((m, 1 \text{H}, \text{H}^3')\), 3.96 \((m, 1 \text{H}, \text{H}^4')\), 3.69 \((s, 6 \text{H}, 2\text{CH}_2\text{O})\), 3.33 \((s, 3 \text{H}, \text{CH}_3\text{-N}=\text{CH}^-)\), 3.20 \((s, 3 \text{H}, \text{CH}_3\text{-N}=\text{CH}^-)\), 3.13–3.12 \((m, 2 \text{H}, \text{H}^5', \text{H}^6')\), 2.54–2.52 \((m, 1 \text{H}, \text{H}^2')\), 2.48–2.46 \((m, 1 \text{H}, \text{H}^7')\), 1.92 \((t, 2 \text{H}, J = 6.00 \text{ Hz}, \text{OCH}_2\text{CH}_3\text{Si})\), 0.10 \((s, 9 \text{H}, \text{Si})\). HRMS \((\text{FAB}+)\) \(m/z\) calc for \(\text{C}_{46}\text{H}_{55}\text{N}_9\text{O}_{10}\text{Si} [M + H]^+\): 968.4686; found, 968.4692.

**Figure 1.** General structure of a nitrogen mustard (NM) and some of their dGuo adducts.

[Image of nitrogen mustard structure]

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Scheme 1. Synthesis of the NM-Fapy-dGuo Phosphoramidite

4H, ArH), 6.27 and 6.23 (two m, 1H, H-1'), 5.31 and 5.12 (s, total 1H, 3'-OH), 4.28 (m, 1H, H-3'), 4.10–4.00 (m, 2H, AcOCH2CH2N), 4.23 (m, 1H, H-4'), 3.72 (s, 6H, 2CH2O), 3.50–3.35 (m, 2H, EtNCH2CH2NCHO), 3.22–3.17 (multiple s, total 6H, 2CH2N-CH), 2.98–2.90 (m, 2H, H-5'), 2.60–2.52 (m, 6H, CH2CH3, AcOCH2CH3, EtNCH2CH2NCHO), 1.98–1.85 (m, 5H, H-2', AcO), 1.33–1.22 (multiple t, total 3H, CH2NCH2). HRMS (FAB+) m/z calculated for C42H54N7O9 [M + H]+, 800.3978; found, 800.3985.

N6-(4-[5-O-(Bis(4-methoxyphenyl)phenylmethyl)-3-O-(N,N-disopropylamino)-2-cyanoethoxy]phosphino)-2-deoxy-α-erythro-pentofuranosylamino)-1,4-dihydro-4-oxo-5-N-[2-(2-hydroxyethyl)-ethylamino]ethylformamidopyrimidine (8). Compound 7 (100 mg, 0.13 mmol) was dissolved in dry CH2Cl2 (20 mL), and a solution of 1M NaOH was added to the gummy residue was dissolved in dry CH2Cl2/CH3OH/pyridine; the CH3OH content was increased by 1%. The mixture of isomers (63.0 mg, 50%). The eluent was initially 97:2:1 CH2Cl2/CH3OH/pyridine, the CH3OH content was increased by 1%. The eluent was initially 97:2:1 CH2Cl2/CH3OH/pyridine, the CH3OH content was increased by 1%.

Oligonucleotide Synthesis. The oligodeoxynucleotides were synthesized on a Perceptive Biosystems Model 8909 DNA synthesizer on a 1 μmol scale using Expedite reagents (Glen Research) with the standard synthetic protocols for the coupling of unmodified bases. The coupling of the NM-Fapy-dGuo phosphoramidite was performed offline for 5 min as previously described.41,43

Enzymatic Digestion and Analysis of Oligonucleotides. The enzymatic digestion of oligonucleotides was carried out in a single step as follows: the oligonucleotide (0.5 A260 units) was dissolved in 70 μL of buffer (pH 7, 10 mM Tris-HCl, 10 mM MgCl2). DNase I (5 units), alkaline phosphatase (1.7 units), and snake venom phosphodiesterase I, type II (0.02 units), were added, and the solution was incubated at 37 °C for 1.5 h. HPLC analysis was performed using solvent gradient 1. Conditions for the HPLC and UPLC-MS analyses are provided in the Supporting Information.

5'-GCT AGC-(NM-Fapy-dGuo)-AG TCC-3' (9). Purified by reversed-phase HPLC using gradient 2 (see Supporting Information for conditions). MALDI-TOF MS (HPA) m/z calculated for [M – H]−, 3775.7; found, 3777.2

5'-ACC AGC TGA GC-(NM-Fapy-dGuo)-AGT CCT AAC AAC-3' (10). Purified by reversed-phase HPLC using gradient 3 (see Supporting Information for conditions). MALDI-TOF MS (HPA) m/z calculated for [M – H]−, 7406.9; found, 7407.7

5'-ACC AGC TGA GC-(8-oxo-dGuo)-AGT CCT AAC AAC-3'. This oligonucleotide was synthesized using commercially available 8-oxo-dGuo phosphoramidite and purified by reversed-phase HPLC using gradient 4 (see Supporting Information for conditions). MALDI-TOF MS (HPA) m/z calculated for [M – H]−, 7289.8; found, 7287.0.

5'-ACC AGC TGA GC-(MeFapy-dGuo)-AGT CCT AAC AAC-3' and 5'-ACC AGC TGA GC-(a-DG)-AGT CCT AAC AAC-3' were prepared previously.44
Oligonucleotide Labeling and Annealing. The oligonucleotides were labeled using T4 polynucleotide kinase and ρ32P-ATP as previously described.44

Time Course of the Endo IV Incision of the NM-Fapy-dGuo Containing Duplexes. These experiments were performed as previously described.44

Kinetics for the Endo IV Incision of the NM-Fapy-dGuo Containing Duplex. These experiments were performed as previously described.44

Time Course Incision of the NM-Fapy-dGuo Containing Duplex by Endo IV at pH 7.0, 7.5, and 8.0. These experiments were performed as previously described.44

Detection of the NM-Fapy-dGuo Containing Duplex after Denaturation and Reannealing. These experiments were performed as previously described.44

Excision of MeFapy-dGuo, NM-Fapy-dGuo, and 8-Oxo-dGuo Containing Duplexes by E. coli Formamido-pyrimidine Glycosylase. The 5′-32P-labeled modified oligonucleotide (200 nM) was annealed to an equal volume of its complementary strand (600 nM) in Tris buffer (50 mM). Oligonucleotide duplex (100 nM, 4 μL) was added to the formamidopyrimidine glycosylase (FPG) reaction buffer (1X: 10 mM Bis-Tris Propane-HCl, 10 mM MgCl2, 1 mM dithiothreitol, 74 μL), followed by addition of FPG (10 nM). Reactions were incubated at 37 °C. Aliquots (5 μL) were removed at the appropriate times, added to 10 μL of 95% formamide loading buffer containing xylene cyanol and bromophenol blue dyes, and heated for 1 min at 90 °C. Aliquots (6 μL) were separated by electrophoresis on a denaturing gel.

Kinetics for the Excision of MeFapy-dGuo, NM-Fapy-dGuo, and 8-Oxo-dGuo Containing Duplexes by E. coli Formamido-pyrimidine Glycosylase. DNA duplex was formed by heating 5′P-labeled oligonucleotide (100 nM in 50 mM Tris) and its complementary strand (1.5 equiv) at 95 °C for 5 min and then slowly cooling to ambient temperature over 1 h. FPG (0.1 nM) was added to varying concentrations of DNA duplex (5′-100 nM for MeFapy-dGuo, 10–180 nM for NM-Fapy-dGuo, and 1–40 nM for 8-oxo-dGuo in FPG reaction buffer (1X)) to a final volume 50 μL. Reactions were run at 37 °C for 10 min. Aliquots (5 μL) were taken every 1 min, added to loading buffer (10 μL), and heated at 90 °C for 1 min. Separation was achieved by PAGE. The kinetics parameters were calculated using KaleidaGraph (v. 4.5, Synergy Software). Reactions were carried out in duplicate.

RESULTS AND DISCUSSION

Phosphoramidite Synthesis. The synthesis of the NM-Fapy-dGuo phosphoramidite reagent is shown in Scheme 1.64

(2-Trimethylsilyl-ethyl)-dGuo (1) was prepared in three steps according to a literature procedure42 and was further protected at the 5′-O- and N6-positions to afford 3 in 43% overall yield from dGuo. Reaction of 3 with bis(2-chloroethyl)ethylamine in trifluoroethanol presumably gives the cationic N7-adduct (4),18,20,26,28,45 which was not isolated. Rather, the reaction mixture was briefly treated with methanolic NaOH followed by careful neutralization with 20% acetic acid to afford protected NM-Fapy-dGuo intermediate 5. Competing alkylation at O6 was observed if this position was not protected. The second chloride, which surprisingly survived the hydroxide treatment, was displaced by cesium acetate, and the O6-protecting group was removed by fluoride ion. Phosphitylation of the 3′-hydroxyl group provided the desired phosphoramidite reagent (8) as a mixture of isomers. The synthesis required nine steps from dGuo and proceeded in ~11% overall yield.

Oligonucleotide Synthesis. Phosphoramidite 8 was used to synthesize 12- and 24-mer oligonucleotides containing the NM-Fapy-dGuo lesion shown in Table 1. An off-line, manual coupling protocol was used to incorporate the modified nucleotide.41,43 Fapy-dGuo lesions with a free 5′-hydroxyl group can rearrange to the pyranose form.41,46 Therefore, the critical step of the oligonucleotide synthesis was the detritylation of the NM-Fapy-dGuo nucleotide. We previously observed that standard DNA synthesis protocols resulted in significant rearrangement to the pyranose form of the MeFapy-dGuo adducts. However, shortening the deprotection cycle minimized the furanose to pyranose rearrangement.41 Oligonucleotides containing the NM-Fapy-dGuo adduct were synthesized using a “short” deprotection cycle. Two products were identified as having the correct mass in a ~1:2.6 ratio (see Figure S10 of the Supporting Information). The major product (later eluting) was assumed to be the furanose form based on our previous work with the MeFapy-dGuo adduct in the same sequence. The two products were observed in a ~1:1 ratio under a long deprotection cycle, which is consistent with the assignment because the long deprotection should favor the isomerization to the pyranose form.

The NM-Fapy-dGuo containing 12-mer oligonucleotide was enzymatically digested to the individual nucleosides. The unmodified nucleosides were readily observed by HPLC analysis in the expected ratio (Figure 2A),47 but NM-Fapy-dGuo was not detected. Substituted Fapy-dGuo nucleosides exist as multiple, slowly interconverting species, which may include the furanose and pyranose forms of the deoxyribose, α- and β-anomers, cis and trans geometric isomers of the formamide, and possibly atropisomers.31,46,48–53 The slow interconversion of the Fapy-isomers often results in poor HPLC chromatographic behavior and diminished detection.44 Therefore, the enzymatic digestion reaction was examined by UPLC-MS6. The extracted ion chromatograms are shown in Figure 2B, in which the neutral loss of the deoxyribose (~116 Da) was monitored. A mass consistent with NM-Fapy-dGuo (400 → 285) was observed along with the unmodified nucleosides. As anticipated, NM-Fapy-dGuo was observed as multiple broad peaks, reflecting its conformational heterogeneity. The enzymatic digestion reaction was also subjected to acid hydrolysis and UPLC-MS analysis (Figure 2C). A broad peak with a mass consistent with the NM-Fapy-Gua base (m/z 285.2) was observed. The broad nature of this peak likely reflects geometric isomers of the formamide group; the geometric isomers of the MeFapy-Gua base could be resolved into two separate peaks by HPLC.

Duplexes containing the MeFapy-dGuo and N7-afatoxin B1-Fapy-dGuo (AFB1-Fapy-dGuo) lesions showed biphasic thermal melting profiles.41,55 It was hypothesized that the two phases represented the α- and β-anomers of the Fapy-dGuo adduct. Attempts to characterize the thermal melting profile of the NM-Fapy-dGuo containing 12-mer gave inconsistent results in our hands. We cannot offer an explanation for this observation.

Incision of the NM-Fapy-dGuo Duplex with E. coli Endonuclease IV (Endo IV). An unusual property of Fapy lesions is that they can isomerize to the unnatural α-anomer. Endo IV incises the S′-phosphodiester bond of an abasic site in
duplex DNA and has been shown to incise α-nucleotides. Endo IV has been used previously to approximate the α/β ratio of Fapy-dGuo, Fapy-dAdo, and MeFapy-dGuo in duplex DNA by the selective incision of the α-anomer. \(^{44,59-61}\) We applied this assay to the NM-Fapy-dGuo containing 24-mer to determine the α/β ratio of the lesion. The modified 24-mer (10) was \(^{32}\)P-labeled, annealed to its complement, and incubated with Endo IV (Figure 3). Approximately 50% of the duplex was incised after a 40 min incubation period at 37 °C, affording a product consistent with phosphodiester hydrolysis at the 5′-side of NM-Fapy-dGuo. The reaction mixture, containing the remaining 50% of the duplex as the β-anomer, was denatured by heating at 90 °C and slowly cooling to reanneal the duplex. This process should re-equilibrate the NM-Fapy-dGuo lesion to its original mixture of anomers. \(^{44,61}\) Additional Endo IV was added, and after 40 min, gel analysis indicated that the total level of incision was ~75%; the 25% increase represents 50% of the duplex remaining after the first incision reaction. The reaction mixture was denatured and reannealed again, and a third portion of Endo IV was added. The third cycle resulted in an additional ~12% of the incised product (~87% total), which represents approximately half of the duplex remaining after the second Endo IV treatment (25%). The results are consistent with a 1:1 mixture of α- and β-anomers after initial annealing of the duplex.

We previously observed that the initial α/β-anomeric ratio of the MeFapy-lesion slowly equilibrated over time. \(^{44}\) The labeled NM-Fapy-dGuo containing 24-mer duplex was incubated at 37 °C and pH 7.5. Aliquots were removed after 0.5 h and then approximately every 24 h and were subjected to Endo IV treatment. The level of incision steadily decreased over time to about 14% after 5 days (Figure 4). The anomerization was also examined at pH 7.0 and 8.0 and was surprisingly insensitive to
pH over this range; this is in contrast to that of the MeFapy-dGuo lesion, which anomerized more slowly at pH 7.5 and not at all at pH 8.0.

The catalytic efficiency ($k_{cat}/K_m$; Table 2) for the Endo IV incision of the NM-Fapy-dGuo containing 24-mer was 0.16 nM$^{-1}$ min$^{-1}$ ($k_{cat} = 5.4 \pm 0.5$ min$^{-1}$, $K_m = 33 \pm 6.8$ nM). The incision efficiency was approximately half of that of the MeFapy-dGuo lesion and ∼4.3-fold lower than α-3-Guo in the same sequence context (Table 2).$^{44}$ The $k_{cat}$ values for the three substrates were similar, and differences in their incision efficiency were largely due to $K_m$. An active site pocket of Endo IV is hypothesized to accommodate α-nucleotides.$^{62}$ The affinity for our three substrates decreases with increasing steric bulk, perhaps reflecting the ability of the active site pocket to accommodate the substrate. We previously reported that the very bulky AFB$_3$-Fapy-dGuo adduct is a poor substrate for Endo IV.

Excision of the NM-Fapy-dGuo Lesion of *E. coli* Formamidopyrimidine Glycosylase (FPG). Fapy lesions are substrates for the base excision repair pathway and are excised by FPG in bacteria and by OGG1, NEIL1, and NTH1 in eukaryotes.$^{35-40}$ Less is known about the excision of N$^5$-substituted Fapy-dGuo lesions. The MeFapy-dGuo, ethyl-Fapy-dGuo, (2-hydroxyethyl)-Fapy-dGuo, (pyridyloxobutyl)-Fapy-dGuo, and Fapy-dGuo lesions derived from a phosphoramidite and sulfur mustard have been reported to be substrates for FPG$^{25,52,63-66}$ whereas AFB$_3$-Fapy-dGuo is not a substrate.$^{66}$ A ring-opened oxidation product of dThd is also a substrate for FPG.$^{67}$ MeFapy-dGuo is a substrate for human$^{68}$ and yeast$^{69}$ OGG1, hNEIL1$^{70}$, and yeast$^{71}$ mouse$^{72}$ and human$^{73}$ NTH1, but it is a poor substrate for hNEIL2$^{74}$ mouse NEIL3$^{74}$, and *E. coli* Endo III and Endo VIII$^{70,72,75}$ when paired with dCyd. Excision by NEIL3 improved markedly in single-stranded DNA.$^{74}$ There is indirect evidence that FPG and hOGG1 will excise the AE-Fapy-dGuo lesion. The overexpression of FPG or hOGG1 was up to 100-fold protective of cells treated with thioTEPA or aziridine, suggesting a role for the AE-Fapy-dGuo lesion in the cytotoxic mechanism of these agents.$^{76-79}$ FPG expression also provided a 10- and 2-fold protective effect against 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and the nitrogen mustard mafosfamide,$^{76,80}$ respectively, suggesting that the corresponding N$^5$-substituted Fapy-dGuo lesions are part of the mechanism of action of these agents as well. The lower protective effect against BCNU and mafosfamide is probably reflective of the greater role of interstrand cross-links in their cytotoxicity.

The excision of MeFapy-dGuo and NM-Fapy-dGuo opposite dCyd from duplex DNA by *E. coli* FPG was examined and compared to that of 8-oxo-dGuo; all three lesions were incorporated into the same 24-mer sequence. Asagoshi et al. previously reported the excision of MeFapy-dGuo and 8-oxo-dGuo from a 25-mer duplex in a 5′-T(NG)G-3′ sequence by FPG and hOGG1.$^{68}$ Both glycosylases had nearly identical catalytic efficiencies for the two substrates opposite dCyd, although the activity for FPG was significantly higher than that of hOGG1. We found that the MeFapy-dGuo and NM-Fapy-dGuo lesions were good substrates for *E. coli* FPG when paired with dCyd (Figure 5). Our $k_{cat}$ and $K_m$ values for the MeFapy-dGuo and 8-oxo-dGuo substrates are in reasonable agreement with those previously reported (Table 3);$^{68}$ however, we found 8-oxo-dGuo to be a better substrate for FPG by ∼2-fold. The excision of NM-Fapy-dGuo was 2.5 and 4.6 times less efficient than that for MeFapy-dGuo and 8-oxo-dGuo, respectively. The excision efficiencies are largely reflective of differences in the $K_m$ values, which roughly correlate with the steric demands of the modification. Consistent with our observation, the $K_m$ for the excision of N$^5$-ethyl-Fapy-dG was reported to be 7-fold higher than that for MeFapy-dGuo.$^{32}$ However, the apparent $K_m$ of nonhydrolyzable cyclopentane analogues of Fapy-dGuo and N$^5$-benzyl-Fapy-dGuo with FPG were nearly identical.$^{81}$

### Table 2. Steady Kinetic Parameter for the Incision of the NM-Fapy-dGuo, MeFapy-dGuo, and α-dGuo Containing 24-mer Duplexes by *E. coli* Endo IV

<table>
<thead>
<tr>
<th></th>
<th>NM-Fapy-dGuo</th>
<th>MeFapy-dGuo</th>
<th>α-dGuo$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{cat}$ (min$^{-1}$)</td>
<td>5.4 ± 0.5</td>
<td>5.5 ± 0.4</td>
<td>4.9 ± 0.5</td>
</tr>
<tr>
<td>$K_m$ (nM)</td>
<td>33 ± 6.8</td>
<td>18 ± 3.8</td>
<td>7.1 ± 1.0</td>
</tr>
<tr>
<td>$k_{cat}/K_m$ (nM$^{-1}$ min$^{-1}$)</td>
<td>0.16</td>
<td>0.31</td>
<td>0.69</td>
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</table>

$^a$This data is reprinted from ref.$^{44}$

## CONCLUSIONS

MeFapy-dGuo and AFB$_3$-Fapy-dGuo have been reported to be persistent lesions from exposure to methylating agents and aflatoxin B$_1$, respectively.$^{82,83}$ This observation suggests that N$^5$-substituted Fapy-dGuo lesions play a role in the carcinogenicity of DNA alkylating agents and secondary tumor development from chemotherapeutic agents such as temozolomide, thioTEPA, BCNU, and nitrogen mustards.

We have site-specifically incorporated an N$^5$-nitrogen mustard Fapy-dGuo lesion into oligonucleotides using the phosphoramidite approach. A DNA duplex containing the NM-Fapy-dGuo lesion is a good substrate for *E. coli* Endo IV and FPG when paired with dCyd. The Endo IV incision indicates that NM-Fapy-dGuo exists as a ∼50:50 α/$\beta$-anomeric ratio after annealing, but it will slowly equilibrate to a ∼14.86 ratio. The NM-Fapy-dGuo lesion will be initially formed as the natural $\beta$-anomer in cellular DNA and will slowly equilibrate to a mixture of anomers over time. Therefore, the α-anomer will be a relevant lesion if NM-Fapy-dGuo persists. The efficient excision of NM-Fapy-dGuo from duplex DNA suggests that FPG could be used as part of an enrichment protocol for the mass spectrometric detection of NM-Fapy-Gua from treated cells, animals, or clinical samples. Oligonucleotides containing NM-Fapy-dGuo will also be useful in repair and replication studies *in vitro* and in cells. Our work compliments previously reported work in which a nitrogen mustard interstrand cross-link model was engineered into oligonucleotides.$^{84}$ It should also be noted that the parent Fapy-dGuo lesion derived from

![Figure 5. Gel analysis of the excision of 8-oxo-dGuo, MeFapy-dGuo, and NM-Fapy-dGuo containing duplexes by *E. coli* formamidopyrimidine glycosylase (FPG).](http://dx.doi.org/10.1021/tx5002354)
oxidative damage has previously been incorporated into oligonucleotides.83—87

■ ASSOCIATED CONTENT

4H NMR spectra of the phosphoramidite and synthetic intermediates, HPLC traces, MS analysis, and kinetic plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

■ DEDICATION

This article is dedicated to Professor Thomas M. Harris on the occasion of his 80th birthday.

■ ABBREVIATIONS

NM, bis(2-chloroethyl)ethylamine; Fapy, formamidopyrimidine; MeFapy-dGuo, N6-(2-deoxy-d-erythro-pentofuranosyl)-6-(2-deoxy-d-erythro-pentofuranosyl)-2,6-diamino-3,4-dihydro-4-oxo-5-N-methylformamidopyrimidine; NM-Fapy-dGuo, N6-(2-deoxy-d-erythro-pentofuranosyl)-2,6-diamino-3,4-dihydro-4-oxo-5-N-[2-(2-hydroxyethyl)ethylamino]-ethyl]-formamidopyrimidine; NM-dGuo, N7-[2-(2-hydroxyethyl)ethylamino]-ethyl]-2-deoxyguanosine; 8-oxo-dGuo, 7,8-dihydro-8-oxo-2′-deoxyguanosine; Endo IV, E.coli endonuclease IV; FPG, E.coli formamidopyrimidine glycosylase; AFB1-Fapy-dGuo, 8,9-dihydro-8(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl-formamido)-9-hydroxyaflatoxin B1; AE-Fapy-dGuo, N6-(2-deoxy-d-erythro-pentofuranosyl)-2,6-diamino-3,4-dihydro-4-oxo-5-N-(2-aminoethyl)-formamidopyrimidine; thioTEPA, 1,1′,1′′-phosphorothioyltriaziridazide; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; HPA, 3-hydroxypropionic acid

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human cells treated with reactive metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Mutat. Res. 600, 138–149.


