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2’-Fluorinated Hydantoins as Chemical Biology Tools for Base Excision Repair Glycosylases

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Abstract

The guanine oxidation products, 5-guanidinohydantoin (Gh) and spiroiminodihydantoin (Sp) are mutagenic and toxic base lesions that are removed by Fpg, Nei, and the Nei-like (NEIL) glycosylases as the first step in base excision repair (BER). The hydantoins are excellent substrates for the NEIL glycosylases in a variety of DNA contexts beyond canonical duplex DNA, implicating the potential impact of repair activity on a multitude of cellular processes. In order to prepare stable derivatives as chemical biology tools, oligonucleotides containing fluorine at the 2’-position of the sugar of 8-oxo-7,8-dihydroguanine (2’-F-OG) were synthesized in ribo and arabino configuration. Selective oxidation of 2’-F-OG within a DNA oligonucleotide provided the corresponding 2’-F-Gh or 2’-F-Sp containing DNA. The 2’-F-hydantoins in duplex DNA were found to be highly resistant to the glycosylase activity of Fpg and NEIL1 compared to the unmodified lesion substrates. Surprisingly, however, some glycosylase-mediated base removal from both the 2’-F-ribo and 2’-F-arabinohydantoin duplex DNA was observed. Notably, the associated β-lyase strand scission reaction of the 2’-F-arabinohydantoins was inhibited such that the glycosylases were “stalled” at the Schiff base intermediate. Fpg and NEIL1 showed high affinity for the 2’-F-Gh duplexes in both ribo and arabino configurations. However, binding affinity assessed using catalytically inactive variants of Fpg and NEIL1 indicated higher affinity for the 2’-F-riboGh containing duplexes. The distinct features of glycosylase processing of 2’-F-ribohydantoins and 2’-F-arabinohydantoins illustrate their utility to reveal structural insight into damage recognition and excision by NEIL and related glycosylases and provide opportunities for delineating the impact of lesion formation and repair in cells.

Graphical Abstract

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Author Contributions

SC, JPR, and SSD designed the study. SC, JPR, JY, BA, JA performed the experiments. All authors contributed to writing the paper and have given approval for the final version of the manuscript.

ASSOCIATED CONTENT

Supporting Information

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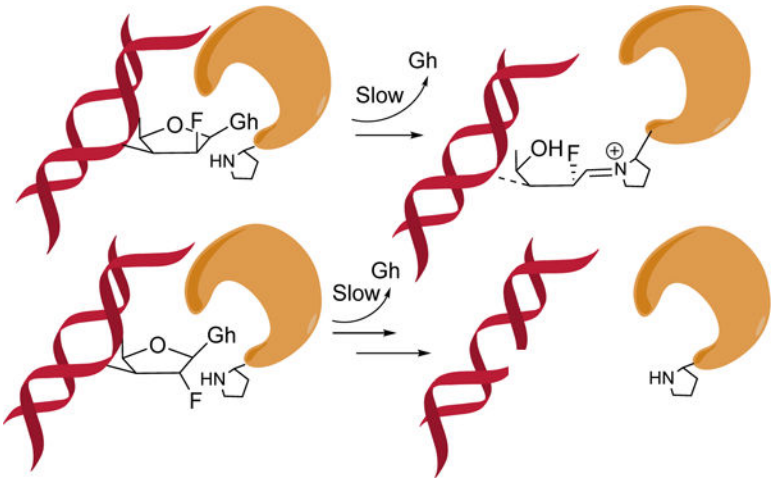
Supplementary figures and tables, full experimental section and compound characterization (PDF).



Introduction

Oxidative stress, due to high levels of reactive oxygen species (ROS), is a common culprit of oxidative DNA damage leading to cancer, neurological disorders and cardiovascular diseases.[1] Guanine bases in DNA are highly sensitive to ROS-mediated oxidation to produce a variety of products.[2] A common biomarker for oxidative stress is the guanine base oxidation product 8-oxo-7,8-dihydro-guanine (OG).[3] Notably, OG is more sensitive than G to oxidation and can yield two secondary oxidation products, 5-guanidinohydantoin (Gh) and spiroiminodihydantoin (Sp).[4–7] Gh and Sp can also be formed directly from G by reaction with potent oxidants, such as singlet oxygen, peroxynitrite, and high-valent metal compounds.[4, 8] The hydantoins are more potently mutagenic than OG, producing G → T and G → C transversion mutations, and the hydantoin lesions are also more efficient blocks to replication and transcription than OG.[4] The consequences of formation of hydantoins in DNA on DNA replication and transcription suggest that these lesions not only erode genomic integrity but impact a wide array of cellular processes.[4]

Oxidized lesions in DNA are primarily repaired via the base excision repair (BER) pathway, which is initiated by damage-specific DNA glycosylases that excise the damaged base from the sugar.[9–11] Depending on the lesion and the glycosylase, further resection of the sugar provides a gap that is replaced and sealed by a DNA polymerase and DNA ligase, respectively.[9] The Fpg/Nei family of BER glycosylases have been found to efficiently recognize and excise Gh and Sp, which includes *E. coli* formamidopyrimidine DNA glycosylase (Fpg or MutM),[12] *E. coil* Endo VIII (Nei),[13] and the mammalian homologues of Nei, endonuclease VIII-like enzymes (NEIL1,2, & 3).[14–16] Fpg, Nei, and NEIL1 are bifunctional glycosylase/lyase enzymes with broad substrate scope that catalyze base excision and associated β- and δ-elimination reactions that produces strand scission at the lesion site.[10–11] Fpg removes Gh and Sp with greater efficiency than OG in duplex DNA.[12, 17] In contrast, Gh and Sp are not substrates for the human OG glycosylase OGG1,[18] but are among the best documented substrates for the NEIL1 glycosylase in duplex DNA.[14]

The human NEIL1 (NEIL1) has many unique features that set it apart from other BER glycosylases. One distinct feature is the presence of two NEIL1 isoforms due to editing of its pre-mRNA by Adenosine Deaminase Acting on RNA 1 (ADAR1).[19] This results in a single amino acid difference at position 242, where the edited (Ed) form of NEIL1 has an arginine and the unedited (UE) enzyme has a lysine.[19] The two isoforms exhibit marked differences in lesion specificity with the unedited form exhibiting enhanced activity for thymine glycol (Tg) removal, while both isoforms exhibit robust activity toward removal of Gh in DNA.[19] The biological impact of NEIL1 recoding remains to be fully elaborated; however, ADAR1 overexpression and transcriptome hyper-editing are associated with inflammation and cancer.[19–24] Other notable properties of NEIL 1 are the ability to remove the hydantoin lesions from non-duplex contexts, such as ssDNA, bubble, bulge, and G-quadruplex DNA.[25–27] NEIL1 is also known to interact with proteins involved in replication, [28] and its activity has been shown to be altered by interactions with non-canonical BER proteins involved in transcription.[4, 29] The promiscuity of NEIL1 in its substrate scope and protein interactions coupled with hydantoin lesion formation under conditions of high oxidative stress suggests a complex interplay between hydantoin BER, DNA replication, and gene transcription.[4, 30] Indeed, unraveling this complexity would be aided by developing new chemical biology tools and approaches for probing the interactions of NEIL1 with hydantoin lesions.

The 2’-fluoronucleotide modification of damaged DNA substrates has been leveraged by many laboratories, including ours, to slow the cleavage reaction of DNA N-glycosylases for use in a variety of biophysical, structural, and cellular applications.[31–39] The installation of the fluorine atom at the 2’-position makes the nucleotide more resistant to base removal by destabilizing the C1’-O4’ oxocarbenium ion transition state (TS) formed during glycosylase catalyzed glycosidic bond hydrolysis (Figure 1).[40–41] The ability to prevent base excision using 2’F versions of damaged substrates has provided structural snapshots of several glycosylases bound to damaged DNA and revealed key features of damage recognition and excision.[37, 39] Electrophoretic mobility shift assays (EMSA) using oligodeoxynucleotides containing noncleavable 2’-fluorothymidine glycol (FTg) showed that the Ed and UE forms of NEIL1 have similar affinities to FTg- containing DNA.[34] These results suggested that the origin of the differential processing of lesions is related to a kinetic step in lesion processing rather than lesion binding affinity. Preventing cellular base excision repair with 2’F nucleoside versions of 5-methylcytosine (5MeC) and further oxidized derivatives has provided a means to evaluate enzymatic and non-enzymatic reactions on these modified bases in cells.[42] These examples suggest that 2’F versions of the hydantoins would be exceedingly useful for unraveling the biology and chemistry of these unique lesions and the impact of BER.

Herein, we report a strategy to prepare 2’-F-hydantoin-containing oligonucleotides, starting from synthesis of the 2’-F-riboOG and 2’-F-arabinoOG phosphoramidite monomers, followed by incorporation into oligonucleotides using solid phase DNA synthesis (Figure 1). The 2’-F hydantoin oligonucleotides were then prepared by oxidation of 2’-F-OG-containing oligonucleotides. We prepared both the arabino and ribo configured 2’-F-hydantoins in order to discern the impact of 2’-F sugar stereochemistry on both recognition and excision by the Fpg/Nei glycosylases. Nucleosides containing the fluorine in the ribo

configuration show a preference for C3'-endo (N-type) RNA-like sugar conformation while 2'-arabino-configured isomers show preference for the C2'-endo (S type) B DNA sugar conformation.[43–46] The presence of the 2'F substituent in the sugar of Gh and Sp was found to dramatically inhibit the efficiency of base excision mediated by Fpg, and NEIL1 glycosylases with both ribo and arabino stereoisomers. Notably, in the case of 2'-F-arabinoGh duplex the associated β-lyase strand scission reaction was impeded, leading to stalling at the Schiff base intermediate and efficient trapping of the covalent complex via reduction with sodium borohydride. Fpg and NEIL1 were found to bind with high affinity to both 2'-F-riboGh and 2'-F-arabinoGh containing duplexes. The distinctly different processing of the 2'-F-hydantoin stereoisomers by these BER glycosylases suggests that these features may be readily exploited in chemical biology studies.

Results and Discussion

Synthesis of 2'-F-ribo and 2'-F-arabino OG- containing oligonucleotides.

Currently, there are no reported routes for synthesis of 2'-F-OG oligonucleotides, despite reference of their use in studies with OG glycosylases.[47] We devised a synthesis of the 2'-F-ribo and 2'-F-arabinoOG phosphoramidites (Scheme 1) for incorporation into oligonucleotides via automated solid phase DNA synthesis. The 9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)guanine (2'-F-arabinoG) nucleoside **1** was synthesized in four steps beginning from 1,3,5-tri-O-benzoyl-α-D-ribofuranose.[48–49] The synthesis of 8-oxo-9-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)guanine (2'-F-arabinoOG) phosphoramidite **9** started with reaction of **1** with saturated bromine water to produce the brominated intermediate **3**. Compound **3** was reacted in a solution with acetic anhydride, glacial acetic acid, and excess sodium acetate, and then deacetylated to produce the 2'-F-arabino-OG nucleoside **5**. The 2'-F-arabinoOG phosphoramidite, **9**, was then prepared by reacting the 2-amino group of **5** with N, N-dimethylformamide dimethylacetal and the 5'-hydroxyl group with 4, 4'-dimethoxytrityl chloride (DMTrCl) to produce **7**, followed by the reaction of the 3'-hydroxyl group with 2-cyanoethyl N, N-diisopropyl chlorophosphoramidite. The 8-oxo-9-(2-deoxy-2-fluoro-β-D-ribo-furanosyl)guanine (2'F-ribo-OG) phosphoramidite **10** was synthesized using similar methods as **9** (Scheme 1) starting with commercially available 2'-F-riboG nucleoside (**2**). The 2'-F-OG phosphoramidites were incorporated into oligonucleotides via standard automated DNA synthesis with 95% coupling yield and their composition was supported by ESI-MS (Supporting Information, Table S1).

Synthesis of Fluorinated Hydantoin Containing Oligonucleotides.

Single-stranded oligonucleotides containing 2'-F-OG were converted to the corresponding 2'-F-Gh and 2'-F-Sp lesions following the approach developed by Burrows and co-workers to prepare Gh and Sp-containing oligonucleotides.[5,50–51] The oxidation reagent, sodium hexachloroiridate, selectively oxidizes OG over G without oxidation of normal bases.[51] The 2'-F-Gh oligonucleotides were formed preferentially from the 2'-F-OG oligonucleotide by Na_2IrCl_6 oxidation at ambient temperature in aqueous solution, while Sp formation predominated at pH 8, and 65 °C. The oxidation products formed from 2'-F-OG under these conditions were purified using anion-exchange HPLC (Figure S22). The oxidation reaction conditions consistently provided higher yields of 2'-F-Gh than 2'-F-Sp. Unfortunately, we

were unable to separate the diastereomers of 2'-F-Sp using HPLC conditions analogous to those used for the corresponding Sp-oligonucleotides.[52] For these reasons, the cleavage and binding analyses with the DNA glycosylases focused more extensively on the 2'-F-Gh-containing oligonucleotides. The formation of 2'-F-Gh and 2'-F-Sp in the DNA oligonucleotides was confirmed by ESI-MS (Table S1).

2'F-Gh-containing duplexes are resistant to the glycosylase/β-lyase activity of BER glycosylases.

We evaluated the ability of the 2'-F substituent to block the glycosylase and β-lyase activity of Fpg and NEIL1 using gel-based assays, similar to those we have detailed previously.[12, 14] We also anticipated an impact of the 2'F-sugar configuration on both the glycosylase and β-lyase activity (Scheme 2), and therefore we devised a quenching strategy to reveal the two separate activities. Briefly, these experiments entailed using a 30-base pair (bp) duplex containing a centrally located OG, 2'-F-riboOG, 2'-F-arabinoOG, Gh, 2'-F-riboGh, or 2'-F-arabinoGh positioned opposite C. The strand-containing the natural or 2'-F-lesion was labeled with a 5'-^{32}P-phosphoryl group and annealed to the complementary strand. The duplexes were incubated with either Fpg or NEIL1 at 37 °C. At various times, aliquots were removed and quenched with either 0.1 N NaOH and denaturing loading dye or with denaturing loading dye alone. The NaOH quench ensured that all abasic (AP) sites produced by the glycosylase activity lead to strand scission at the lesion site, and therefore reveal the extent of hydantoin removal. In samples quenched with denaturing loading dye, glycosylase and β-lyase reactions at the 2'F-nucleotide are required to observe strand scission. The DNA cleavage products were separated from the unreacted substrate by polyacrylamide gel electrophoresis (PAGE) and visualized by storage phosphor autoradiography.

Different levels of cleavage with and without base quenching were observed that were dependent both on the 2'-F lesion and the glycosylase. Minimal Fpg mediated-cleavage with and without base quenching was observed with the 2'-F-riboOG and 2'-F-arabinoOG DNA duplex. These observations indicated that OG is not removed from 2'-F-OG by Fpg. In contrast, Fpg-mediated cleavage of 2'-F-riboGh and 2'-F-arabinoGh was observed (Fig. 2) Approximately 10–30% of the 2'-F-Gh oligonucleotide was cleaved by Fpg in 60 min, while under similar reaction conditions Fpg catalyzes complete removal of the natural Gh and Sp lesions within 1 min.[17] These results indicate that the rate of Gh base excision mediated by Fpg has been significantly impeded by addition of the 2'F substituent in 2'-F-Gh consistent with the expected destabilization of the oxocarbenium ion TS.[31] Similar results to those with Fpg were observed with Ed and UE NEIL1 (Fig. 2B). Notably, however the extent of NEIL1-mediated cleavage of both the 2'-F-riboGh and 2'-F-arabinoGh was significantly greater than observed with Fpg (Figure 2b). In addition, there was no discernable difference between the extent of cleavage of 2'-F-Gh by Ed and UE NEIL1. Specifically, the extent of NEIL1-mediated cleavage of 2'-F-Gh duplex DNA after 60 min as judged by the extent of cleavage with base-treatment (Fig. 2D) was significant (60–70%), indicating the presence of the 2'-F-substituent does not completely block the glycosylase activity of NEIL1. It is again noteworthy, that NEIL1-catalyzed Gh base removal under similar conditions goes to completion, and such reactions typically are complete within the first manual timepoint (1 min) (Fig. 2B).[14] The inability of the 2'-F substitution with the hydantoin lesions to

completely ablate base excision is likely due to the high intrinsic base removal activity of the Fpg/Nei glycosylases toward these lesions. Notably, previous work showed that the T4 endonuclease V cleaved the glycosidic linkage at the 2'-F -riboT of the 5'-nucleotide of a thymine dimer, albeit at a reduced rate compared to a natural thymine dimer.[53]

The comparison of the extent of cleavage at the 2'F-Gh-containing duplex with and without NaOH quenching indicates the ability of the bifunctional glycosylase to catalyze the β-lyase activity on the 2'-F-AP site produced upon Gh base excision. This analysis showed no difference in extents of cleavage mediated by Fpg and NEIL1 on the 2'-F-riboGh-containing DNA duplex with the two types of quenching methods, whereas NaOH quenching revealed significantly increased extents of cleavage of 2'F-arabinoGh relative to the dye alone quench. The difference was particular striking with edited NEIL1 where the extent of β-lyase activity was 10–20% of the glycosylase activity.

These results establish that the Fpg and NEIL1-catalyzed-β-lyase reaction is sensitive to the configuration of the fluorine at the C2' position of the sugar. Indeed, Mazumder et al. reported that Endonuclease III catalyzes a syn β-elimination reaction by abstraction of the pro-S 2-hydrogen in DNA.[54] For 2'-F-arabinoGh, the hydrogen atom in the pro-S position has been replaced by the fluorine atom; thus, by analogy to Endonuclease III, the NEIL1-catalyzed β-elimination reaction may not readily occur due to non-optimal positioning of active site catalytic base residues (Scheme 2). In previous work with T4-endonuclease V, only the 2'-F ribo configuration of the thymine dimer was studied; however, the results were similar to that reported here with Fpg and NEIL1 processing of 2'-F arabinoGh. In the case of the thymine dimer substrate, the 2'-F sugar conformation is more constrained such that the optimal conformation leading to the beta-elimination may be less accessible. Importantly, these results point to a stereoelectronic effect imparted by the 2'-F substitution that impacts the efficiency of enzyme-catalyzed β-elimination reactions.

2'-F-hydantoin sugar configuration impacts extent of Sodium borohydride trapping.

A common method to detect formation of the Schiff base intermediate in bifunctional glycosylase/lyases is the use of sodium borohydride ($NaBH_4$) reduction to form a stable covalent enzyme-DNA complex that may be detected under denaturing conditions of SDS-PAGE. This strategy was used to evaluate the extent that Fpg and NEIL1 glycosylases remove the base from the 2'F-hydantoin-containing duplex DNA to produce a Schiff base intermediate (Scheme 2, Figure 3). A comparison of the amount of covalent complex trapped in the reaction with the 2'-F-ribo versus 2'-F-arabinohydantoin duplexes shows substantially greater amounts of enzyme-DNA complex with the arabino configuration for all three enzymes (Figure 3, lanes e, f). The amount of trappable Schiff base intermediate correlates directly with the extent of cleavage observed at the fluorinated nucleotide (Figure 2). Notably, the largest amount of Schiff-base intermediate is detected with NEIL1 and the 2'-F-arabinoGh and 2'-F-arabinoSp duplexes, consistent with the larger difference between the glycosylase and β-lyase activity.

The decreased ability to catalyze the β-elimination reaction and the ability to more efficiently trap the Schiff base intermediate with sodium borohydride indicates that the glycosylases are stalled at Schiff base intermediate with the 2'-F-arabinohydantoin duplexes.

This is particularly striking with NEIL1 that was capable of removing the hydantoin base from the arabino-configured F-hydantoins to a significant extent and form large amounts of the covalent Schiff intermediate, but exhibited minimal ability to catalyze the β-lyase activity. The ability of the 2'-F-arabinoGh-containing DNA to transiently covalently capture the glycosylase suggests this feature may be used to effectively inhibit NEIL glycosylases in cells by forming a covalent adduct. Alternatively, this feature may be useful in developing affinity probes to pull-down NEIL1 and associated proteins from cellular extracts.

Affinity of bifunctional glycosylases towards 2'-F-Gh-containing DNA.

Electrophoretic mobility shift assays (EMSA) were used to measure the relevant dissociation constants (K_d) of Fpg and edited NEIL1 with ribo and arabino-FGh containing duplexes. Briefly, these experiments entailed titration of the enzyme into a solution of 5'-^{32}P-end-labeled duplex at 25 °C, followed by analysis using native PAGE to separate and detect the free DNA duplex from the glycosylase-DNA complex. A representative image of the EMSA and plots of percent protein bound DNA versus glycosylase concentration are shown in Figure 4, and K_d values are summarized in Table 1. The relative K_d values indicate that Fpg binds to both 2'-F-arabino and 2'-F-riboGh duplexes with high affinity (0.04 ± 0.01 and 0.021 ± 0.008 nM, respectively). When comparing affinity of Fpg towards 2'-F-arabinoGh versus 2'-F-riboGh, no difference was seen, despite observed differences in β-lyase activity. This suggests that the high affinity with the 2'-F-arabinoGh duplex may be due in part to the stalled Schiff base intermediate. Affinity studies using a catalytically inactive variant of the enzyme (E3Q Fpg)[17] were performed to evaluate the impact of the covalent trapping of the 2'-F-arabinoGh/Fpg complex as the Schiff base intermediate on the observed Kds. When comparing the stereoisomer affinities in this situation, it was found that the affinity of E3Q Fpg for the 2'-F-riboGh duplex exhibits a 10-fold higher affinity (0.39 ± 0.01 nM) than with the duplex containing 2'-F-arabinoGh (4.0 ± 0.6 nM). The difference in the affinities with a catalytically inactive variant support that the high affinity of Fpg to a 2'-F-arabinoGh duplex is due in part to the persistence of a Schiff base intermediate.

Both isoforms of NEIL1 bound to the fluorinated lesions with nM affinity with minimal difference between the edited or unedited isoforms or the fluorinated nucleotide configuration. The similarity in affinity of both isoforms of NEIL1 towards the 2'-F-Gh lesion duplexes is of particular interest. In previous work, we observed an approximate 3-fold increase in the rate of Gh removal by Ed over UE NEIL1.[19] The similarity in affinity of the two isoforms with 2'-F-Gh suggests that the differences in Gh lesion removal are complex, and not simply due to differences in binding; in addition, some caution in terms of interpreting the results herein must be made given that the 2'-F-Gh lesion is not completely resistant to NEIL1 cleavage. Notably, removal of Tg was the most dramatically different between the isoforms,[19] and similar affinity for the stereoisomers of 2'-F-Tg was also previously observed.[34] In addition, structural studies of Ed and UE NEIL1 bound to a Tg duplex have suggested UE NEIL1 more effectively promotes Tg base tautomerization leading to higher activity for Tg removal.[55] Indeed, this illustrates another layer of complexity in NEIL1-catalyzed lesion excision and further underscores the need for additional tools and approaches to dissect out various steps in the lesion recognition and process.

To discern the impact of catalytic activity on the observed binding affinities, EMSA were performed in a manner similar to Fpg using a catalytically inactive and truncated from of NEIL1 (Δ56 K54L edited and unedited NEIL1).[56–57] Similar to the results comparing the relative affinity of Fpg and its catalytically inactive form, a decrease in the affinity of NEIL1 towards 2'-F-arabino Gh was seen compared to its affinity towards 2'-F-riboGh. In the case of the unedited isoform of Δ56 K54L NEIL1, an approximate 7-fold decrease in its affinity towards 2'-F-arabinoGh compared to the active enzyme (5.5 versus 0.8 nM, respectively) was observed. A roughly two-fold increase in K_d was observed in comparing inactive, edited NEIL1 to its active variant (1.5 versus 0.8 nM, respectively). One consequence of these changes in K_d with the inactive NEIL1 isoforms is the observation of higher affinity of the edited enzyme over the unedited enzyme for the 2'-F-arabinoGh DNA. These results underscore that the lesion removal efficiency differences between the two isoforms may be a result of differences imparted at various stages in nucleotide recognition and excision. The differences between the isoform lesion excision may be subtle and potentially dynamic. Indeed, only the unedited isoform exhibits sensitivity to the 2'F sugar configuration; the edited isoform of Δ56 K54L NEIL1 binds with similar high affinity to both the2'-F-arabinoGh and 2'-F-riboGh duplex.

Fpg and NEIL1 exhibited high affinity for the 2'-F-Gh:C-containing duplexes but were also able to mediate Gh base removal to some extent. Accordingly, the K_d values measured with the active enzyme are not true K_d values for the enzymes to the substrate; rather, the determined K_d values represent binding to a mixture of substrate and product. Thus, in order to determine the extent of cleavage occurring under the conditions of the EMSA, the extent of glycosylase mediated cleavage and percentage of bound duplex for Fpg were determined in parallel at each enzyme concentration used in the EMSA (Figure 5). These experiments entailed splitting the samples from the K_d titration; one sample was subject to native PAGE for EMSA, and the other sample was quenched, and then run on denaturing PAGE similar to glycosylase assays to determine the extent of cleavage at the lesion site.

The analysis of titration curves for Fpg with the 2'-F-riboGh:C duplex indicates that the measure K_d values represents the dissociation constant of Fpg with the substrate since, at the approximate midpoint of the EMSA titration, the extent of Fpg-mediated cleavage at the 2'-F-riboGh lesion is less than 15% of the substrate. In contrast, with the 2'-F-arabinoGh:C duplex, the amount of bound complex corresponds directly to the amount of cleavage suggesting that the observed K_d is due to a mixture of substrate and product. The tracking of the two curves together suggests that the affinity for the product after base removal is as high as for the substrate 2'-F-arabinoGh duplex. Taken together with the differences in glycosylase and glycosylase/lyase activity with 2'F-arabinoGh, and the ability to effectively trap a Schiff base intermediate with 2'-F-arabinoGh, our results suggest that high affinity with these duplexes is due in large part to the stalled β-lyase activity.

Concluding Remarks and Potential Applications

Herein we report the first efficient and convenient method of synthesis of oligonucleotides containing 2'-F-Gh and 2'-F-Sp in both the arabino and ribo configurations from the corresponding 2'-F-OG precursor. The hydantoins Gh and Sp are known to be excellent

substrates for the Fpg/Nei/NEIL family of glycosylases. The installation of F at the 2'-position of the Gh nucleotide was shown to significantly reduce the efficiency of enzyme-catalyzed base excision relative to the native lesion; notably, however, Gh excision was not completely ablated. Our results suggest that the configuration of the 2'-F hydantoin significantly impacts the ability to be recognized and excised by Fpg and NEIL1. In the duplex containing 2'-F-arabinoGh, Gh removal by the BER glycosylases is more efficient than from the corresponding 2'-F-riboGh duplex. However, the enzyme-catalyzed β-lyase strand scission of the glycosylases is substantially stalled with the 2'-F-arabinoGh -containing DNA. This suggests that the F substituent may be oriented in the position of the H normally abstracted by basic residues of the bifunctional glycosylase/lyase enzyme that mediate strand scission. As a consequence, the glycosylase becomes "trapped" to the F-AP site as the Schiff base intermediate. Fpg and NEIL1 were shown to exhibit high affinity for both 2'-F-ribo and 2'-F-arabinoGh-containing duplexes. The high affinity for the 2'-F-arabinoGh is likely due in part to the formation of the stalled β-lyase complex. The high affinity of Fpg and NEIL1 with the "RNA-like" ribo-configured 2'-F-Gh implies that the C3' endo sugar pucker may facilitate rather than hinder lesion recognition and nucleotide flipping into the enzyme active site. This is in contrast to the expectation for a preference of the B DNA C2' endo sugar pucker expected for the 2'-arabino configured sugar. These results provide interesting mechanistic insight into the Fpg/NEIL family of glycosylases, but also provides avenues to exploit the distinct features of the enzyme interaction with ribo versus arabino configured 2'-F-Gh oligonucleotides in various types of chemical biology applications.

The high affinity of Fpg and NEIL1 to the 2'-F-riboGh duplex indicates that ribo-configured 2'F-hydantoins will be useful in applications to probe features of lesion base recognition within DNA by providing glimpses of the complex prior to lesion excision. Notably, however, since there is some base excision observed with 2'-F-riboGh, experimental conditions may have to be optimized or performed with a mutated glycosylase to completely ablate base excision. Alternatively, a similar strategy could be used for prepare 2',2''-F-Gh which would be anticipated to be even more resistant to the glycosylase activity. High affinity complexes with the 2'-F-riboGh may aid in generation of diffraction quality crystals of the glycosylases bound to DNA.[39] The fluorinated hydantoin lesions will also be useful for analyzing features of nucleotide flipping by DNA glycosylases. For example, incorporation of a fluorescent base analogue, such as 2-aminopurine, adjacent to the 2'-F-riboGh nucleotide could be used to monitor changes in Gh environment upon enzyme binding, as has been illustrated by Stivers and co-workers with 2'-F-U and UDG.[58] An alternative strategy for monitoring nucleotide flipping was recently reported by Drohat and co-workers that capitalizes on the installed F in the 2'-F nucleotide to monitor changes in ^{19}F NMR chemical shift.[59] Specifically, using 2'-F-U or 2'-F-T-containing duplex DNA, the equilibrium between the "stacked" versus "flipped" nucleotide (K_{flip}) in the presence of TDG was determined by the signal intensity for the two distinct ^{19}F chemical shifts. Both approaches using 2'-F-Gh or 2'-F-Sp would be particularly useful in studies with NEIL1 to probe differences in nucleotide flipping between the edited and unedited isoforms.[19] The amino acid in NEIL1 that is recoded (K242 to R242) due to ADAR1-dependent mRNA editing is located in the lesion recognition loop[19] and this loop has distinctly different

conformations in the free versus bound enzyme.[55] The presence of Lys or Arg may alter loop flexibility and therefore impact the ability of the enzyme to extrude and excise the lesion. These types of experiments would also be useful in teasing apart differences in Gh recognition and excision by NEIL 1, 2 and 3 in canonical duplex DNA and noncanonical DNA contexts, such as G-quadruplexes[4]. Insight into features of the distinct context dependent preferences of NEIL1, 2 and 3 glycosylases would provide key information as to the biological implications of NEIL family enzymes recognition and excision of hydantoin lesions.

Both 2'-F-arabino and 2'-F-ribohydantoins may also be leveraged as chemical biology probes in cells. DNA containing 2'-F-hydantoins may be useful in cell assays to stall BER, and therefore allow the opportunity to determine the extent of repair mediated by other repair mechanisms, such as nucleotide excision repair.[60–61] 2'-F-OG and 2'-F-hydantoins nucleosides and isotope labeled versions of these nucleotides may also be used in cellular incorporation and enzymatic processing studies in a manner analogous to those reported by Carell and co-workers with 2'-F-ribo 5-methyl C nucleosides and oxidized derivatives.[42] Note these studies used the 2'-F-ribo-configured nucleosides since the the 2'-F-arabino nucleosides were found to be toxic.[36] Our finding that the 2'-arabino-F-hydantoins incorporated into DNA may be trapped as a covalent intermediate with the BER glycosylases suggests this feature may lead to toxicity in cells. Indeed, the ability to target NEIL enzymes in cells, and form a transient covalent complex suggests that F-hydantoin-containing oligonucleotides could be potent NEIL glycosylase inhibitors. This strategy would be a unique way to address the wide-spread interest in developing inhibitors for DNA repair enzymes as a cancer chemotherapeutic strategy.[62] These diverse examples illustrate the wide array of chemical biology applications for 2'F hydantoins.

Supplementary Material

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Acknowledgment

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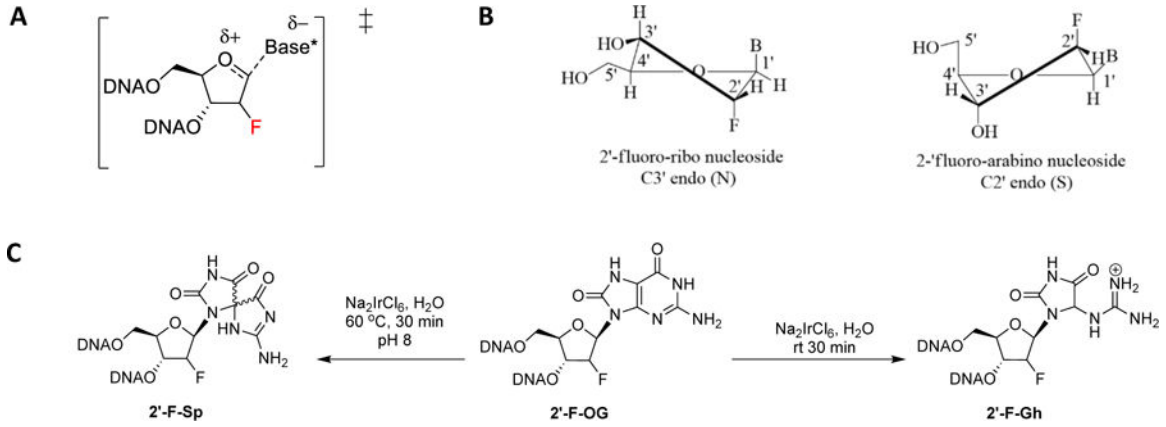


Figure 1.

2’-F-ribohydantoins and 2’-F-arabinohydantoins as probes for DNA glycosylases. (A) The presence of the 2’-F substituent is expected to destabilized the transition state for glycosidic bond cleavage, and therefore decrease the rate of enzyme catalyzed base removal (B) The expected sugar conformations for 2’-F-ribo versus 2’-F-arabino nucleosides. (C) Selective oxidation of 2’-F-OG within oligonucleotides provides a convenient strategy to make oligonucleotides containing 2’-fluorinated derivatives of the hydantoin lesions Gh and Sp.

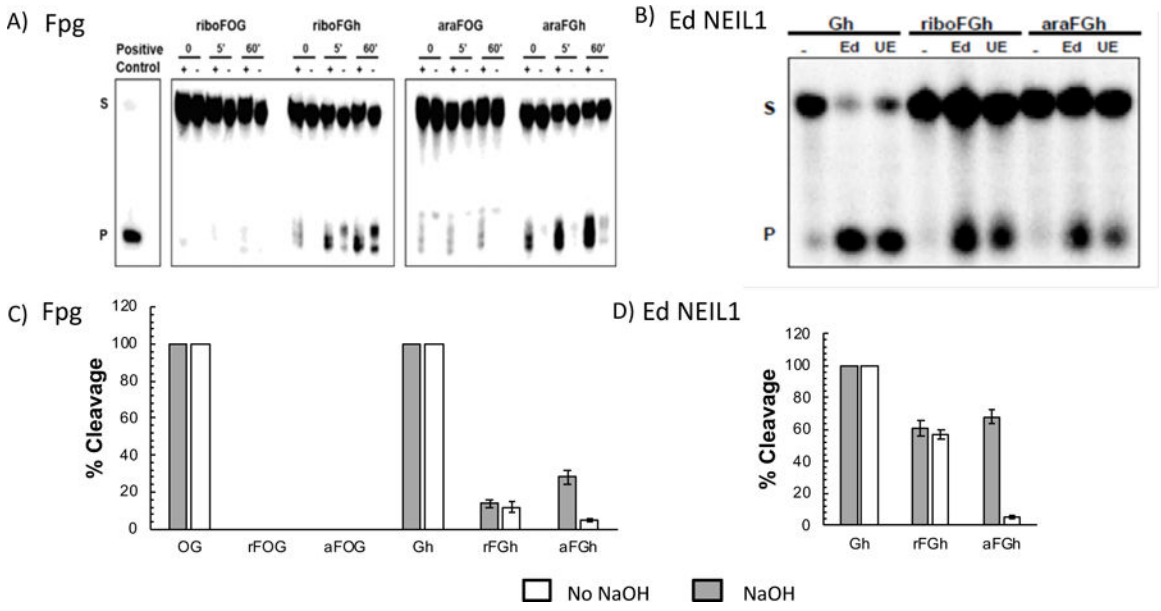


Figure 2. Diminished glycosylase and β-lyase activity of Fpg and NEIL1 on 2'F-ribo and 2'-F-arabinoGh lesions in DNA.

(A) Representative autoradiogram of Fpg in reaction with X:C-containing duplexes (X = 2'-F-riboOG, 2'-F-riboGh, 2'F-arabinoOG, and 2'-F-arabinoGh). S represents the 30-nt strand resulting from the substrate, while P represents the 15-nt strand of the product. The positive control is the corresponding 30-bp OG:C duplex incubated with Fpg for 60' and quenched with 0.1 M NaOH. Other lanes indicate reaction of Fpg with the respective lesions for the indicated period of time. (+) indicates treatment with 0.1M NaOH after the indicated period of time (glycosylase activity), and (−) indicates no treatment with base (lyase activity). Multiple bands are observed due to presence of products from β and δ-lyase activity. Level of magnification is high to allow observation of small extents of cleavage of the 2'F-nucleotide. (B) Representative autoradiogram of Edited (Ed) and Unedited (UE) NEIL1 with 30-bp X:C-containing duplexes (X = Gh, 2'-F-riboGh, and 2'-F-arabinoGh). All samples treated with 0.1M NaOH after 5' incubation. (C, D) Bar graph quantitation of the extent of cleavage after 60' mediated by bifunctional glycosylases Fpg (C) and Ed NEIL1 (D) on FOG versus FGh duplexes relative to the natural lesions. All product bands arising from β and δ-elimination were included in the gel quantitation.

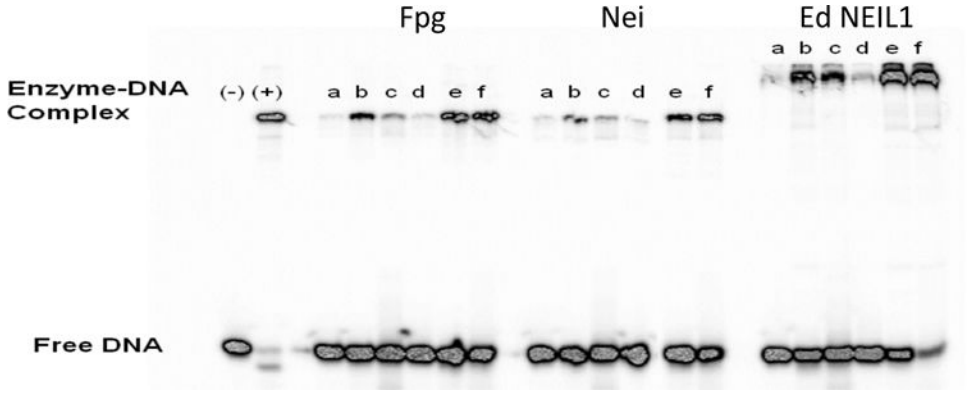


Figure 3.

Storage phosphor autoradiogram of SDS-PAGE for $NaBH_4$ trapping assay. The 30-bp duplex contained 2'-F-riboOG (a), 2'-F-riboGh (b), 2'-F-riboSp (c), 2'-F-arabinoOG (d), 2'-F-arabinoGh (e), or 2'-F-arabinoSp (f) opposite C. The (+) represents the enzyme control (OG:C incubated with Fpg), while (−) represents the no enzyme control (OG:C duplex alone).

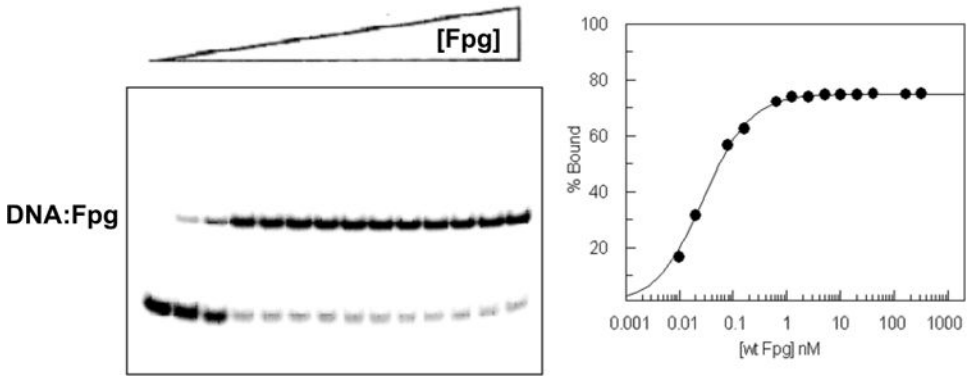


Figure 4. Dissociation constant determination with 2'-F-hydantoin duplexes and Fpg glycosylase using EMSA.

Representative storage phosphor autoradiogram of native PAGE gel and corresponding plot of the binding isotherm for Fpg binding to 2'-F-arabinoGh:C-containing 30-bp duplex. The [DNA duplex] was 10 pM, and [Fpg] ranged from 10 pM to 330 nM.

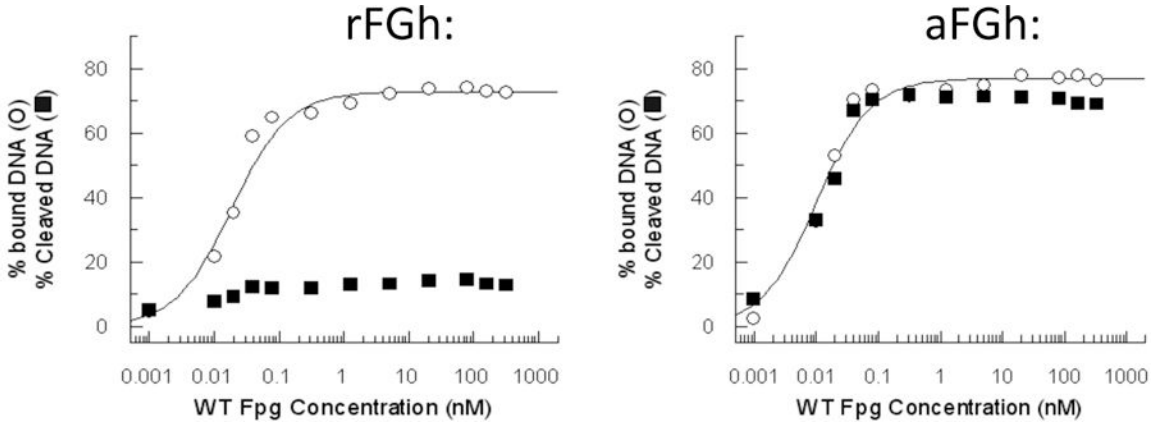
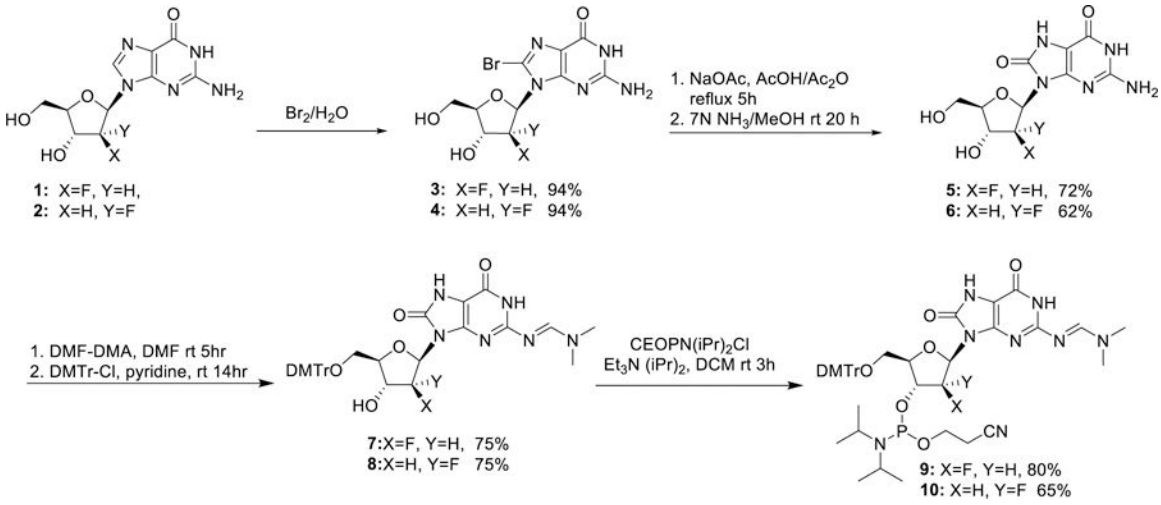


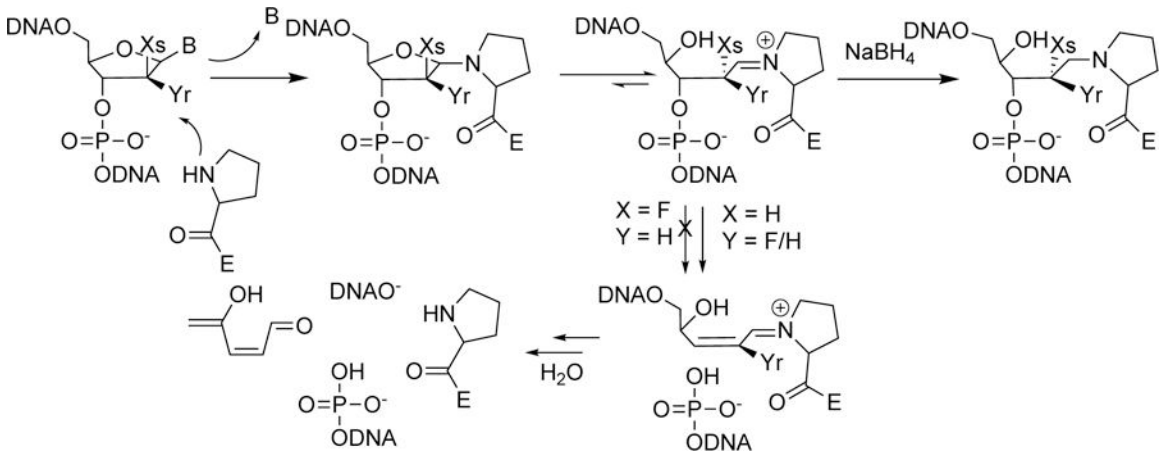
Figure 5:

Representative plot of percentages of cleavage and binding of Fpg as a function of Fpg concentration with 2'F-riboGh (rFGh) and 2'-F-arabinoGh (aFGh) opposite C in 30-bp DNA duplex.



Scheme 1:

Synthesis of 2'-F-ribo and 2'-F-arabino OG phosphoramidite monomers. The 2'-F-ribo and 2'-F arabino OG phosphoramidites were prepared starting with 2'-F-riboG or 2'-F-arabinoG nucleosides (500 mg) in overall yields of 28% and 41% (synthetic details described in the Supporting Information).



Scheme 2. Glycosylase and β-Lyase Reactions of Fpg/Nei enzyme family.

Base excision reactions utilize a N-terminal proline as the nucleophile. The ring-opened iminium tautomer is subject to 2'-proton abstraction to ultimately lead to β-elimination. Above **t**he X or Y positions may be F. In the case of both X and Y being H, the pro-S or pro-R designation is indicated by the subscript. Previous work has suggested that Endo III removes the pro-S H, and by analogy, we propose that replacement of the proS H with F, as expected for the 2'-F arabino configuration, placing the H in a non-optimal position for abstraction leading to stalling of the β-lyase activity.

Table 1.

Apparent dissociation constants (K_d) for Fpg and NEIL1 with 2'-F-riboGh (rFGh) and 2'-F-arabinoGh (aFGh) in 30-bp duplexes at 25 °C. In the case of WT Fpg and NEIL1, the 2'-F-Gh is a substrate and therefore these do not represent true Kds to the substrate, but are rather representative of the apparent Kd to a mixture of substrate and product (covalent complex). E3Q Fpg is an inactive form of Fpg, while Δ56 K54L are truncated inactive variants of NEIL1. The salt buffer concentration with E3Q Fpg was lowered to 30 mM to facilitate observation of a shifted E3Q-DNA complex. In all other experiments, the buffer salt concentration is 150 mM NaCl; additional details may be found in the Supporting Information.

Dissociation constants (nM)		
Enzyme	**aFGh:C**	**rFGh:C**
Fpg	0.04 +/− 0.01	0.021 +/− 0.008
E3Q Fpg (30 mM NaCl)	4.0 +/− 0.6	0.39 +/− 0.01
Unedited NEIL 1	0.8 +/− 0.1	1.2 +/− 0.5
Edited NEIL 1	0.8 +/− 0.2	1.3 +/− 0.3
Δ56 K54L Uned. NEIL1	5.5 +/− 0.9	1.2 +/− 0.2
Δ56 K54L Ed. NEIL1	1.5 +/− 0.3	1.7 +/− 0.2