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Structure and thermodynamic insights on acetylaminofluorene-modified deletion DNA duplexes as models for frameshift mutagenesis

Anusha Sandineni^{1,‡}, Bin Lin^{2,‡}, Alexander D. MacKerell Jr.², and Bongsup P. Cho^{1,*}

¹Department of Biomedical and Pharmaceutical Sciences, College of Pharmacy, University of Rhode Island, Kingston, RI 02881

²Department of Pharmaceutical Sciences, School of Pharmacy, University of Maryland, Baltimore, MD 21201

Abstract

2-Acetylaminofluorene (AAF) is a prototype arylamine carcinogen that forms C8-substituted dG-AAF and dG-AF as the major DNA lesions. The bulky *N*-acetylated dG-AAF lesion can induce various frameshift mutations depending on the base sequence around the lesion. We hypothesized that the thermodynamic stability of bulged-out slipped mutagenic intermediates (SMIs) is directly related to deletion mutations. The objective of the present study was to probe the structural/conformational basis of various dG-AAF-induced SMIs formed during a translesion synthesis. We performed spectroscopic, thermodynamic, and molecular dynamics studies of several AAF-modified 16-mer model DNA duplexes, including fully paired and –1, –2, and –3 deletion duplexes of the 5′-CTCTCGATG[FAAF]CCATCAC-3′ sequence and an additional –1 deletion duplex of the 5′-CTCTCGGCG[FAAF]CCATCAC-3′ *Nat* sequence. Modified deletion duplexes existed in a mixture of external B and stacked S conformers, with the population of the S conformer being ‘GC’ –1 (73%) > ‘AT’ –1 (72%) > full (60%) > –2 (55%) > –3 (37%). Thermodynamic stability was in the order of –1 deletion > –2 deletion > fully paired > –3 deletion duplexes. These results indicate that the stacked S-type conformer of SMIs are thermodynamically more stable than the conformationally flexible external B conformer. Results from the molecular dynamics simulations indicate perturbation of base stacking dominate the relative stability along with contributions from bending, duplex dynamics, solvation effects that are important in specific cases. Taken together, these results support a hypothesis that the conformational and thermodynamic stabilities of the SMIs are critical determinants for the induction of frameshift mutations.

INTRODUCTION

Genomic instability has been implicated in many human diseases including cancer and aging.¹ DNA mutations may be due to damage from environmental factors, such as UV radiation or certain chemicals, as well as random mistakes during replication for cell division. Accumulation of DNA damage contributes to frameshift mutations involve the gain or loss of one or more base pairs relative to the original sequence, thereby altering the information content of the genome. The underlying molecular mechanisms of frameshift mutations are not well understood, but those induced by aromatic amines have been studied

*To whom correspondence should be addressed: bcho@uri.edu, Telephone #: 401-874-5024 (Office); 401-874-5766 (FAX).
equally contributed

Supporting Information. Modeling details, MALDI-TOF and ESI mass, imino proton NMR, and CD spectra, and tables describing additional CD and molecular dynamics data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

extensively.^{2,3} For example, it is well known that a 'GC' deletion in the *E. coli* *NarI* hot spot (5'---GGCGC--3') results from a slipped mutagenic intermediate (SMI) formed during replication.⁴⁻⁹ As shown in Figure 1a, an incorporation of the correct nucleotide dC (blue) opposite a modified dG (red) stalls the replication fork, causing a slippage of the growing primer strand. Continued extension leads to a looped-out bulge structure in the template strand, resulting in a daughter strand that is two bases shorter than the template strand.

Aromatic amines are widely present in the environment as byproducts of fossil fuel combustion, tobacco smoke, dyes, and charred meat. These molecules have been implicated in the etiologies of various sporadic human cancers.¹⁰ The prototype aromatic amine 2-acetylaminofluorene (AAF) is converted *in vivo* into a highly electrophilic nitrenium ion, which in turn reacts directly with cellular DNA to form DNA adducts.¹¹⁻¹³ The most commonly formed are C8-substituted dG adducts: dG-AAF (Fig. 1b) and the *N*-deacetylated dG-AF. Although these two lesions are structurally similar, they exhibit different mutagenic and repair properties. For example, the dG-AF is largely nonmutagenic and is correctly replicated by a high-fidelity polymerase in *E. coli*.^{2,3} This effect may be due to its flexibility to accommodate both *syn*-stacked (S) and *anti*- (B-type) glycosidic conformations.¹⁴⁻¹⁹ On the other hand, the bulky *N*-acetylated dG-AAF lesion exists primarily in the highly distorting *syn*-glycosidic stacked (S)- or wedge (W)-conformations and strongly blocks the polymerase, thus resulting in frameshift mutations.^{16-18,20} In mammalian cells, however, the two lesions mostly result in point mutations.²¹⁻²³

Using the *E. coli* *lacI* gene, Schaaper *et al.* found a greater frequency of -2 followed by -1 frameshifts and a few -3 deletions.^{6,7,24} Schorr and Carell recently conducted primer extension studies with the human bypass polymerase η on variations of the *NarI* hot spot sequences.²⁵ Structural instability ensued after addition of the correct dC opposite dG-AAF triggered a slippage of the dC primer end to form various bulges, depending on the base sequence around the lesion. For example, dG₃-AAF in the *NarI* sequence (5'-G₁G₂CG₃CC-3') induced the -2 frameshift exclusively. Changing G₂C to AT (5'-G₁ATG₃CC-3') resulted in a -3 deletion mutation, presumably due to the presence of weak A:T base pairs around the lesion. Contiguous Gs around the lesion (5'-G₁G₂GG₃GG-3') yielded a strong -1 deletion mutation. These results suggest that the bulky dG-AAF:dC pair facilitates a slippage and the stability of the resulting looped-out SMI structure may be important for the manifestation of the frameshift mutations.

We hypothesized that AAF-induced SMIs do not adopt a single conformation, but rather exist as a mixture of three major conformational categories depending on the location of the planar carcinogen: namely, a stacked (S) conformer into the bulge, an externally bound B-type conformer, and intermediate conformers between S and B. Our working hypothesis was that the combined thermodynamic stabilities of an SMI play critical roles in determining the outcomes of specific deletion mutations.²⁶ To that end, we prepared several model AAF-modified bulge duplexes and their respective unmodified controls (Fig. 1c). Using these duplexes, we conducted systematic spectroscopic (¹⁹F NMR, ICD) and thermodynamic (UV-melting, DSC) studies. We also performed extensive MD simulations to obtain molecular explanations for the experimental findings. The results show that the thermal and thermodynamic stabilities of bulky adduct-induced SMIs are key factors for determining the propensity to form different frameshift mutations.

EXPERIMENTAL PROCEDURES

Caution

Aminofluorene analogs are known mutagens and suspected human carcinogens and therefore must be handled with caution.

Crude oligodeoxynucleotides (oligo, 10 μ mol scale) in desalted form were purchased from Eurofins MWG Operon (Huntsville, AL, USA). All HPLC solvents were purchased from Fisher Inc. (Pittsburgh, PA, USA).

Preparation of FAAF modified oligonucleotides

FAAF-modified 16-mer template oligo (Fig. 1c) were prepared using the general procedures described previously.^{14, 16, 19, 27, 28} For example, 0.5 ~ 1.0 mg of *N*-acetoxy-*N*-2-(acetyl amino)-7-fluorofluorene in absolute ethanol was added drop wise to a pH 6.0 sodium citrate buffer (10 mM) containing approximately 200 ods of an unmodified oligo (5'-CTCTCG₁ATG₂CCATCAC-3') and placed in a water-bath shaker for 5 min at 37°C. Figure 2a shows a typical HPLC chromatographic profile on a reverse phase column before purification. Unreacted oligo appeared at 11.7 min (peak 1) and modified oligos (peak 2,3,4) in the 15 – 25 min range. Peak 2 and 3 were confirmed as G₁- and G₂-mono-FAAF-adduct, respectively, on the basis of the UV absorption intensity in the 300–320 nm region (Fig. 2b) and nuclease digestion mass spectrometry analyses. Peak 3 eluting at 25 min was assigned as a di-FAAF-modified oligo (Fig. 2b, see below, Results). The HPLC system consisted of a Hitachi EZChrom Elite unit with a L2450 diode array detector and a Phenomenex Luna C18 column (150×10 mm, 5.0 μ m). The HPLC solvent gradient system involved 3–15% acetonitrile for 15 min followed by 15 ~ 30% acetonitrile for 25 min in pH 7.0 ammonium acetate buffer (100 mM) with a flow rate of 2.0 mL/min. The desired G₂-modified oligo (5'-CTCTCG₁ATG₂[FAAF]CCATCAC-3') was annealed with an appropriate complementary sequence to form model duplexes at the various sequence settings (Fig. 1c). An identical set of unmodified control duplexes were similarly prepared. We reported previously the preparation and characterization of the other modified oligo (5'-CTCTCG₁GCG₂[FAAF]CCATCAC-3') used for preparation of the 'GC'-1 deletion duplex (Fig. 1c).²⁹

Characterization of FAAF modified oligonucleotides

The purified FAAF-modified 16-mer oligos were characterized by initial enzyme digestions followed by a combination of matrix assisted laser desorption ionization-time of flight (MALDI-TOF) and electrospray ionization (ESI) mass spectrometry.^{30, 31}

A MALDI matrix solution was prepared by dissolving a 1:1 ratio of 3-hydroxypicolinic acid (3-HPA) and di-ammonium hydrogen citrate (DHAC). 1 μ L of each analyte and the matrix solution were mixed to produce a spotting solution, which was applied on the MALDI plate and air dried. A 1 μ L solution containing 100 pmol of an oligo was used in both snake venom phosphodiesterase (SVP) and bovine spleen phosphodiesterase (BSP) digestion experiments. SVP and BSP remove one nucleotide at a time from the 3'- or 5'- end, respectively. For the SVP digest, 0.5 ~ 2 μ L of SVP solution (10×10^{-2} units/ μ L) was added to 1 μ L of the oligonucleotide solution, 6 μ L of 100 mM ammonium citrate, and 6 μ L of deionized water. In the BSP digestion, 1–2 μ L BSP (1×10^{-2} units/ μ L) solution was added to 1 μ L of the oligo solution and 7 μ L of deionized water.

The digest solution was heated to 37°C for the SVP digestion, but kept at room temperature for the BSP digestion. A 1 μ L sample of the digest solution was removed at regular time intervals until the digestion rate was significantly reduced, and the reaction was quenched by mixing the aliquot with 1 μ L of matrix (3-HPA and DHAC in a 1:1 ratio). The sample was spotted on the MALDI plate and dried for immediate analysis. All MALDI-MS spectra were obtained using Shimadzu Axima Performance MALDI-TOF mass spectrometer equipped with a 50 Hz nitrogen laser. The spectra were obtained in a positive ion reflectron mode.

The molecular weights and the position of FAAF attachment were also verified by ESI-TOF mass spectrometry as described previously.^{29, 31} All LC/MS spectra were acquired using a

Waters SYNAPT quadrupole time-of-flight mass spectrometer (Milford, MA, USA) operated in the negative ion and V-modes.

UV-melting

UV melting experiments were conducted using a Cary100 Bio UV/VIS spectrophotometer equipped with a 6×6 multi-cell block and 1.0-cm path length. Sample cell temperature was controlled by a built-in Peltier temperature controller. Samples with a total concentration in the range of 0.5 ~ 10 μ M were prepared in solutions containing 0.2M NaCl, 10mM sodium phosphate and 0.2 mM EDTA at pH 7.0. Thermomelting curves were constructed by varying temperatures of the sample cell (1 °C/min) and monitoring absorbance at 260 nm. A typical melting experiment consisted of forward/reverse scans and was repeated five times. Thermodynamic parameters were calculated using the program MELTWIN version 3.5, as described previously.³²

Circular Dichroism (CD)

CD measurements were conducted on a Jasco J-810 Spectropolarimeter equipped with a Peltier temperature controller. Typically, 6.5 μ M of each strand was annealed with an equimolar amount of a complementary sequence. The samples were dissolved in 300 μ l of a pH 7.0 buffer (0.2 M NaCl, 10 mM sodium phosphate, 0.2 mM EDTA) and placed in a 1.0 mm path length cell. The samples were heated at 85°C for 5 min and then cooled to 15°C, over a 10 min period to ensure complete duplex formation. The CD was scanned from 200 to 400 nm at a rate of 50 nm/min. Spectra were acquired every 0.2 nm with 2 s response time were the averages of 10 accumulations and were smoothed using 17-point adaptive smoothing algorithms provided by Jasco.

¹⁹F-NMR

Approximately 20 μ ols of a FAAF-modified 16-mer oligo was annealed with an equimolar amount of respective complementary sequence to produce a model duplex (Fig. 1c). The samples were then dissolved in 300 μ L of a pH 7.0 NMR buffer containing 10% D₂O/90% H₂O, 100 mM NaCl, 10 mM sodium phosphate and 100 mM EDTA, and filtered into a Shigemi tube through a 0.45 μ m membrane filter. All ¹H- and ¹⁹F-NMR results were recorded using a HFC probe on a Varian NMR spectrometer operating at 500.0 and 476.5 MHz, respectively. ¹⁹F-NMR spectra were acquired in the ¹H-decoupled mode and referenced relative to CFCl₃ by assigning external C₆F₆ in C₆D₆ at -164.9 ppm. ¹⁹F-NMR spectra were measured between 5 and 78°C with increment of 5–10°C. Computer line shape simulations were performed as described previously using WINDNMR-Pro (version 7.1.6; *J. Chem. Edu.* Software Series; Reich, H.J., University of Wisconsin, Madison, WI, USA).¹⁵

Differential Scanning Calorimetry (DSC)

DSC measurements of all five FAAF-modified duplexes were obtained by a Nano-DSC from TA Instruments (Lindon, UT, USA) using the general procedures reported previously.^{29, 33} Template-primer solutions were prepared by dissolving desalted samples in a pH 7.0 buffer solution consisting of 20 mM sodium phosphate and 0.1 M NaCl. All sample solutions were run at 0.1 mM concentration. In a typical scan, a 0.1 mM template-primer solution was scanned against buffer from 15°C to 90°C at a rate of 0.75°C/min. Raw data were collected as microwatts vs. temperature. At least five repetitions were obtained. A buffer vs. buffer scan was used as a control and subtracted from the sample scan and normalized for heating rate. The area of the resulting curve was proportional to the transition heat, which, when normalized for the number of moles of sample, is equal to the transition enthalpy, ΔH .

Molecular Modeling

In order to gain structural and energetic insights into the experimental findings at the molecular level, we modeled all the FAAF-modified and unmodified deletion DNA duplexes and subjected them to extensive MD simulations. d[CTCTCG₁ATG₂CCATCAC] was built using the program 3DNA³⁴ as canonical B DNA for the unmodified full duplex, where G₂ is assumed to be in the *anti* conformation. Coordinates were then read into CHARMM³⁵ and patched to modify G₂ with FAAF. To build the model bulged structures (Fig. 1c), the partner C of G₂ on the complementary strand was deleted and the resulting gap was filled by linking its two neighboring nucleotides, yielding a B-type bulge -1 deletion, in which the modified G has an *anti*-glycosidic conformation. The -2 deletion was modeled by using NMR structure 1AX6³⁶ as template via a DNA homology modeling protocol implemented in CHARMM. The resulting conformation was an S-type -2 deletion because the modified G in 1AX6 has a *syn*-glycosidic bond. To create the S-type -3 deletion the -2 model was taken and the partner T of the 7th adenine was removed with the broken strand then reconnected. The S-type conformers for the full duplex and -1 deletion were made by rotating the glycosidic torsion of G₂ by 180° from their respective B-type models using CHARMM. The B-type conformers for -2 deletion and -3 deletion were also made by rotating the glycosidic torsion of G₂ of their respective S-type conformers by 180°. For the full duplex a W-type model was modelled by employing a base-flipping protocol³⁷ on G₂ starting from its S-type conformer to swing FAAF into the minor groove, a wedge conformation by definition. From these modeling efforts, 18 starting structures were generated, consisting of modified or unmodified full duplex, -1 deletion, -2 deletion, or -3 deletion of both B-type and S-type, plus modified and unmodified version of W-type full duplex. All the models were subjected to extensive energy minimization and equilibration in TIP3P water³⁸ and neutralizing sodium ions. 50 ns MD simulations of each system were performed using the program GROMACS 4.5.5³⁹ with the latest CHARMM36 nucleic acid force field.⁴⁰ Full details of the molecular modeling and MD simulation protocols are given in the Supporting Information. All simulations achieved adequate stability after 10 ns as judged by their RMS differences with respect to the starting structures. Trajectories from 10 to 50 ns were used for all analyses. The terminal two nucleotides at each end of the duplexes were excluded from analyses, unless noted, due to terminal fraying in the simulations. Curves+⁴¹ and CHARMM were used to perform structure and interaction energy analyses. PyMOL⁴² and VMD⁴³ were used for visualizing trajectories and preparing structural images.

RESULTS

Model Systems

The model 16-mer template (5'-CTCTCG₁ATG₂CCATCAC-3') is patterned after the sequence used by Schorr and Carell in their primer kinetic studies²⁵ using polymerase η , except that G₂ was adducted with the fluorinated *N*-acetylaminofluorene (FAAF) (Fig. 1b). The utility of fluorine tagged aromatic amine DNA adducts as an effective structure probe has well been documented.^{15, 27, 28, 44} The modified template was annealed with various primers to create four model duplexes: full duplex (16/16-mer), -3 deletion (16/13-mer), -2 deletion (16/14-mer) and -1 deletion (16/15-mer)(Fig. 1c). We also studied the "GC" version of the 16/15-mer -1 deletion duplex, in which the two A:T base pairs between G₁ and G₂ are replaced with two G:C base pairs (5'-CTCTCG₁GCG₂CCATCAC-3')(Fig. 1c). The same 16-mer *NarI* sequence in the fully paired duplex was used previously as a substrate for structural and nucleotide excision repair studies.²⁹ We also investigated the structure of the *N*-deacetylated FAF-containing truncated 12/10-mer inner core (CTCG₁GCG₂CNATC: G₂=FAAF, N=C or T) duplexes.²⁶ The dC -2 deletion duplex (N=dC) was found to adopt exclusively an intercalated conformer, whereas the dT/-2

(*N*=dT) counterpart existed as multiple conformers. The results provide structural evidence for the greater propensity of the G₂-lesion to form SMI in the dC over the dT –2 deletion duplex.

Characterization of FAAF-modified oligonucleotide templates

Figure 2a shows the HPLC profile of a work-up mixture after 20 min of reaction between a 16-mer oligo (5'-CTCTCG₁ATG₂CCATCAC-3') and the activated FAAF carcinogen (see Experimental Procedures). Unreacted oligo appeared at 11.7 min (peak 1) and three modified oligos (peaks 2–4) followed in the 15 ~ 25 min time period. The shoulder intensity in the 290–320 nm of peak 4 (diadduct) was twice as the two early eluting ones, which confirms peaks 2 and 3 as mono-FAAF modified oligos (Fig. 2b).³²

Figure 3A shows the MALDI-TOF spectra of the 5'→3' exonuclease digestion fragments of peak 3 at different time intervals. The ions observed at *m/z* 5,016 at 0 time interval (Fig. 3A, trace a) represents the molecular weight of the FAAF modified 16-mer oligo template (5'-CTCTCG₁ATG₂CCATCAC-3') before digestion. An increase in incubation time leads to the digestion of subsequent 5'-unmodified bases. The 5'-nuclease has decreased activity at the FAAF lesion site as evidenced by significant peak intensity at *m/z* 2,897 and 2,593 for the 5'-T(FAAF)G₂CCATCAC-3' and 5'-(FAAF)G₂CCATCAC-3' fragments, respectively (see insets for theoretical MW values). Figure 3B shows the results for the 3'→5' exonuclease digestion. As expected, decreased 3'-digestion activity was noted at *m/z* 2,929 after 30 s (trace b), which is consistent with the 5'-CTCTCG₁ATG₂(FAAF)-3' fragment. The 5'→3' and 3'→5' digests of peak 3 were also subjected to ESI-QTOF. The ions observed at *m/z* 863 and *m/z* 1,295 in Supporting Information Figure S1a are the (M-3H)³⁻ and (M-2H)²⁻ ions from the fragment 5'-G₂(FAAF)CCATCAC-3'. Similarly, the ions at *m/z* 975 and *m/z* 1,463 in the 3'→5' digest correspond to the (M-3H)³⁻ and (M-2H)²⁻ ions formed from the fragment 5'-CTCTCG₁TAG₂(FAAF)-3' (Supporting Information Figure S1b). Taken together, these results confirm peak 3 as G₂-FAAF modified 16-mer template.

The early eluting mono adduct peak 2 was characterized similarly. As expected, the 5'→3' nuclease activity (Supporting Information Figure S2) was decreased at 60 min for *m/z* 3,540, representing an ion formed from the 5'-G₁(FAAF)ATG₂CCATCAC-3' fragment. By contrast, the 3' digestion (Supporting Information Figure S3) was very efficient, but halted at *m/z* 1,982, consistent with the 5'-CTCTCG₁(FAAF)-3' fragment ion. The ESI spectra of the 5'→3' digest exhibited the (M-3H)³⁻ of the 5'-G₁(FAAF)ATG₂CCATCAC-3' fragment at *m/z* 1,178 ion is the (M-3H)³⁻ (Supporting Information Figure S4a). The 3'→5' digest shows ions at *m/z* 990, which corresponds to the (M-2H)²⁻ ion formed from the fragment 5'-CTCTCG₁(FAAF)-3' (Supporting Information Figure S4b). These results confirm peak 2 as FAAF-G₁.

UV-melting

Figure 4 shows the UV-melting profiles of FAAF-modified duplexes (red) relative to their respective unmodified controls (blue) at 10 μM. All of the duplexes showed monophasic and sigmoidal curves with a strong linear correlation (*R*² > 0.9) between *T*_m⁻¹ and ln*C*_t, confirming their typical cooperative helix-coil melting transition. Thermal and thermodynamic parameters calculated from these curves are summarized in Table S1. It is clear that the full and –3 deletion duplexes were destabilized thermally (Δ*T*_m) and thermodynamically (ΔΔ*G*_{37°C}) upon FAAF modification: full duplex (–7.7°, 4.5 kcal/mol) > –3 deletion (–5.7°, 2.0 kcal/mol). By contrast, –2 and –1 deletion duplexes displayed a strong stabilization: 'AT' –1 deletion (13.0° C, –8.5 kcal/mol) > –2 deletion (9.5° C, –7.2 kcal/mol). The 'GC' –1 deletion duplex displayed thermal and thermodynamic stabilization (13.3° C, –7.1 kcal/mol) similar to that of the 'AT' counterpart.

Differential Scanning Calorimetry (DSC)

Figure 5 shows DSC plots of excess heat capacity C_p^{ex} vs. temperature for all five FAAF-modified duplexes relative to their unmodified controls. These curves are transformed into the corresponding thermodynamic histograms (Fig. 6), and the results are tabulated in Table 1. The median peak of each bell curve indicates T_m , at which half of the duplexes melt, whereas the areas under the curve represent the increased enthalpy (ΔH) of duplex formation.³³ Consistent with the UV melting data, the 'AT' -1 deletion duplex was most stabilized ($\Delta\Delta G_{37^\circ C} = -6.3$ kcal/mol, $\Delta T_m = 15.2^\circ C$), followed by -2 deletion duplex ($\Delta\Delta G_{37^\circ C} = -3.5$ kcal/mol, $\Delta T_m = 10.8^\circ C$). As expected, the fully paired duplex was most destabilized ($\Delta\Delta G_{37^\circ C} = 3.5$ kcal/mol, $\Delta T_m = -8.0^\circ C$) followed by -3 deletion duplex ($\Delta\Delta G_{37^\circ C} = 0.8$ kcal/mol, $\Delta T_m = -3.1^\circ C$).

Unlike fully-paired duplexes where stacking usually promotes increased entropy upon modification (Table 1), the mostly stacked (72–73% S) modified -1 deletion duplexes exhibited decreased entropy relative to the the control deletion duplexes ('AT'; $\Delta\Delta S = -50.8$ eu and 'GC' $\Delta\Delta S = -31.0$ eu). The decreased entropy, however, was compensated by large enthalpies to produce a net gain in overall free energy ('AT'; $\Delta\Delta G_{37^\circ C} = -6.3$ kcal/mol and 'GC' $\Delta\Delta G_{37^\circ C} = -5.6$ kcal/mol). The -3 deletion duplex (52% B-type), however, displayed a slight entropy gain ($\Delta\Delta S = 8.4$ eu), but was compensated ($\Delta\Delta H = 3.5$ kcal/mol) to produce a small loss of free energy ($\Delta\Delta G_{37^\circ C} = 0.8$ kcal/mol). Largely stacked (60%) fully-paired duplexes are known to have disrupted Watson-Crick base pairing at the lesion site, thus resulting in enthalpy reduction ($\Delta\Delta H = 13.9$ Kcal/mol). Here too enthalpy-entropy compensation afforded a loss of free energy ($\Delta\Delta G_{37^\circ C} = 3.5$ kcal/mol).⁴⁵

Thermodynamic parameters obtained from UV melting (ΔH_{VH}) vs. DSC (ΔH_{cal})

The enthalpy values (ΔH_{cal}) obtained from DSC are the model-independent direct enthalpies. On the other hand, the values (ΔH_{VH}) from UV melting curves as well as any other method based on the van't Hoff method are model-dependent which assumes a simple two state transition. One can conclude that DNA melting occurs in a two-state manner in the case of $\Delta H_{VH} = \Delta H_{cal}$. However, in the present work ΔH_{VH} values are consistently greater than ΔH_{cal} for all DNA constructs examined (Table 1 and Table S1). This indicates that the melting transition of FAAF-modified bulge duplexes involves not just a cooperative two-state transition, but also some type of multi-molecular heterogeneous oligomerization process, possibly including aggregation. It should also be noted that ΔH_{cal} is concentration dependent (active or folded concentrations) while ΔH_{VH} is concentration independent. The observed conformational heterogeneity of active concentrations is consistent with the ¹⁹F NMR results below. The reverse trend was observed for T_m , *i.e.*, DSC > T_m . This is probably due to differences in concentrations used in the measurements: UV (5–10 μ M) (Supporting Information Table S1) and DSC (0.1 mM)(Table 1), *i.e.*, a higher concentration of duplex will have a higher T_m . However, the non-matching of T_m could also be due to differences in scan rates (deg/min) and salt concentrations.

Induced Circular Dichroism (ICD)

Figure 7 shows CD spectral overlays of all five FAAF-modified duplexes (solid lines) relative to their respective unmodified controls (dotted lines) at 30°C. All duplexes exhibited a (+)275nm/(-)250nm S-shape CD curve characteristic for a typical B-form DNA duplex. A small negative ICD ellipticity around 290 nm was noted^{16, 46} for fully-paired and -1 'AT'-modified duplexes.

A slight increase in the positive intensity (hyperchromicity) at 275 nm was noted for -2 and -1 (both 'AT' and 'GC') deletion duplexes. This is likely due to lesion-induced duplex stability caused by increased stacking of the planar aromatic carcinogen in the bulge (see

Thermodynamics above). The opposite (hypochromicity) was observed for the fully-paired and -3 deletion duplexes with the effect much greater for the latter. Again, the trend is in agreement with the differences in conformational population (inserted S-type *vs.* external B-type) as well as the thermodynamic results.

The modified duplexes also displayed significant blue shifts relative to their respective unmodified controls, signifying adduct-induced DNA bending.²⁹ For example, protein-induced DNA bending is known to exhibit significant blue CD shift at 275 nm of regular B-type DNA⁴⁷ which indicates bending of the DNA backbone. Initially, we probed the bending of unmodified deletion duplexes relative to the fully-paired unmodified counterparts. No wavelength shift was observed except for -3 deletion duplex which exhibited 5 nm of blue shift (data not shown). Similarly, we compared modified deletion duplexes relative to their unmodified counterparts (Fig. 7). All except for -3 deletion exhibited blue shifts upon FAAF-modification: full duplex ($\Delta_{G^*G} = 5$ nm) > 'AT' -1 deletion $\sim$ 'GC' -1 deletion ($\Delta_{G^*G} = 4$ nm) > -2 deletion ($\Delta_{G^*G} = 2-3$ nm). We have shown previously that FAAF in the CG**C* context exists mostly (61%) in the stacked conformation.²⁹ Hence, the greater blue shift observed for a fully-paired duplex could be attributed to a major structural disturbance at the lesion site. The significant blue shifts observed in deletion duplexes could also indicate a stacked-induced bending of DNA, *i.e.*, 'AT' and 'GC' -1 (72-73% S) followed by -2 (55% S) deletion duplexes. An exception was the FAAF-modified -3 deletion duplex, which displayed a shift to longer wavelengths ($\Delta_{G-G^*} = 3$ nm)(Supporting Information Figure S5). This may be due to its relatively high B-type conformation (52%) compared to other deletion duplexes. When compared to the fully-paired unmodified duplex, however, the -3 deletion duplex displayed a blue shift of 3 nm (Supporting Information Figure S5). One caveat is that bending surely is a factor, but adduct-induced twist and rise could also change base stacking interactions.

Dynamic ¹⁹F NMR: conformational heterogeneity

Figure 8 shows dynamic ¹⁹F NMR spectra of all five FAAF-modified duplexes (see Supporting Information Figure S6 for full temperature range dynamic ¹⁹F NMR spectra). While signal patterns vary, all exhibited sharp single signals around -115 ppm at coalescence temperatures, signifying duplex melting, *i.e.*, 70, 60, 65, 75, 78° C for full, -3, -2, 'AT' -1, 'GC' -1 deletion duplexes, respectively.

Our previous ¹⁹F NMR studies of fully paired FAAF-duplexes in various flanking sequence contexts (TG*A, CG*C, CG*G, GG*C) have revealed a mixture of B-, S- and W-conformations in the -115.0 ~ -115.5 ppm, -115.5 ~ -117.0 ppm and -116.5 ~ -118.0 ppm ranges, respectively.^{16, 29} Consequently, we assigned the -116.0 and -118.1 ppm signals at 5°C of the fully paired duplex as the S- and W-conformations, respectively (Fig. 8a). We have also accumulated an extensive collection of ¹⁹F NMR spectra of fluorinated aminofluorene-modified duplexes in various sequence settings and found that in the base-displaced stacked (S) conformer fluorine is always shielded (upfield) relative to that of the external binding B-type conformer.^{14, 15, 26, 29, 44, 48, 49} ¹⁹F shielding is a hallmark for the van der Waals interaction and the ring current effect caused by the carcinogen moiety within the stacked and bulge duplexes (S-type conformation).⁴⁴ We have used a similar strategy to assign the signals in the ¹⁹F NMR spectra of deletion duplexes, *i.e.*, the signals (~ -115 ppm) near the coalescence temperatures arise from denatured single stranded modified exposed fluorine and non-stacked external B-type conformers, and shielded signals arise from a buried fluorinated carcinogen with excess van der Waals interactions with neighboring base pairs as occurs in S-type conformations.

The 'GC' -1 deletion duplex displayed two major signals at 10°C (Fig. 8e). The signal at -116.0 ppm could be assigned as the B-type conformer because of its close proximity to the

coalescent signal (ss, single strand). The major signal at -116.5 was resistant to melting and its sharp signal persisted even at 70°C ! This must be an inserted S-type conformation, undergoing a melting transition in the $70\text{--}75^{\circ}\text{C}$ range. Moreover, the unusually high coalescence melting (78°C) is in good agreement with greater thermal and thermodynamic stability (Table 1 and 2).

A similar ^{19}F NMR pattern was obtained for the 'AT' -1 deletion duplex, *i.e.*, B (-115.6 ppm, purple) and S (-116.5 ppm, red) at 10°C (Fig. 8d). A strong signal at -116.8 ppm (marked *) is located in between S and B and coalesced to S around 40°C . This signal could be an intermediate conformer between B and S conformers (see below), but is unique to the 'AT' -1 duplex. Other than that, the two -1 duplexes exhibited similar dynamic patterns. The greater conformational stability seen in NMR for the 'GC' over 'AT' -1 deletion duplex is in good agreement with the thermodynamic results above and probably due to the better hydrogen-bonding capability of the $5'$ -G:C over A:T.

The -3 and -2 deletion duplexes also displayed two major ^{19}F signals around -115 ppm (Fig. 8 b,c) However, these signals are collectively shifted downfield by about 1 ppm relative to those of the -1 duplexes, suggesting an altered electronic environment, *i.e.*, an open and flexible bulge. Again, the downfield and, ring current effect, and coalescence behaviour. Additional signals marked with asterisk could be intermediate conformers.

Supporting Information Figure S7 compares ^{19}F NMR spectra of all the modified deletion duplexes at 30°C . The percent population ratios were calculated by line simulations (red). The population of S conformers was in order of 'GC' -1 (73%) $\sim$ 'AT' -1 (72%) $>$ full (60%) $>$ -2 (55%) $>$ -3 (37%) for the deletion duplexes. The S/W population ratio for the full duplex was 60:40 (not shown). Lesion-induced conformational change in the bulge structures is also evident from their imino proton spectra at 5°C (Supporting Information Figure S8). In all cases, a mixture of broad imino signals was observed for the Watson-Crick hydrogen bonds ($12\text{--}14$ ppm) as well as those at and near the lesion site ($10\text{--}12$ ppm). As shown in Supporting Information Figure S9, unlike the fully-paired duplex the high field imino proton signals of the -3 deletion duplex disappeared rapidly as the temperatures increase, indicating lesion-induced conformational flexibility at and near the lesion site. In contrast, the -1 deletion duplexes showed strong persistence of the high field imino protons even at higher temperatures. These imino proton results indicate a tightly packed structure at the lesion site, consistent with the thermodynamic stabilization and increased stacking with strongly positive CD around 270 nm.

Molecular Dynamics (MD) Simulations

To provide molecular explanations for the experimental results described above, we modeled all the FAAF-modified deletion DNA duplexes as well as their unmodified counterparts as controls, then subjected those models to MD simulations. Each deletion duplex was modeled in both B-type and S-type states. The full duplex was modeled with an extra W-type, as observed in ^{19}F NMR experiment (Fig. 8a). B type simulations were initiated with an anti-glycosidic bond while the S and W type simulations were initiated with syn-glycosidic bond.²⁹ During the simulations of the W type unmodified system, the guanine rotated about the glycosidic bond to assume an anti conformation with the duplex relaxing to a B-form duplex conformation. Simulations were subjected to a range of analyses to identify molecular contributions to the stabilities of the studied duplexes. From these analyses, base pairing, base stacking, DNA bending, solvent accessible surface area (SASA) and hydration energies were indicated to contribute to duplex stability, as described below. Selected images of the different bulge duplexes are shown in Figure 9.

Base Pairing—DNA base pairing was calculated by applying a cutoff of 3.5 Å for the N1-N3 distance for all base pairs excluding the terminal 2 nucleotides at each end. The results are shown in Supporting Information Table S2. The amount of Watson-Crick interactions are in the vicinity of 90% in most cases, indicating that most of base pairs are well maintained throughout the simulations. The -3 deletion shows a significantly higher amount of base pairing, with the exception of the unmodified S state, although the -3 deletion is less stable than the full duplex. With the -1 and -2 deletions there is more base pairing in the unmodified deletions, even though the modified species are more stable for those deletion duplexes. Given the lack of correlation with thermodynamics from the UV melting or DSC experiments, base pairing is not contributing to the impact of the presence of the bulges or the FAAF adduct on stability.

Base Stacking—Base stacking energies were calculated as the sum of the interaction energies between neighboring base pairs. Results in Supporting Information Table S3 on the total stacking energies can be compared to the experimental enthalpies in Table 1 (DSC) and Supporting Information Table S1 (UV). Upon going from the full duplex to the deletion duplexes there is a significant decrease in stacking with the order of the changes being $-3 > -2 > -1$. These results mirror the ordering in the change in enthalpy going from the full duplex to the unmodified deletion duplexes in the DSC experiments (Table 1), and the ordering is the same for the modified species though the experimental enthalpy of the -1 deletion becomes more favorable. The good agreement in terms of ordering between the calculated base stacking energies and the experimental enthalpies is not surprising considering that stacking is the dominant contributor to helix stability⁵⁰ and upon going from full to -1 to -2 to -3 each system has one base less than the previous thus lower stacking energies. The only exception is that for the modified full duplex where the calculated base stacking energy is more favorable than that of the modified -1 deletion case, while the DSC experiments shows the modified -1 deletion to have more enthalpic stabilization (Table 1). This suggests that in the case of the -1 deletions additional interactions, such as solvation may be contributing to the enthalpic stabilization.

Additional analysis of stacking was performed to understand its contribution to the change in duplex stability due to the presence of the adduct. To quantitatively interpret the enthalpy differences ($\Delta\Delta H$) between the modified and the unmodified duplexes, the stacking energies were calculated per base, thereby yielding the impact of the presence of the adduct on the stability. In addition, the base stacking energies were weighted by the population of each species determined from the ¹⁹F-NMR experiments according to the following equation:

$$\Delta E_i = \frac{\sum_{j=B,S,W} E_{ij}^{\text{mod}} w_j}{\sum_{j=B,S,W} w_j} - E_i^{\text{unmod}} \quad (1)$$

where ΔE_i is the change in stacking energy associated with FAAF modification from the simulations of the different B, S and W states, i indicates full duplex, -3 deletion, -2 deletion, and -1 deletion DNA duplexes, E_{ij}^{mod} is the per base stacking energy of the j conformer of modified i DNA duplex, where j is the B, S, or W conformer. w_j is the weight of each conformer obtained from the populations observed in the ¹⁹F NMR experiment (Fig. 8). E_i^{unmod} is the total stacking energy of unmodified i DNA duplex.

As there is evidence that the unmodified deletion duplexes have a significant amount of conformational heterogeneity⁵¹ and their populations cannot be readily determined from

experiments due to the lack of fluorine atom tag, the change in stacking energy was calculated using both the anti and syn unmodified duplexes as reference states. This is equivalent to assuming 100% B or 100% S for unmodified deletion duplexes and represents extreme cases that will yield final values of ΔE_{stack} that will bracket the value if the unmodified state is comprised of anti/syn heterogeneity. The results based on this analysis are shown in Figure 10 and listed under column ΔE (ranking) in Supporting Information Table S3. Notably, the ordering of the results is consistent with the experimental $\Delta\Delta H$ values though the absolute values are not directly comparable to the experimental data. This is expected because other factors besides base stacking contribute to the change in enthalpy. Nonetheless, when either the anti or syn conformation is used as reference, the calculated ΔE_{stack} reproduces the ranking of the impact of FAAF modification on $\Delta\Delta H$ for the full duplex and different DNA deletion duplexes correctly. Thus, the present MD results indicate that the changes in stacking are dominating the experimentally observed change in the enthalpy due to the presence of the bulges and associated with FAAF modification.

DNA bending—Average total bending of DNA full duplex and deletions calculated by Curves+ are listed in Supporting Information Table S4. We note that Curves+ has the capability to deal with deletion duplexes with gaps in the DNA strands as required for the present study. Analysis of the bending data indicates that there is no clear trend with respect to the extent of bending upon going from the full duplex to the deletion duplexes. However, addition of the FAAF modification led to a decrease in bending in the B and S conformers of the fully-paired duplex, while an increase in bending occurred in the W conformer of the full duplex and all the deletion duplexes. The increase in bending in the W conformer may contribute a net gain of entropy if it is assumed that increases in bending lead to an increase in system entropy in DNA duplexes.⁵² The increase in bending may also contribute to the experimentally observed increase in entropy of stabilization of the -3 deletion duplex. However, this is not consistent with the -2 and -1 bulges, which undergo a net entropy loss due to the adduct. Clearly, alternative phenomena are contributing to the change in entropy. The bending results are also mostly consistent with the results from CD experiments above, in which all but -3 deletion duplex displayed significant blue shifts upon FAAF modification. The red CD shift in the -3 deletion appears to be due to its relatively high percentage of B-type conformation, which has much lower bending than its S-type conformation.

Additional analysis focused on dynamics properties of duplexes. Analysis of root mean square fluctuations (RMSF) of the duplexes was initially undertaken according to

$$\text{RMSF} = \sqrt{\frac{1}{N} \sum_{i=1}^N (x_i - \bar{x}_i)^2}$$
 where x_i is the atom coordinates. Relative average RMSF values with respect to the full duplex show the bulges to have increased mobility over the full duplex in the majority of cases (Supporting Information Table S5); this is consistent with the more favorable entropy seen in the DSC and UV melting experiments. Exceptions were the modified B state, where the fluctuations were lower for the -2 and -3 bulges and similar for the -1 bulge. These results indicate that the less favorable entropy associated with FAAF modification observed in the experiments for the -1 and -2 bulges may be due to, in part, changes in the flexibility of the duplexes.

Solvation effects—Solvation can also impact both enthalpic and entropic aspects of duplex stabilities. To focus on the possible contribution of solvation effects on the bulge regions we computed the solvent accessible surface area (SASA) of the central common five base pairs. The results are listed in Supporting Information Table S6. Overall, the SASA decreased upon going from full to -1 to -2 to -3 deletion duplexes, as expected due to the

omission of the nucleotides. The differences correspond to approximately $-415 \text{ \AA}^2$, $-249 \text{ \AA}^2$ and $-111 \text{ \AA}^2$ for the -3 , -2 and -1 bulges relative to the full duplex, respectively. This ordering is consistent with the magnitude of both the enthalpy and entropy changes in the bulges relative to the full duplex from the DSC experiments above (Table 1). The omission of the nucleotides would lead to less favorable hydration, contributing to the loss of enthalpy, which would be compensated by an increase in entropy due to the increased mobility of water that is no longer hydrogen bonding with the DNA. However, the effect is smaller than the SASA that would be lost associated with deletion of a nucleotide. This is indicated by the percent (%) per nucleotide being less than 100%, where 100% corresponds to that average SASA of each nucleotide from the unmodified full duplex simulation. This effect is largest in the -1 duplex, where the average % per nucleotide is 68 up to the -3 bulge where the % per nucleotide is 86. Thus, the -3 bulge samples conformations that lead to solvent interactions similar to that of nucleotides in a duplex. Such behavior may lead to the thermodynamic changes associated with FAAF modification being similar in the -3 bulge and the full duplex.

Hydration energies were computed as the average interaction energies over the trajectories between the central 5 base pairs of the DNA duplexes and explicit water molecules using fshift and vswitch truncation between $10 \text{ \AA}$ and $12 \text{ \AA}$ for the electrostatic and Lennard-Jones terms. The results are compared with the SASA data above (Supporting Information Table S7). Consistent with the SASA analysis, the hydration energies are less favorable as the bulge becomes larger. However, when the results are compared on a % per nucleotide basis relative to the full unmodified B conformer the values are systematically larger than with the analogous SASA values. For example the average over the four duplexes for each deletion duplex are 124, 106 and 94 % for the -3 , -2 and -1 deletion duplex, respectively, versus values of 86, 77 and 69 % for the SASA. These results suggest that the change in solvation associated with the bulges is having a larger impact on the change in enthalpy than on the expected compensatory change in entropy. This may represent a general effect contributing to the overall destabilization of the unmodified bulges relative to the full duplex. However, neither the SASA or hydration energies correspond with the impact of FAAF modification on the full duplex and bulges. This is where differential stacking plays a larger role leading to the more favorable enthalpy upon FAAF modification of the -2 and -1 deletion duplexes.

Intermediate conformations between S and B

We performed clustering analysis on the trajectories. The largest cluster in each trajectory is the classic B or S conformer, while the conformations present in the smaller clusters could be intermediate conformations. We also computed the solvent accessible surface area (SASA) of the fluorine atom during the simulations (Supporting Information Figure S10). In the B (*anti*) conformers, the atom is fully exposed to the solvent during the majority of the simulation time, having maximal SASA. In the S (*syn*) conformers, the fluorine is stacked between the base pairs and therefore its SASA is smaller. However, it is clear that a range of intermediate conformations exist, including highly solvent exposed conformations. Cluster analysis identified additional clusters in the case of the -1 , -2 and -3 deletion simulations. From the second largest cluster for each system the representative conformation was obtained and is shown in Figure S10. These structures correspond to clusters sampled roughly 10% of the simulation time. Thus, they may represent lower probability structures that may contribute the ^{19}F NMR spectra in Figure 8.

DISCUSSION

The solution structures of arylamine-modified DNA duplexes, including AF- and AAF, have been studied in various sequence settings.^{14, 17, 19, 53} In the fully paired duplexes, these

lesions exist largely in a mixture of the major groove anti-glycosidic B and the intercalated syn-glycosidic S conformers.^{14, 15, 17, 19} Additionally, the bulky *N*-acetylated AAF adopts a W conformer, in which the carcinogen is placed in the narrow minor groove.¹⁶ The relative population balance of these conformers is governed primarily by the nature of the base sequences surrounding the lesion. The resulting conformational heterogeneity has been shown to be associated with different mutational and repair outcomes. Milhe and coworkers conducted a ¹H NMR study on AAF modified 11/10-mer -1 deletion duplexes in the CG*C context.⁵¹ They found that approximately 70% of the aminofluorene moiety was located externally (e.g., B-type) and conformational heterogeneity prohibited further analysis of the remaining conformers. This is clearly in contrast to the present result, which showed 73% of S conformation for the 16/15-mer 'GC' -1 deletion duplex in the same CG*C context. However, a AAF on the 12/10-mer -2 deletion duplex exhibited S-type conformation,⁵⁴ in good agreement with our result in the present study. Similar NMR studies on -1 and -2 deletion duplexes modified by the *N*-deacetylated dG-AAF,^{36, 55} showed exclusively S-type conformations. All these cases, greater thermal stabilities ($\Delta T_m = 11\text{--}15^\circ$) have been observed upon adduct formation.^{17, 56}

We hypothesized that the dG-AAF-modified deletion duplexes exist as a combination of the external-binding B, the inserted S, and some intermediate conformations between them (Fig. 9). Our working hypothesis was that dG-AAF induces various sequence-dependent slippages during replication, and that the conformational and thermodynamic stabilities of the resulting SMIs are key factors for determining the different types of frameshift mutations.

Our dynamic ¹⁹F-NMR results (Fig. 8) showed that dG-FAAF existed in varying ratios of B- and S-types, along with a few intermediate conformers. A greater population of the S-type conformation was observed for the 'GC' (73%) and 'AT' (72%) -1 duplexes as compared to the -2 (55%) and -3 (37%) deletion duplexes. This result was probably due to the favorable stacking of the intercalated aminofluorene moiety in the bulges. The increased positive CD at 275 nm was caused by an increase of a-a stacking interactions. A blue CD shift indicated distortion and bending of the lesion-induced DNA (Fig. 7), which was also observed in the MD simulations for the deletion duplexes (Supporting Information Table S2).

Fully paired arylamine-modified duplexes are known to destabilize thermally and thermodynamically relative to the unmodified controls.¹⁷ The dG-FAAF produced a mixture of complex S/B/W-conformers (Fig. 9). The bulky *N*-acetyl group was apparently responsible for producing up to 40% W-conformer in the fully paired 16-mer duplex (Fig. 8a).^{16, 29} The destabilizing effect of the dG-FAAF lesion was related to the difference in the conformational populations. Thus, the 60% S-conformeric full duplex disrupted the Watson-Crick base pairs at the lesion site, resulting in thermal ($\Delta T_m = -8.0^\circ\text{C}$) and enthalpic destabilizations ($\Delta\Delta H = 13.9\text{ kcal/mol}$). Our previous conformational and thermodynamic studies of the *NatI* 16-mer full duplex also revealed 61% S conformations, which resulted in thermal ($\Delta T_m = -8.3^\circ\text{C}$) and enthalpic destabilizations ($\Delta\Delta H = 24.7\text{ kcal/mol}$). It is worth noting that full duplexes containing benzo[*a*]pyrene (10*R*-(+)-*cis-anti*-B[*a*]P-*N*²-dG) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (C8-dG-PhIP) adopt thermally destabilized base-displaced intercalated conformations ($\Delta T_m = -12$ and -10°C , respectively).⁵⁷ The same B[*a*]p lesion in the -1 deletion duplex, however, dramatically enhances the thermal stability ($\Delta T_m = +19^\circ\text{C}$) of this duplex, and is completely resistant to nucleotide excision repair. The C8-dG-PhIP adduct in the same setting results in less thermal stabilization ($\Delta T_m = +3^\circ\text{C}$) and shows moderate repair efficiency.⁵⁷

In contrast to the fully-paired duplex, the S-conformeric bulged structures resulted in thermal and thermodynamic stabilizations upon adduct formation. For example, a dramatic

thermal stabilization has been observed for several bulge duplexes modified by the bulky arylamine and benzo[a]pyrene carcinogens.^{17, 56} Modeling studies suggested that the *syn*-glycosidic conformeric SMI was more stable than the *anti*-external conformation.⁵⁸ This *syn*-SMI was stabilized by favorable interactions between the carcinogen and flanking base pairs inside the bulge pocket. The highly stacked nature of the 'GC' (73%) and 'AT' (72%) -1 deletion duplexes resulted in enthalpic stabilization ($\Delta\Delta H = -15.2$ kcal/mol and $\Delta\Delta H = -21.9$ kcal/mol, respectively). As expected, the 55% S conformeric -2 deletion duplex displayed intermediate thermal ($\Delta T_m = -10.8^\circ$) and enthalpic stabilizations ($\Delta\Delta H = -9.6$ kcal/mol). A similar, but truncated (12/10mer), *NarI* -2 duplex displayed thermal ($\Delta T_m = 11.7^\circ$) and enthalpic stabilizations ($\Delta\Delta H = -5.3$ kcal/mol). This is surprising in light of the calculated empirical thermodynamic parameters from SantaLucia *et al.*, which showed significant loss of interactions between Watson-Crick dinucleotide steps.⁵⁹ As expected, the highly flexible B-conformeric (52%) -3 deletion duplex exhibited enthalpic destabilization ($\Delta\Delta H = 3.5$ kcal/mol).

Our MD simulation results suggest a model in which the changes in stability associated with the presence of bulges and the impact of the FAAF modification on the duplexes were dominated by changes in stacking, that are related to experimental enthalpic changes (Figure 10), and changes in configurational entropy as evidenced by the change in RMS fluctuations, that are related to entropic changes (Table 1 and S5). It is difficult to relate experimental thermodynamic data to simulated properties; the enthalpic and entropic terms are both influenced by numerous factors, including, most notably, solvation. For the enthalpic contributions, the SASA and hydration energies were calculated from the simulations (Tables S6 and S7). The hydration effects contributed to the enthalpic destabilization associated with the presence of the bulges relative to the full duplex, although they did not appear to contribute significantly to the impact of the adducts on duplex stability. However, the behavior of the -3 bulge solvation differences, based on SASA, mimicked to an extent those occurring in the fully-paired duplex. This phenomenon may contribute to the similar thermodynamic contributions to stability that were observed in the -3 species as compared to the -1 and -2 bulges.

Schorr and Carell²⁵ conducted primer extension studies with the human bypass pol η on several dG-AAF-modified *NarI* sequences. The addition of the correct dC opposite the lesion during replication triggered the dC primer end to slip and resulted in the formation of various SMIs, depending on the base sequence context around the lesion. The dC located 3' to the AAF lesion was found to be critical for the misalignment/shifting process. Strong -2 deletion was observed in the *NarI* 5'---CGGCG[AAF]CC---3' or 5'---CGGCG[AAF]CT---3' sequences. When all of the dC bases around the lesion were replaced by dG (i.e., 5'---CGGGG[AAF]GG---3'), only the -1 slippage was obtained with a high yield. A rare -3 deletion occurred when the sequence was modified appropriately to exclude 3 bases. In other words, the efficiency of the frameshifting process was in order of $-1 > -2 > -3$ deletions.

In full agreement with the pol η primer extension assay data, our ¹⁹F NMR results showed that the AAF-modified opposite -1, -2, and -3 deletion duplexes had 72%, 55%, and 37% intercalated, S-type bulge conformations. The highly stacked S-conformeric -1 and -2 deletion duplexes displayed tight compactness of the lesion in the bulge, and consequently displayed greater thermal and thermodynamic stabilities. Previous modeling studies found that the AAF-modified SMI was quite stable relative to its normally extended counterpart in the presence of the *syn*-carcinogen conformation.⁵⁸ In the -3 deletion duplex, however, the lesion was not stacked well, resulting in greater conformational flexibility (Fig. 9). These results indicate that the optimum space required to incorporate the AAF lesion is the -1 followed by the -2 bulge. The question is then how structures of such perturbed deletion

duplexes in the translesion synthesis could possibly be accommodated in different polymerases, which contain the usual palm, finger, thumb and little finger domains with the active site in the palm domain and DNA bound between thumb and little finger. To accommodate the structurally altered SMIs the thumb and little finger domains would have to move away from each other to create the space required for the deletion duplexes. However, Biertümpfel *et al.* showed a very rigid molecular splint of human pol η to hold the duplex region in the B-form,⁶⁰ making it inefficient at extending primers after the bulky cisplatin lesions, which necessitates a second translesion DNA polymerase to complete bypass *in vivo*.⁶¹ By contrast, Alt and coworkers have shown a set of structural features (e.g., an open apo-enzyme conformation) that enable pol η to replicate across the strongly distorting cisplatin DNA lesions.⁶² Similarly, the prokaryotic DNA pol II has a nice pocket in the DNA duplex region that can accommodate a -2 deletion.⁶³ Indeed, Becherel and Fuchs have shown that -2 deletions in the *NarI* hot spot sequence require DNA Pol II.⁶⁴ It should be noted that Xu and coworkers have obtained some interesting structural insights on how a Y-family DNA polymerase may accommodate a slippage structure, however in this case it involved the spacious prokaryotic polymerase Dpo4.^{65, 66}

In conclusion, a frameshift is triggered by the insertion of a correct dC opposite the bulky AAF lesion at the replication fork, which can be accommodated by either the *anti*- or *syn*-glycosidic conformation. The structural and conformational instabilities of such an accommodation facilitate the development of various misalignments, which depend on the nature of the base sequences that are in close proximity to the modified dG. The data in the present study provide the general structures of bulky adduct-induced SMIs and the impact of their thermal and thermodynamic stabilities on the propensity to elicit different frameshift mutations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

dG-AAF-adduct	<i>N</i> -(2'-deoxyguanosin-8-yl)-2-acetylaminofluorene
dG-FAAF-adduct	<i>N</i> -(2'-deoxyguanosin-8-yl)-7-fluoro-2-acetylaminofluorene
DSC	differential scanning calorimetry
ICD	induced circular dichroism
MALDI-TOF	matrix assisted laser desorption/ionization-time-of-flight
SASA	solvent accessible surface area
SMI	slipped mutagenic intermediate

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Figure 1.

(a) Mechanisms for slippage induced -2 deletion on *NarI* sequence; (b) Chemical structures of dG-AAF and dG-FAAF; (c) Model duplexes used in the present study.

Figure 2.

(a) HPLC chromatogram of a reaction mixture derived from treatment of the 16-mer sequence (5'-CTCTCG₁ATG₂CCATCAC-3') with an activated FAAF (*N*-acetoxy-*N*-2-(acetylamino)-7-fluorofluorene). Mono- (G₁, G₂) and di-FAAF adducts are eluted in the 15-25 min range. (b) On-line photodiode array UV spectra of unmodified, mono-, and di-FAAF adducts.

Figure 3. MALDI-TOF mass spectra of peak 3 in Figure 2a; (A) after 5'→3' exonuclease digestion (BSP enzyme) (B) after 3'→5' exonuclease digestion (SVP enzyme) in Reflectron mode at various time intervals. Insets provide theoretical MW of the corresponding fragments.

Figure 4.

UV-melting curves of FAAF modified duplexes (red) and their respective unmodified controls (blue) all at 10 μ M in 0.2 M NaCl, 10 mM sodium phosphate, and 0.2 mM EDTA at pH 7.

Figure 5. Differential scanning calorimetry (DSC) curves in 20 mM phosphate buffer containing 0.1 M NaCl at pH 7: FAAF-modified duplexes in red and unmodified controls in blue.

Figure 6. Comparative thermodynamic histograms of FAAF-modified duplexes with respect to their unmodified controls: $\Delta\Delta H = \Delta H$ (modified duplex) – ΔH (control duplex), $\Delta\Delta S = \Delta S$ (modified duplex) – ΔS (control duplex) and $\Delta\Delta G = \Delta G$ (modified duplex) – ΔG (control duplex).

Figure 7. CD Spectral overlays of (a) fully-paired (b) –3 deletion (c) –2 deletion (d) ‘AT’ –1 deletion, and (e) ‘GC’ –1 deletion duplexes at 30 °C. FAAF modified duplexes (solid lines) and unmodified control duplexes (dotted lines).

Figure 8.

Dynamic ^{19}F NMR spectra of FAAF-modified duplexes in various sequence settings: (a) fully-paired, (b) -3 deletion, (c) -2 deletion, (d) 'AT' -1 deletion, and (e) 'GC' -1 deletion duplexes. Dynamic ^{19}F NMR spectra of FAAF-modified duplexes in various sequence settings: (a) fully-paired, (b) -3 deletion, (c) -2 deletion, (d) 'AT' -1 deletion, and (e) 'GC' -1 deletion duplexes. S, B, and W denote S-, B-, and W-conformers. ss indicates denatured FAAF-modified single strands.

Figure 9. Selected molecular dynamics images of the fully-paired and various deletion duplexes. The presented conformations were selected from the largest cluster based on RMSD clustering analysis performed in CHARMM.

Figure 10.

Calculated relative stacking energy (ΔE , Table S3) and experimental enthalpy (ΔH_{cal}) (Table 1) histograms of FAAF-modified duplexes. Lower bound is the stacking energy corresponding to unmodified S conformation as reference state in Equation (1), upper bound is corresponding to unmodified B conformation as reference state in Equation (1). See Table S3 for details.

Table 1

Thermal and thermodynamic parameters derived from Differential Scanning Calorimetry (DSC)

	$-\Delta H$ kcal/mol		$-\Delta S$ eu		$-\Delta G_{37^\circ C}$ kcal/mol		T_m °C		$\Delta\Delta H^c$ kcal/mol	$\Delta\Delta S^d$ eu	$\Delta\Delta G_{37^\circ C}^e$ kcal/mol	ΔT_m^f °C
	Unmod	Mod	Unmod	Mod	Unmod	Mod	Unmod	Mod				
Full duplex	116	102	321	287	16.3	12.8	66.8	58.8	13.9	33.8	3.5	-8.0
-3deletion	77	73	215	206	10.3	9.5	54.9	51.8	3.5	8.4	0.8	-3.1
-2 deletion	88	98	254	274	9.5	13.0	49.4	60.2	-9.6	-20.0	-3.5	10.8
'AT' -I deletion	100	122	285	336	11.3	17.6	54.0	69.2	-21.9	-50.8	-6.3	15.2
'GC' -I deletion	90	106	250	281	12.8	18.4	61.7	77.7	-15.2	-31.0	-5.6	16.0

^aThe average standard deviations for $-\Delta G$, $-\Delta H$, and T_m are ± 0.4 , ± 3.0 , and ± 0.4 , respectively. All template-primer solutions (0.1 mM) were prepared by dissolving desalted samples in a pH 7.0 buffer solution consisting of 20 mM sodium phosphate and 0.1 M NaCl.

^b T_m values is the temperature at half the peak area.

^c $\Delta\Delta H = \Delta H$ (modified duplex) - ΔH (control duplex).

^d $\Delta\Delta S = \Delta S$ (modified duplex) - ΔS (control duplex).

^e $\Delta\Delta G = \Delta G$ (modified duplex) - ΔG (control duplex).

^f $\Delta T_m = T_m$ (modified duplex) - T_m (control duplex).