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DNA base sequence effects on bulky lesion-induced conformational heterogeneity during DNA replication

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ABSTRACT

4-Aminobiphenyl (ABP) and its structure analog 2-aminofluorene (AF) are well-known carcinogens. In the present work, an unusual sequence effect in the 5′-CTTCTG1G2CTCTCATT-3′ DNA duplex is reported for ABP- and AF-modified G. Specifically, the ABP modification at G1 resulted in a mixture of 67% major groove B-type (B) and 33% stacked (S) conformers, while at the ABP modification at G2 exclusively resulted in the B-conformer. The AF modification at G1 and G2 lead to 25%:75% and 83%:17% B:S population ratios, respectively. These differences in preferred conformation are due to an interplay between stabilizing (hydrogen bonding and stacking that is enhanced by lesion planarity) and destabilizing (solvent exposure) forces at the lesion site. Furthermore, while the B-conformer is a thermodynamic stabilizer and the S-conformer is a destabilizer in duplex settings, the situation is reversed at the single strands/double strands (ss/ds) junction. Specifically, the twisted biphenyl is a better stacker at the ss/ds junction than the coplanar AF. Therefore, the ABP modification leads to a stronger strand binding affinity of the ss/ds junction than the AF modification. Overall, the current work provides conformational insights into the role of sequence and lesion effects in modulating DNA replication.

INTRODUCTION

ABP: N-(2′-deoxyguanosin-8-yl)-4-aminobiphenyl; AF: N-(2′-deoxyguanosin-8-yl)-2-aminofluorene; AAF: N-(2′-deoxyguanosin-8-yl)-2-acetylamino fluorene

DNA is damaged by many agents in our environment. For example, aryamines are carcinogens derived from cigarette smoke, incomplete combustion of diesel exhaust, and overcooked meat, among others (1,2). These aromatic chemicals do not directly interact with DNA; however, they are metabolized in vivo by cytochrome P450 and subsequently activated by cellular enzymes, such as N-acetyltransferase or sulfotransferase to form DNA adducts (3,4). For example, 4-aminobiphenyl (ABP) is a well-known human bladder carcinogen and a potent urinary-bladder carcinogen in experimental animals and is activated by cellular N-acetyltransferase to produce C8-substituted dG-ABP as a major DNA lesion (5,6). The mutations of the p53 tumor suppressor gene in bladder carcinomas of patients who are occupationally exposed to ABP match with the ABP-mutational hotspots of the p53 gene in cells treated to ABP (7). The structurally similar liver carcinogens 2-aminofluorene (AF) and N-2-acetylamino fluorene (AAF) produce similar C8-dG adducts (Figure 1A). AF could be error-free by correctly base pairing with an incoming dC (8), whereas AAF blocks the replication process and requires recruitment of lesion bypass polymerases for translesion synthesis (TLS) (9). In Escherichia coli, the TLS of AF produces point and frameshift mutations, whereas bypass of the AAF lesion is frequently accompanied by a frameshift mutation (10).

These bulky DNA adducts have been extensively studied and shown to adopt at least three major conformations in the duplex setting: major groove B-type (B), base-displaced stacked (S), or minor groove wedge (W) (Figure 1B) (11–13). In the S-conformation, the carcinogen stacks between the bases and disrupts base pairing in the double helix, whereas the B-type conformer places the carcinogen in the major groove of the DNA helix that maintains the traditional Watson–Crick base pairings. The W-conformer was originally found in dA mismatch duplexes, and the hydrophobic lesion is placed in the narrow minor groove (14–17). The S/B/W-population ratios have been shown to be influenced by neighboring sequence contexts for the ABP-dG, AAF-dG and AF-dG lesions, which consequently have different repair and mutational efficiencies (11,12,15,17–20). Similarly, bulky DNA lesions at the single-strand (ss)/double-strand (ds) replication fork can

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Figure 1. (A) Structures of ABP \([N-(2'-deoxyguanosin-8-yl)]-4-aminobiphenyl\], AF \([N-(2'-deoxyguanosin-8-yl)]-2-aminofluorene\], and AAF \([N-(2'-deoxyguanosin-8-yl)]-2-acetylaminofluorene\] and their fluoro models, FABP \([N-(2'-deoxyguanosin-8-yl)]-4'-fluoro-4-aminobiphenyl\], FAF \([N-(2'-deoxyguanosin-8-yl)]-7-fluoro-2-aminofluorene\], and FAAF \([N-(2'-deoxyguanosin-8-yl)]-7-fluoro-2-acetylaminofluorene\], as well as the definitions of key dihedral angles (\(\theta\), \(\varphi\) and \(\xi\)). (B) Major groove views of arylamine-conformational motifs: B-, S- and W-conformers. (color code: arylamine- red CPK, modified dG- blue, complementary dC- orange).

potentially exist in multiple conformations, which may affect mutational outcomes.

The mutagenicity of DNA adducts is related to how the polymerase processes each of the damaged duplex conformations. When a replicative polymerase encounters a bulky DNA lesion, DNA synthesis pauses or stops to allow the recruitment of bypass polymerases. This in turn affects whether the synthesis is accurate or error prone. For example, AF- and AAF-modified duplexes favor the S-conformer due to the 3′-flanking purine base (15,17,19,21–23). AAF at G3 in the NarI sequence (\(5'-\text{G1G2CG3CN}-3'\)) induces a greater frequency of −2 frame shift mutations, with the G3-mutational frequency regulated by the nature of the N base, \(N=\text{C or T}\) (23–25). Miller et al. reported a significant reduction in nucleotide incorporation rates by Klenow fragment (Kf) exo− at positions neighboring and downstream of the lesion site (26). We investigated the conformational effect of AF on nucleotide insertion efficiencies catalyzed by Kf exo− on TG*A and CG*A systems. The results showed that AF in the CG*A duplex sequence adopts a greater population of S-conformer than the TG*A sequence. The potentially S-conformatic CG*A at the ss/ds junction thermodynamically favors A over C opposite the lesion site (22).

Additionally, we previously reported an unusual 3′-flanking T effect on a duplex sequence (\(5'-\text{CCATCG*CNACC}-3'\), \(N=\text{T or A}\), i.e., the FABP modification at G* exhibited a 40%/60% B:S in the G*CT context whereas 100% B in the G*CA sequence context. Though not as dramatic, a similar trend was observed for the AF and AAF lesions (11).

In the present paper, another unusual fluorine-labeled FABP (FABP)-induced sequence effect on a 16mer duplex sequence (\(5'-\text{CTTCTG1G2TCCTCATTC}-3'\)) was investigated (Figure 1). FABP-modification at G1 exhibited a 67%/33% ratio of B:S conformations, whereas the B-conformation was adopted exclusively at the G2 modification. We conducted a series of conformational [NMR, induced circular dichroism (ICD), molecular dynamics (MD) simulations], thermodynamic [UV-melting, differential scanning calorimetry (DSC)], and binding [surface plasmon resonance (SPR), isothermal titration calorimetry (ITC)] experiments for two 16mer DNA templates [(\(5'-\text{CTTCTG1G2TCCTCATTC}-3'\)) and (\(5'-\text{CTTCTG1G2TCCTCATTC}-3'\)); G1*/G2* = FABP or FAF (fluorine-labeled AF)]. The data showed that the B-conformer is a thermodynamic stabilizer and the S-conformer is a destabilizer in full duplex settings.
However, the opposite was true for the ss/ds sequence contexts. Lesion stacking increased the binding affinity of the complementary strands in the order of FABP > FAF and TG*GT > TGG*G. The twisted nonplanar FABP was a better stacker than the planar FAF in ss/ds systems, which contrasts the situation in the duplexes. Overall, the present study provides valuable insights into the important distinction between ss/ds and full ds sequence contexts, and the flanking base sequences and lesion stacking in modulating bulky lesion-induced conformational heterogeneity.

MATERIALS AND METHODS

Caution: 4-Aminobiphenyl derivatives are known human bladder carcinogens. Aminofluorene analogues are animal carcinogens; therefore, must be handled with caution.

Oligodeoxynucleotides (oligo, 10 μmol scale) in desalted form were obtained from Integrated DNA Technologies (Iowa) and were purified by using a reverse phase HPLC. The HPLC system comprised a Hitachi EZChrom Elite HPLC with a L-2450 diode array detector and a Phenomenex Luna C18 column (150 × 10 mm, 5.0 μm) (Phenomenex, Torrance, CA, USA). All HPLC solvents were purchased from Fisher Inc. (Pittsburgh, PA, USA).

Preparation of lesion-modified oligodeoxynucleotides

FABP-modified 16mer templates (5′-CTTCTG1G2TCCTCATTCTC3′) were prepared according to the published procedures (25). For each lesion, we obtained two mono-adducts [d(5′-CTTCTG1G2TCCTCATTCTC3′)] and [d(5′-CTTCTG1G2TCCTCATTCTC3′)] (G* = FABP) and one di-adduct [d(5′-CTTCTG1G2TCCTCATTCTC3′)]. Briefly, 5–10 mg of N-acetoxy-N-trifluoroacetyl-7-fluoro-4-aminobiphenyl dissolved in 500 μl absolute ethanol was added to 10 mL sodium citrate buffer (10 mM, pH 6.0) containing ~200 μls of unmodified 16mer oligo and incubated in a 37°C water bath shaker for 1 h. Unreacted carcinogens were removed by extraction with anhydrous diethyl ether. An acetoni-trile gradient of 14–25% in triethylammonium acetate (TEAA) buffer (100 mM, pH 7.0) for 30 min was used to separate the two mono-adduct strands from di-adduct and unreacted 16mer oligos. Supplementary Figure S1A shows the HPLC profile and photodiode array UV spectra of FABP-modified 16mers. The combined collection of the two mono-adduct oligos was lyophilized and stored in -80°C. FAF-modified 16mer oligos were prepared similarly according to published procedures (27) and the resulting HPLC profile is shown in Supplementary Figure S2A.

MALDI-TOF mass spectrometry characterization of mono-adducts

The FABP- and FAF-modified mono-adducted 16mer oligos above were digested by 3′–5′ exonuclease enzyme digestion and characterized by MALDI-TOF mass spectrometry following published procedures (28). Briefly, 1 μL (0.1 unit) of snake venom phosphodiesterase (SVP) was added to a solution of 1 μL of modified oligo (~100–200 pmol), 6 μl of ammonium citrate (100 mM, pH 9.4), and 6 μl of water for 3′–5′ digestion. In each case, 1 μl of the reaction mixture was withdrawn at regular time intervals and was quenched by mixing with 1 μl of matrix (3-hydroxypicolinic acid and diammonium hydrogen citrate in a 1:1 ratio). All MALDI spectra were obtained using Shimadzu Axima Performance MALDI-TOF mass spectrometer equipped with a 70 Hz nitrogen laser in positive ion reflectron mode (15).

UV melting

UV thermal melting experiments were performed on a Cary100 Bio UV-Vis spectrophotometer (Santa Clara, CA) equipped with a 6 × 6 multi-cell chamber (1.0 cm path
length) and connected with a built-in Peltier temperature controller. Samples were prepared in the buffer (containing 0.2 M NaCl, 10 mM sodium phosphate and 0.2 mM EDTA at pH 7.0) in the range of 0.8–5.6 μM. Samples were scanned forward/reverse five times during melting experiments. Thermodynamic parameters were calculated using the program MELTWIN version 3.5 according to previous reports (25).

Differential scanning calorimetry (DSC)

DSC calorimetry measurements were performed on a Nano-DSC from TA Instrument (Lindon, UT, USA) according to previous reports (29). Solutions at 0.1 mM containing unmodified or modified templates with various primers were dissolved in a pH 7.0 buffer (0.1 M NaCl, 20 mM sodium phosphate), and the solutions were incubated at 95°C for 5 min and cooled at room temperature for 3 h to ensure duplex formation. After 15 min degassing, samples were loaded into the instrument cells. A buffer scan was used as control and then subtracted from the sample scan and normalized for heating rate. All samples were scanned against the control buffer from 15 to 85°C at a rate of 1°C/min by measuring five repetitions. Raw data were collected as microwatts versus temperature. ΔH is an integration of ΔCpex over temperature T. Tm was the temperature at half the peak area. Thermodynamic parameters (ΔG and ΔS) were determined according to previously described procedures (30,31).

Circular dichroism (CD) spectra

Circular dichroism (CD) experiments were conducted on a Jasco J-810 spectropolarimeter (Easton, MD) equipped with a Peltier temperature controller. Typically, a modified template (20 μM) was annealed with an equimolar amount of a primer in 400 μl buffer (containing 0.2 M NaCl, 10 mM sodium phosphate, and 0.2 mM EDTA, pH 7.0). The samples were hybridized by heating to 95°C for 5 min and slowly cooled to room temperature for 3 h to ensure duplex formation. Spectra were acquired every 0.2 nm from 200 to 400 nm at a rate of 50 nm/min for five repetitions and smoothed with 25-point adaptive algorithms provided by Jasco (17).

Dynamic 19F-NMR

Approximately 0.1 mM of full and ss/ds duplex samples (Figure 2) were dissolved in 250 μl of NMR buffer (pH 7.0) containing 10% D2O/90% H2O, 100 mM NaCl, 10 mM sodium phosphate, and 100 μM EDTA. Solutions were filtered into a Shigemi tube through a 0.22 μm membrane filter. All 1H- and 19F-NMR experiments were performed on a Varian NMR spectrometer with a HFC probe operating at 500.0 and 476.5 MHz, respectively (13,18,32). Imino proton spectra was obtained at different temperatures (5–50°C) using phase-sensitive jump-return sequences and referenced to DSS. 19F-NMR spectra were recorded with increment of 5–10°C (from 5 to 60°C) in the 1H-decoupled mode and referenced to C6F6 in C6D6 at –164.9 ppm. Computer line shape simulations were processed as reported previously using MestReNova 10.0.2 (MestReNova Research, Spain) (13,33).

Surface plasmon resonance (SPR)

SPR binding measurements were conducted using a Biacore T200 instrument (GE Healthcare, Marlborough, MA, USA). Two isomeric 16mer biotinylated DNA template strands (5′-biotin-CTTCTG1*G2TCCTCATTC-3′ and 5′-biotin-CTTCTG1*G2TCCTCATTC-3′; G* = FABP or FAF) were each modified with FABP or FAF (21,27,29,34). HPLC gradient conditions for biotinylated FABP-modified oligos were 16%–25% gradient on acetonitrile for 55 min in TEAA buffer (100 mM, pH 7.0) with a flow rate of 3.0 ml/min (Supplementary Figure S3A). The biotin-FABP-modified sequences were characterized by MALDI-TOF-MS using 3′-5′exonuclease enzyme digestion (Supplementary Figure S3B and C).

Carboxymethylated CM 5 chip was activated by amine coupling kit according to published procedures (21,29,34). Streptavidin (SA, 50 μg/ml dissolved in sodium acetate buffer, pH 4.5) was coated on flow cells 2, 3 and 4 after the injection of EDC/NHS mixture for 7 min. Flow cell 1 was left as blank. Ethanolamine (1 M) was injected over the surface to block unreacted esters. The unbound SA was removed by five pulses of 50 mM NaOH and three pulses of running buffer. The surface was stabilized for 20–30 min in running buffer before DNA coating. Flow cells 2, 3 and 4 were coated around 300 RU of unmodified DNA sequence (TG1G2T), FABP modified (TG1*G2T) and (TG1G2*T) DNA sequences by the manual control mode, respectively. Samples of 9mer to 16mer primer sequence were prepared separately in HBS-P+ buffer (10 mM HEPES, 150 mM NaCl, 0.05% surfactant P20 at pH 7.4) and injected over the chip surface with 100 s contact time and 360 s dissociation time at a flow rate of 15 μl/min at 25°C.

Due to distinct molecule weights, the primers (9mer to 16mer) were used at different concentrations to gain steady-state associations: 300 nM (9mer), 500 nM (10mer), 500 nM (11mer), and 750 nM (12, 13 and 16mer). After the system reached a steady state, excess primers were washed off by the HBS-P+ buffer, and the system was regenerated by adding NaOH (50 nM, 30 s). We were unable to measure the usual KD (kD/kd) kinetics of duplex formation for each elongation. Instead, we used dissociation rates (kd), which are concentration-independent, to estimate binding strengths for complementary strands.

Dissociation constants (kd) were analyzed in kD-alone fitting mode by Scrubber software (version 2.0, Myszka and collaborators, BioLogic Software). The goodness of the fit was determined from the residual standard deviation.

Isothermal titration calorimetry (ITC)

VP-ITC microcalorimeter from MicroCal Inc. (Northampton, MA, USA) was performed to measure full duplex formation of unmodified and modified templates (2.5 μM) with their complementary (25 μM) sequences prepared in 10 mM phosphate buffer (pH 7.0), containing 0.2 M NaCl and 0.2 mM EDTA. The samples were degassed thoroughly
under vacuum using ThermoVac (MicroCal, Northampton, MA, USA) for 20 min. ITC experiments consisted of 25 consecutive injections and each injection involves 10 μl of complementary sequence with injection time 10 and 240 s intervals between each injection with 307 rpm stirring at 25°C. The integrated and normalized areas of raw data were plotted as ΔH of injectant against the molar ratio. The titration data were fitted using a single-site binding model provided in ORIGIN7.0 software supplied by the manufacturer. ΔH, ΔS, K and N (reaction stoichiometry) of 16mer full duplex formation were obtained (35,36).

**RESULTS**

**Experimental DNA sequences**

Chemical structures of ABP and AF, and their fluorine analogues, FABP and FAF, are shown in Figure 1A. In the present study, 16mer (5′-CTTCTG1G2TCCTCATTC-3′) was applied for the n – 1 and n TLS duplexes. NMR results indicate that the fully paired FABP modified 16mer duplexes exhibited 67%/33% of B:S and 100%B population ratios for G1 and G2 modifications.

As shown in Figure 2, the two 16mer template strands (TG1*G2T or TG1G2*T: G* = FABP or FAF) were each annealed with various lengths of complementary strands (9 to 16mer) to generate two discrete TLS systems (n – 2, n – 1, n + 1, n + 2, n + 5 for TG1*G2T and n – 1, n, n + 1, n + 2, n + 3, n + 6 for TG1G2*T, respectively; n is the lesion site). In the present study, we used these TLS model systems consistently for 19F-NMR, CD, DSC, UV melting, and SPR experiments. Our strategy is to investigate the effects of the B-/S-conformational heterogeneity on thermodynamic and duplex binding parameters. The results will help us understand the uniqueness of flanking base sequence in the TGGT context in promoting lesion-induced conformational heterogeneity.

**MALDI-TOF characterization**

FABP- and FAF-modified 16mer oligos were characterized by exonuclease enzyme digestion followed by MALDI-TOF mass spectrometry, according to published procedures (15,28,29). Supplementary Figure S1B shows the MALDI-TOF MS spectra of the 3′–5′ SVP exonuclease digestions of peaks 1 and 2 from FABP-HPLC profile (Supplementary Figure S1A) at different time points (0, 0.5, 2 and 5 min). Briefly, the m/z of 4,960.6 at 0 min represents the control mass to charge ratio of the modified template without SVP. Within 0.5 min of 3′–5′ exonuclease digestion (Supplementary Figure S1B), the lower masses showed up corresponding to the 15mer to 6mer fragments. The fragment at m/z = 1944.6 (theoretical 1945.3), which was the most detectable mass between 2 and 5 min digestion, was assigned to a 6mer containing the FABP lesion. These results confirmed that peak 1 from the HPLC profile was the FABP-modified-G1 sequence (5′-CTTCTG1[FABP] TCCTCATTC-3′, TG1*G2T). Peak 2 from HPLC profile was characterized similarly. Supplementary Figure S1C shows the control molecular ion m/z of 4960.2 at 0 min. The m/z 2273.6 ion peak in the 0.5–5 min of digestion corresponded to the 7mer fragment containing FABP-modified guanine. These results confirm peak 2 as the FABP-modified-G2 sequence (5′-CTTCTG1G2[FABP] TCCTCATTC-3′, TG1G2*T).

The FAF-modified 16mer templates have been characterized similarly. Supplementary Figure S2B–C shows the MALDI-TOF MS spectra of the 3′–5′ SVP exonuclease digestion of the FAF-modified 16mer oligo

**Molecular modeling**

Nucleoside model. The rotational barrier about the nucleoside dihedral angles (θ, φ and ξ; Figure 1A) for both the anti and syn orientations of FAF and FABP-dG was determined by constraining each dihedral angle in 10° increments from 0 to 360° and performing B3LYP/6–31G(d) optimizations. The interdependence of the conformational energy about θ and φ was investigated, while the conformation about ξ for FAPB-dG was only investigated using the lowest energy conformation about θ and φ. All quantum mechanical calculations were performed using Gaussian 09 (revision D.01) (37).

ds DNA model. The initial undamaged helix (5′-CTTCTG1G2TCCTCATTC-3′) was generated using the NAB module of AMBER 14 (38). The helix was modified to position the FABP-dG or FAF-dG adduct at G1 or G2 such that the lesion was paired opposite dC. At each position, three orientations of the lesion site were considered to generate the B, S and W adducted DNA conformations. The resulting duplexes were neutralized with Na+ ions and solvated in a TIP3P octahedral water box with sides 8.0 Å from the solute. All natural nucleotides were modeled with the AMBER ff14SB force field (39,40). FABP-dG and FAF-dG parameters were assigned according to the GAFF (41) and AMBER ff14SB force fields using ANTECHAMBER 1.4 (Supplementary Table S1) (42), and partial charges were plotted as ΔΔH, ΔΔS, ΔΔK and ΔΔN (reaction stoichiometry) of 16mer

Ewald algorithm was used for long-range electrostatic interactions, with a nonbonded cutoff of 8.0 Å. All simulations and analysis were completed with Amber 14 using the pmemd and cpptraj modules, respectively (38).
Dynamic 19F-NMR spectroscopy

Dynamic 19F-NMR experiments were performed to measure the extent of conformational heterogeneity during primer elongation. Figure 3 and Supplementary Figure S4 respectively show the 19F-NMR spectra of the FABP- and FAF-modified duplex series as a function of temperature (5–60°C). The same sequence systems were used for CD, DSC UV-melting, and SPR experiments for comparison. Briefly, FABP-G1*-16mer duplex showed a mixture of S- (33%) and B- (67%) conformations at 25°C (highlighted in Figure 3A). By contrast, the corresponding FABP-G1*-16mer duplex exclusively revealed B-conformer (100%) with no sign of conformational heterogeneity.

**FABP-TG1*G2*T.** In Figure 3A, no indication of conformational heterogeneity at n = 2 (9mer) and n = 1 (10mer) was found, whereas the 3′-end of the primer did not reach the lesion site. A slight heterogeneity was observed at n = 11 (11mer). Interestingly, however, it disappeared at n + 1. At n = 2 (16/13mer), we observed complex 19F signals indicating the presence of multiple conformations. These 19F signals were coalesced into a sharp single peak around –116.5 ppm upon around and >50°C, this result indicates duplex melting. The conformational pattern was steadied at fully paired 16mer duplex (n = 5), which showed a ratio of 33% S and 67% B populations at 25°C (highlighted in Figure 3A).

The 19F-signals were assigned according to the general protocols based on relative chemical shifts, chemical exchange patterns, and H/D isotope effects (27). We have investigated a number of 19F-NMR spectra of arylamine-modified duplexes in various sequence settings and found that the fluorine of the S-type conformer resonates always upfield relative to that of the external binding B-type conformer and they are in slow exchange and eventually coalesce into a denatured ss. In addition, the 19F tag in the B-conformation is fully exposed to the solvent in the major groove area, whereas that of the S-conformer is shielded from van der Waals interactions with neighboring base pairs. In addition, the fully exposed 19F tag of the major groove binding B-conformer always exhibits significant deuterium isotope effect (0.18 ppm) going from 10% to 100% D2O (Supplementary Figure S5A–B). This result is in contrast to the buried 19F of the stacked conformer, which shows no isotope effect (0.08 ppm) (Supplementary Figure S5A).

**FAF-TG1*G2*T and FAF-TG1G2*T.** Supplementary Figure S4 shows the dynamic 19F-NMR spectra for the FAF-modified series. The results indicate both FAF-TG1*G2*T and FAF-TG1G2*T duplexes adopted more S-conformation than the FABP series. FAF-TG1*G2*T had approximately 42% more S than the FABP counterpart TG1*G2*T (75% S and 25% B) in the full duplex, whereas the S increased from 0% to 17% in the FAF/TG1G2*T. These results reflected the planar nature of FAF relative to FABP preferring to intercalate/stack. Moreover, the B-conformer exhibits a significant deuterium isotope effect (<0.18 ppm), going from 10% to 100% D2O, whereas the stacked conformer shows little isotope effect (0.04 ppm) (Supplementary Figure S6A and B).

**Induced circular dichroism (ICD)**

Supplementary Figure S7 shows CD overlays of unmodified and FABP-modified 16mer templates with increasing primer length. The S curve consisted of +α at 270 nm, and the B-form DNA duplex consisted of −α at 250 nm. Given the conformational heterogeneity, FABP-modified TLS duplexes exhibited lesion ICD between 290 and 360 nm (19, 25). This result is in contrast to the unmodified control series, which showed no such ICD. The ICD gradually increases along with the primer elongation from 9mer to 16mer. In the fully paired 16mer duplex, the CD data agrees with the above 19F-NMR data, which showed a B/S-conformeric TG1*G2*T and exclusively B-conmeric TG1G2*T on fully paired 16mer duplexes. The predominant B-conformer of TG1G2*T highly affects the bending of DNA helix around the adduct site. G1 and G2 FABP-modified duplexes displayed slight blue shifts (~0.3 nm) relative to the unmodified controls, indicating adduct-induced DNA bending (28). Similar results were obtained for FAF-modified series (Supplementary Figure S8).

**UV melting experiments**

The UV-melting profiles of all the ss/ds duplex models were measured at the 0.8–4.0 μM range for FABP and FAF samples. The UV monophasic and sigmoidal melting curves between InCt and Tm (R2 > 0.9) confirmed that the duplexes adopted a typical helix-coil transitions (13). Supplementary Figure S9 shows the plots of melting (Tm) for FABP and
Dynamic $^{19}$F-NMR spectra (5–60°C) of FABP-modified duplexes with primer elongation. (A) FABP-modified G$_1$ (TG*GT) showing a 67% B- and 33% S-conformational mixture in full duplex. (B) FABP-modified G$_2$ (TGG*T) exhibiting 100% B-conformer in full duplex. For all the duplexes, the $^{19}$F signals around 60°C coalesced into a sharp single peak around -116.5 ppm indicating a denatured duplex. * indicates unassigned minor conformers.

FAF lesions at the TG$_1$*G$_2$T and TG$_1$G$_2$*T systems (red dash lines and blue dash lines, respectively) relative to the unmodified controls (dark gray solid lines) as a function of primer elongation for TLS systems. Thermal and thermodynamic parameters calculated from UV melting curves are summarized in Supplementary Tables S2 and S3. For all the modified and unmodified duplexes, $T_m$ increases with the elongation of primers. However, modified systems showed stalls around the lesion sites.

The lesion effect in the TG$_1$*G$_2$T system was minimal at one and two bases before the lesion sites ($\Delta$1$T_m$ of 0.35 and 0.67°C for 9mer and 10mer, respectively). This finding is in contrast to that of the TG$_1$G$_2$*T system, which showed minimal lesion effect at those sites (9mer $\Delta$1$T_m$ of 1.45°C). However, the effect was significant when the primer elongation was opposite the lesion [11mer and 10mer for TG$_1$*G$_2$T ($\Delta$1$T_m$ = −2.54°C) and TG$_1$G$_2$*T ($\Delta$1$T_m$ = −5.21°C), respectively]. In full duplex (16mer), TG$_1$*G$_2$T showed greater negative thermodynamic effect to TG$_1$G$_2$*T ($\Delta$1$T_m$ of −7.68°C to −6.29°C), which can be due to the S-conformer (see $^{19}$F-NMR results above).

**DSC**

DSC experiments were performed for the FABP-modified TLS models (TLS/TG$_1$*G$_2$T versus TLS/TG$_1$G$_2$*T). Figure 4 shows DSC thermogram overlays of excess heat capacity $C_p^{\text{ex}}$ versus temperature. $T_m$ is the maximum point of the curves, and transition enthalpy ($\Delta H$) is the area under the curves. DSC results are summarized in Table 1 (29,44).

**Control TLS.** Figure 4A shows the DSC thermograms for the control TLS system. For the natural DNA, $T_m$ and $\Delta H$ values increased progressively with increasing primer length from 9mer ($T_m$ = 42.9°C, $\Delta H$ = −71.4 kcal/mol) to 13mer ($T_m$ = 59.8°C, $\Delta H$ = −87.2 kcal/mol) and increased significantly at full duplex (16mer, $T_m$ = 63.1°C, $\Delta H$ = −98.5 kcal/mol).

**TG$_1$/FABP/G$_2$T Duplex.** In FABP-G$_1$ profiles (Figure 4B), the 9mer primer, which is two bases ($n-2$) before the lesion ($n$), showed a minimal lesion effect, i.e. $T_m$ = 43.3°C compared with the $T_m$ of the control duplex of 42.9°C. However, 10mer ($n-1$) to 16mer ($n+5$) primers were stalled, i.e. their $T_m$ values were clustered between 51.0 and 53.0°C. The 16mer full duplex showed a significant increase in enthalpy $\Delta H$, indicating a full duplex formation.

**TG$_1$G$_2$/FABP/T Duplex.** DSC patterns for the TG$_1$G$_2$*T system (Figure 4C) were similar to those of the TG$_1$*G$_2$T. Curves for 10mer ($n$) to 13mer ($n+3$) were clustered between $T_m$ 45.0°C and 53.0°C, which is lower than that of the TG$_1$*G$_2$T counterpart. The increase in $\Delta H$ was not as incremental as that of $T_m$. In general, the exclusively B-conformeric 16mer TG$_1$G$_2$*T full duplex showed greater $T_m$ and $\Delta H$ than the G$_1$ counterpart that contains a mixture of B- and S-conformers (see $^{19}$F-NMR above).
Figure 4. DSC heat capacity $\Delta C_p$ versus temperature (15–85°C) for primer elongation (9mer to 16mer) of the (A) control, (B) FABP-modified G1 (TG*GT) and (C) FABP-modified G2 (TGG*GT). (D) Full duplex overlays of unmodified and FABP G1/G2-modified sequences. FABP-G1 and FABP-G2 show smaller $T_m$ and $\Delta H$ than the control/unmodified in full duplex exhibiting that S-conformer (FABP-G1) might produce thermodynamic destabilization in full duplex.

Table 1. Thermal and thermodynamic parameters derived from DSC

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<th>Sequence</th>
<th>$\Delta H^a$ (kcal/mol)</th>
<th>$\Delta S^a$ (eu)</th>
<th>$\Delta G_{37°C}^a$ (kcal/mol)</th>
<th>$T_m^a$ ($^\circ$C)</th>
<th>$\Delta \Delta H^b$ (kcal/mol)</th>
<th>$\Delta \Delta G_{37°C}^c$ (kcal/mol)</th>
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$^a$ $T_m$ value is the maximum point of the DSC curve.
$^b$ $\Delta \Delta G = \Delta G$ (modified duplex) – $\Delta G$ (control duplex).
$^c$ $\Delta \Delta H = \Delta H$ (modified duplex) – $\Delta H$ (control duplex).
$^d$ $\Delta T_m = T_m$ (modified duplex) – $T_m$ (control duplex).
finding suggests that the B-conformation can be a thermodynamic destabilizer during primer elongation and yet the S-conformer might produce thermodynamic destabilization in the full duplex. TG1*G2T and TG1G2*T duplexes in TLS settings show consistently smaller $T_m$ and $\Delta H$ compared with the unmodified controls (Figure 4D).

**ITC for duplex formation**

ITC provides the nature of duplex formation through a sigmoidal curve and the respective thermodynamic parameters ($\Delta H$, $\Delta G$, $\Delta S$) through plots of heat capacity ($C_p$) versus molar ratios. The results indicate that the lesion destabilizes the duplex formation.

Figure 5 shows ITC thermograms of duplex formation for FABP- and FAB-modified TGGT sequences at 25°C. In all cases, unmodified controls (black solid line) showed well-defined sigmoidal curves with sharp changes in heat capacity at starting and ending points. The results indicate the typical cooperative 1:1 binding process (e.g. molar ratio of $N = 1$) expected for formation of Watson–Crick-based DNA duplexes. By contrast, the ITC curves for modified full duplexes were not well defined in terms of stoichiometry and overall shape, probably due to heterogeneity. Supplementary Table S4 summarizes the ITC-derived thermodynamic parameters $\Delta H$, $\Delta S$, $K$ and $N$ for unmodified and modified sequences, for example, the binding constant order is TGGT ($49.9 \times 10^7$ M$^{-1}$) > TG1*G2T ($18.7 \times 10^7$ M$^{-1}$) > TG1G2*T ($5.9 \times 10^7$ M$^{-1}$) in FABP case. In general, the $\Delta H$ and the binding constant of the modified sequences are consistently lower than that of the controls, and the FABP shows more variances in shapes than the FAF counterpart.

**SPR on DNA duplex formation**

SPR experiments were conducted to probe the effect of conformational heterogeneity on duplex formation and dissociation. The results were obtained in a buffer-only environment without a polymerase. With different concentrations used for each complementary strand, we measured dissociation rates ($k_d$) to evaluate their binding strengths (see Materials and Methods).

Supplementary Figure S10 shows the entirety of SPR results, and Table 2 lists the respective dissociation rate constants. Supplementary Figure S11 shows the overlay of normalized SPR sensograms for FABP and FAB lesions on the unmodified (green) and the TG1*G2T (blue)/TG1G2*T (purple) sequences with increasing length of complementary strands. As expected, in each complementary sequence, SPR data show gradual reduction in $k_d$ values with elongation in unmodified and modified sequences.

Rapid duplex formation took place for all duplexes; however, different dissociation rates were observed for modified duplexes. In general, slower dissociation indicates greater duplex binding with thermodynamic stability. This appears to be generally true with post-lesion duplexes (16/11 to 16/16mer). The 16/9mer duplexes, which represents the pre lesion $n = 2$ and $n = 1$ site for G1 and G2, respectively, showed generally faster dissociation (Figure 6A, Table 2): TG1G2[FABP]T ($7.1 \times 10^{-2}$ s$^{-1}$) < TG1[FABP]G2T ($12.0 \times 10^{-2}$ s$^{-1}$) < unmodified TG1G2T ($16.5 \times 10^{-2}$ s$^{-1}$). As for the 16/10mer (Figure 6B), however, dissociation was dramatically slower for TG1[FABP]G2T ($3.43$ s$^{-1}$) than the unmodified ($0.515$ s$^{-1}$) and TG1G2[FABP]T ($2.370$ s$^{-1}$). These results are consistent with duplex stability in the order of TG1*G2T > unmodified > TG1G2*T in FABP case. The 16/9mer is one base before ($n = 1$) the B/S-conformer TG1G2*T; and 16/10mer is one base ($n = 1$) before the TG1*G2T duplex. The results suggest that the modified $n = 1$ duplex may form a stable S-conformer at the ss/ds junction, resulting in slow dissociation. Similar SPR patterns were observed for the FAB lesion (Supplementary Figure S11B); however, general dissociation rates were slightly faster compared to the FABP lesion. This finding indicates that FABP is a better stacker in the ss/ds duplex setting.

**Molecular modeling**

Molecular modeling was used to understand the conformation of the lesion, and the strength of discrete interactions between the lesion and the surrounding environment. Through using various model sizes, we can better understand the effects of the different compositions of the FABP and FAB bulky moieties on the conformational preferences in the DNA environment.

**Nucleoside model.** To investigate the difference in the preferred orientation of the bulky moiety with respect to the nucleobase for FAF-dG and FAB-dG, the conformational energy was mapped as a function of the dihedral angles that dictate rotation about the C–N–C linker (i.e. $\theta$ and $\varphi$, Figure 1) for both the anti and syn orientations of the lesions (Supplementary Figure S12). Due to the symmetry within the FABP bulky moiety, the calculations revealed one minima for anti-FABP that positions the first ring of the bulky moiety planar to the adducted G, while the bulky moiety is twisted by 70° with respect to the adduct G in the two additional local minima. The rotational barrier between these minimum energy orientations is low (< 2.5 kcal/mol). Based on the lowest energy orientation of anti and syn-FABP-dG, the two rings in the FABP moiety were determined to preferentially adopt a twisted orientation (i.e., $\xi$, the angle between the two rings is ~40°, Supplementary Figure S14). Nevertheless, the rotational barrier within the bulky moiety is small, being only ~2.5 kcal/mol.

FABP displays the same conformational preference about the $\theta$ and $\varphi$ dihedral angles as FABP-dG (Supplementary Figure S12), although the rotational barrier with respect to $\theta$ is slightly (~1 kcal/mol) higher for syn-FABP-dG than syn-FABP-dG. Since the FABP bulky moiety is not symmetrical like FABP, the two minima with respect to the $\varphi$ dihedral angle represent distinct conformations (Supplementary Figure S13). Specifically, $\varphi = 0°$ at the methylene linker towards N7, while $\varphi = 180°$ at the N9 linker. The rotational barrier about $\varphi$ (up to ~7 kcal/mol) is substantially higher than about $\theta$ (up to ~3.5 kcal/mol). Due to the nature of the methylene linker between the two rings, the bulky moiety of FAB-dG is planar in contrast to the twisted bulky moiety of FABP-dG. This
Figure 5. ITC binding thermograms of duplex formation for (A) FABP- and (B) FAF-modified 16mer TGGT full duplexes at 25°C.

Table 2. Dissociation off-rate constants ($k_d, \times 10^{-2}, s^{-1}$) from SPR

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<th>Unmodified</th>
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<th>FAF-G₂</th>
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<td>0.041</td>
<td>0.021</td>
<td>0.082</td>
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</table>

*Errors are within the limits of 5%.

Figure 6. Normalized SPR sensorgrams of FABP-modified 16mer TG₁G₂T sequences with 9mer and 10mer primers, and progression of lesion conformational flexibility (hatched lines) and conformational rigidity (solid lines). (A) 9mer and (B) 10mer. Slower dissociation shows better stability of the primer-template duplexes. Unmodified (green); G₁-modified (blue); G₂-modified (purple). HBS-P+ buffer is the dissociation solution. The green unmodified trace cannot be labeled as either $n$ or $n-1$ because there’s no lesion present.
difference will likely influence the adducted DNA conformational preference.

**DNA model.** To gain more information about the preferred conformation of FABP- and FAF-dG at the G1 and G2 positions in the 16mer duplexes, MD simulations were performed with the lesion opposite dC for duplexes adopting the B- or S-conformation. All simulations showed a stable rmsd throughout the 100 ns simulation (Supplementary Table S5). Regardless of the lesion, duplex conformation or modification position, minimal distortions were observed to the overall helical structure and the flanking base pairs formed canonical Watson-Crick hydrogen bonds throughout the simulation (Supplementary Table S6 and Supplementary Figures S15 and S16). Furthermore, a consistent conformational preference occurred at the lesion site in terms of the glycosidic orientation or the orientation of the bulky moiety with respect to the adducted dG (as defined by the χ, θ and ϕ dihedral angles) regardless of the lesion or position (Supplementary Table S5).

In the B-conformer of the FABP-modified 16mer duplexes, the adduct maintains Watson-Crick hydrogen bonding with the pairing dC (Supplementary Table S7), and the bulky moiety is positioned in major groove parallel to the adducted G regardless of the lesion position (θ and ϕ ≈ 0°, Supplementary Table S5 and Figure 7A). There is free rotation about the linker between the two bulky moiety rings (i.e. standard deviation in ξ of up to ~11°, Supplementary Table S5). The preference about θ, ϕ and ξ for FABP-dG in the helix is consistent with the inherent conformational preference predicted for the nucleoside model. Regardless of whether the lesion is at G1 or G2, the hydrogen-bonding and stacking interactions between the adducted and flanking base pairs are similar (average interaction strength of approx. ~98 kcal/mol, Supplementary Table S8), and the duplexes are destabilized by the solvation of the non-polar bulky moiety by a similar extent (SASA ≈ 155 Å², Supplementary Table S5). Due to the similar stabilization (lesion site interactions) and destabilization (solvent accessibility of the bulky moiety) effects, the MMPBSA binding energies of the modified 16mer duplexes are within 2 kcal/mol, with the G1 modification being less stable (Figure 7A). Overall, there is little difference in the structure and stability of the B-conformeric duplex modified at G1 and G2.

In the FABP-modified S-conformeric duplex, the pairing dC is positioned in the major groove and forms a hydrogen bond with the DNA backbone of the 3' flanking base for up to 33% of the simulation (Supplementary Table S7). In the S-conformer, the bulky moiety intercalates into the helix; however, the bulky moiety position is sequence dependent (Figure 7B). Specifically, at G1, the adducted G is positioned in the major groove to afford stacking between both rings of the bulky moiety and the flanking bases (approx. ~24 kcal/mol, Supplementary Table S8). Furthermore, the lesion position is stabilized by a hydrogen bond between the linker N–H and O4' of the adducted nucleotide (for 63% of the simulation, Supplementary Table S7). Consequently, this positioning of the lesion reduces the SASA of the hydrophobic bulky moiety compared to the B-conformeric duplex (by ~120 Å², Supplementary Table S5). To maintain the lesion site stacking interactions, the two rings in the FABP bulky moiety maintain a planar relative orientation (ξ = 174.4 ± 22.7°, Supplementary Table S5). In contrast, at G2, the adducted dG stacks with the flanking bases and the bulky moiety is positioned towards the minor groove, which results in minimal intrahelical stacking with the terminal ring of the bulky moiety. This permits free rotation about the linker between the two bulky moiety rings (ξ = 53.2 ± 138.8°, Supplementary Table S5). Although significant stacking interactions occur between the lesion and the flanking bases (approx. ~24 kcal/mol, Supplementary Table S8), the bulky moiety position at G2 does not shield FABP from solvent to the same extent as at G1 (difference of ~30 Å²). Furthermore, at G2, due to the position of the lesion, a unique hydrogen bond occurs between the linker N–H of FABP-dG and N2 of G1 (24% occupancy). Since this interaction is not as persistent and has a longer distance (by ~0.4 Å) than the hydrogen bond with the linker at G1 (Supplementary Table S7), this may contribute to the destabilization of the helix for G2* relative to G1*. Due to the decreased stabilization (reduced hydrogen bonding) and greater destabilization (increased solvent exposure of FABP) at G2, the binding energy of the G2 modified 16mer is 6.5 kcal/mol less than that of the G1 modified duplex (Figure 7B).

For FAF-dG adducted DNA in the B-conformation, the lesion maintains Watson-Crick hydrogen bonding with the pairing dC (Supplementary Table S7), and the bulky moiety is positioned in the major groove and parallel to the adducted dG (θ and ϕ ≈ 0°, Supplementary Table S5 and Figure 7C). At G2, FAF-dG is stabilized by stacking and hydrogen-bonding interactions to a similar extent as seen for FABP-dG (approx. ~98 kcal/mol, Supplementary Table S8). However, at G1, weaker stacking interactions occur between the adducted and flanking base pairs (by ~3.5 kcal/mol). Furthermore, although the SASA of the FAF-moiety is inherently larger than that of FABP (Supplementary Table S5), the SASA of FAF-dG at G1 is greater than the SASA of the lesion at G2 (by ~10 Å²). Therefore, due to decreased stabilization of the lesion site through hydrogen bonding and stacking interactions, and increased destabilization due to greater solvent exposure of the hydrophobic moiety, the helix binding energy of the FAF-modified duplex in the B-conformer with the lesion at G1 is 6.8 kcal/mol less than that at G2 (Figure 7C).

When the FAF-modified 16mer adopts the S-conformer, the pairing dC is positioned in the major groove and forms a hydrogen bond with the backbone of the 3' flanking base (up to 71% of the simulation, Supplementary Table S7 and Figure 7D), regardless of the modification position. In the S-conformer, both G1 and G2 the bulky moiety adopts a planar geometry and FAF-dG is intercalated into the helix such that both the adducted G and bulky moiety stack with the flanking bases (θ and ϕ ≈ 0°, Supplementary Table S5 and Figure 7D). As a result, the SASA of the hydrophobic moiety (~69 Å², Supplementary Table S5) and the magnitude of the lesion site interactions (approx. ~68 kcal/mol, Supplementary Table S8) are independent of the damage site. As a result, the binding energies for the duplexes are similar, with the G1 modification being 2 kcal/mol less stable than the G2 counterpart. It is also noteworthy that the stacking interactions at the lesion site for the FAF-modified
duplex in the S-conformer are up to 6 kcal/mol stronger than those in the FABP-modified helices and the flanking bases in the S-conformer (Supplementary Table S5).

**DISCUSSION**

**Unusual flanking sequence effects on the TG1G2T sequence**

In this study, we present a striking flanking sequence effect on a 16mer fully paired DNA duplex 5′-CTTCTG1G2TCCTCATTC-3′, in which lesion modification at G1 (TG1*G2T sequence) and G2 (TG1G2*T sequence) yielded a dramatic difference in conformational heterogeneity. Dynamic 19F-NMR results indicate that the FABP-modification at G1 and G2 resulted in 67:33% of B:S and 100% B population ratios, respectively. Modification with the planar FAF in the same sequence contexts exhibited greater S-conformer population: 25%:75% B:S and 83:17% B:S population ratios for the G1 and G2 positions, respectively. This represents a significant increase in the S-conformation from 33% to 75% and from 0% to 17% going from FABP to FAF in the TG1*G2Ta n dTG1G2*T sequence context, respectively. The greater S-conformer population of FAF over FABP at duplex DNA can be attributed to their aromatic coplanarity, i.e. the single-carbon atom methylene bridging between two aromatic rings in FAF restrains the twisting of the biphenyl. Indeed, nucleoside modeling showed that the FABP-dG lesion is twisted by ∼40°, while FAF-dG is inherently planar.

This sequence effect is analogous to our recent reporting on a 11mer DNA duplex sequence 5′-CCATCG*NACC-3′ (N = T or A), in which the FABP modification exhibited a 40:60% ratio of B:S and 100% B population ratios, and the FAF modification resulted in 10:90% and 34:66% B:S in the G*CT and G*CA sequence context, respectively (27). These 3′-next flanking sequence effects influence primer kinetics, DNA replication, and repair (11). Lesion stacking was primarily responsible for the greater population of the stacked S-conformer compared with that of the G*CA counterpart; the S-conformation decreased the binding affinity of the complementary strands. These adduct-induced conformational heterogeneities are caused by 3′-next flanking sequence effects, which is different from the TG1G2T context considered in the present study, which corresponds to flanking sequence effects.

Our calorimetric results showed a greater thermodynamic stability for the B-conformer G2 over the S/B-conformeric G1 in the full duplex, which indicates that the B-conformer is a thermodynamic stabilizer in fully paired duplex DNA. As shown in ITC curves (Figure 5), lesion modification has reduced enthalpic energies (∼13% and ∼17% average for FABP- and FAF-G1/G2, respectively) during duplex formation, as expected. The lesion destabilizes the duplex formation. Correlating the ITC data with the conformation heterogeneity is difficult; hence, the difference in ring planarity had a major effect on the shape of binding isotherms (C), i.e., FABP has greater sequence differences compared with FAF.

Modeling data indicates that the conformational preference of FABP-dG is due to a destabilization of the S-conformer at G2 compare to the complex at G1. Specifically, the S-conformer of FABP-dG modification is positioned with the adducted G in the major groove at G1, while the bulky moiety was positioned in the minor groove at G2. This differential positioning of FABP-dG in the S-conformer lead to reduced stabilization (hydrogen bonding) and greater destabilization (greater SASA of the hydrophobic bulky moiety) at G2 and provides a structural explanation for the reduced S-conformer at G2. Conversely, the conformational preference of FAF-dG is due to a destabilization of the B-conformer at G1 relative to G2. As seen for the S-conformer of FABP-dG modified DNA, the destabilization of the B-conformer with modification at G1 is due to the reduced stabilization (stacking) and greater destabilization (greater SASA of the hydrophobic bulky moiety) at G2, which provides a structural explanation for the observed reduced B-conformer contribution at G1. Nevertheless, re-
gardless of the position or modification, MD predicts the adducted DNA duplex to be more stable in the B-conformer at the G₁ and G₂ positions than the S-conformer, which explains the thermodynamic data. Finally, comparison of the lesion site interactions for FABP-dG and FAF-dG modified DNA reveals that planar FAF affords stronger stacking between the lesion and flanking base pairs (by up to 6 kcal/mol) than twisted FABP. This rationalizes the greater occurrence of the S-conformation for FAF-dG adducted DNA than FABP-dG adducted DNA.

Thermodynamic effect on lesion heterogeneity during primer elongation

We studied six discrete DNA replication model duplexes prepared by annealing the 16mer TG₁*G₂T and TG₁G₂*T (G* = FABP or FAF; Figure 2) modified template with appropriate primer strands. These experiments were performed in buffer solutions without a polymerase. The main objective of these experiments is to study the effect of the lesion-induced S/B-heterogeneity on their thermal and thermodynamic stabilities during the primer elongation that mimics a TLS.

The thermodynamic results show a systemic increase in \( T_m \) and \( \Delta H \) with primer elongation from 9mer to 16mer in unmodified control duplexes. However, the FABP modification generally as a lower \( T_m \) and \( \Delta H \), as well as changed their calorimetric curve patterns for the TG₁*G₂T and TG₁G₂*T systems. As mentioned above, for full duplexes, the exclusively B-conformeric G₂* was more thermally stable than G₁*. However, this finding is not observed during the primer elongation from 10mer to 13mer, in which average \( T_m \) of TG₁G₂*T are lower those of TG₁*G₂T. These results imply that the B-conformer is a thermodynamic stabilizer in the duplex setting but is a destabilizer during primer elongation. In other words, the S-conformer may be a thermodynamic stabilizer in the TLS process (as presented below).

The impact of lesion heterogeneity on ss/ds duplex formation

We performed SPR experiments to investigate the effect of lesion heterogeneity on strand binding. SPR is an excellent real-time technique that allows monitoring the formation and dissociation of DNA duplexes, DNA-RNA hybridization, and triplex formation. Supplementary Figure S11 shows an overlay of SPR sensograms as a function of various complementary strands (9mer to 16mer). Sensograms from SPR for each primer elongation and dissociation rate \( k_{\text{off}} \) (Table 2) was used to evaluate lesion and sequence effects. This finding is due to the different concentrations of each primer elongation; thus, conducting a global binding analysis is difficult for \( K_D \) affinity values (see Results). Instead, we utilize \( k_{\text{off}} \) dissociation rates (Table 2) to examine lesion effects.

The binding strength for each primer elongation was increased with increasing extent of Watson–Crick hydrogen bonding. As a result, dissociation rates are steadily reduced with primer elongation. Not surprisingly, the binding strength for the unmodified templates was generally greater than that of the modified ones throughout the DNA replication process. Initially, the effect was relatively minimal at prelesion sites (\( n - 1 \) and \( n - 2 \) for G₁* and G₂*, respectively) (Figure 6A, 9mer). According to NMR results, the FABP-G₁* lesion with the 9mer (16/9mer) is likely to exist in a B/S-mixture that can be readily accommodated in the flexible n – 2 environment. As for G₂*, this represents \( n - 1 \), in which the mostly B-conformer can exist at the 16/9mer ss/ds junction. The 16/9mer sequences appeared to be slightly more stabilized than the controls. However, the effect of lesion modification on dissociation was clearly seen around the lesion sites. The 16/10mer corresponds to \( n - 1 \) and \( n \) for G₁* and G₂*, respectively. The effect on dissociation was in the order of \( n - 1 \) G₁* > control > \( n \) G₂*. The most significant lesion effect of G₁* at \( n - 1 \) is probably due to the presence of S-conformer population that can stabilize the ss/ds replication fork (see above). This situation is illustrated in Figure 6B. For G₂*, the primer has advanced up to the lesion site (\( n \)); hence, such lesion-induced stacking cannot attain the same stability. As a result, \( n \) G₂* (pink) was less stable than that of the unmodified control (green). The opposite was true for the 16/11mer, in which the binding stability at G₁* was reduced at the lesion site presumably due to the clash with the new primer. This is not the case for the B-conformeric G₂* at \( n + 1 \), which did not change much other than the usual gain of binding energy through elongation. The trend remained unchanged for the rest of the elongation (\( n + 2 \), \( n + 3 \)) and for the full (\( n + 16 \)) duplex.

As shown in Supplementary Figure S11, the nature of the lesion (FABP versus FAF) had noticeable effect on dissociation rates, except that FAF dissociates generally faster than FABP (Table 2). A similar situation was noted in our previous G*CA/G*CT examples (27). Although no polymerase was involved, these SPR results are in line with the NMR/thermodynamic/modeling results on the corresponding duplex given that the greater S-conformation of the G₁* sequence posed a major hindrance toward the binding affinity. In other words, the lesion effect was propagated primarily upstream of the template strand and gradually diminished.

We conducted systematic \(^{19}\)F-NMR, CD, DSC, SPR and molecular modeling studies for the elongation of the TG₁*G₂T and TG₁G₂*T sequences modified by FABP and FAF. Model systems mimic a hypothetical TLS process involving the bulky structurally-related arylamine lesions. DNA adducts in the TG₁*G₂T duplex exhibited greater populations of the S-conformer compared with that in the TG₁G₂*T duplex. Bulky lesions exhibited unique sequence-dependent conformational heterogeneities at various elongation positions, including the replication fork that differentially contributes to template-primer bindings and thermodynamics. We found that the B-conformer is a major thermodynamic stabilizer in the duplex settings and the S-conformer is a destabilizer. The opposite was observed during the primer elongation process involving ss/ds duplex sequences, i.e. the S-conformation promotes lesion stacking with nascent base pairs at the ss/ds replication fork. The results are supported by SPR sensograms, which revealed that the bulky adduct in the stacked conformation increases the binding affinity of the complementary strands in the order of FABP > FAF and TG₁*G₂T > TG₁G₂*T. Interestingly, unlike the coplanar FABF, the twisted FABP is a better stacker at the ss/ds junction than in fully paired...
duplexes. The extent of primer dissociation rates across the lesion were influenced by the flanking DNA sequences and the stacking ability of the lesion. Future studies include examination of the effects of the conformational heterogeneity in DNA replication and repair.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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