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Structure of the 1,4-Bis(2'-deoxyadenosin-N⁶-yl)-2*R*,3*R*-butanediol Crosslink Arising from Alkylation of the Human *N-ras* Codon 61 by Butadiene Diepoxide[†]

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Abstract

The solution structure of the 1,4-bis(2'-deoxyadenosin-N⁶-yl)-2*R*,3*R*-butanediol crosslink arising from N⁶-dA alkylation of nearest neighbor adenines by butadiene diepoxide (BDO₂) was determined in the oligodeoxynucleotide 5' - d(CGGACXYGAAG)•d(CTTCTCGTCCG)-3'. This oligodeoxynucleotide contained codon 61 (underlined) of the human *N-ras* protooncogene. The crosslink was accommodated in the major groove of duplex DNA. At the 5'-side of the crosslink there was a break in Watson-Crick base pairing at base pair X⁶•T¹⁷, whereas at the 3'-side of the crosslink at base pair Y⁷•T¹⁶, base pairing was intact. Molecular dynamics calculations carried out using a simulated annealing protocol, and restrained by a combination of 338 interproton distance restraints obtained from ¹H NOESY data and 151 torsion angle restraints obtained from ¹H and ³¹P COSY data, yielded ensembles of structures with good convergence. Helicoidal analysis indicated an increase in base pair opening at base pair X⁶•T¹⁷, accompanied by a shift in the phosphodiester backbone torsion angle β P5'-O5'-C5'-C4' at nucleotide X⁶. The rMD calculations predicted that the DNA helix was not significantly bent by the presence of the four-carbon crosslink. This was corroborated by gel mobility assays of multimers containing non-hydroxylated four carbon N⁶,N⁶-dA crosslinks, which did not predict DNA bending. The rMD calculations suggested the presence of hydrogen bonding between the hydroxyl group located on the β-carbon of the fourcarbon crosslink, and T¹⁷ O⁴, which perhaps stabilized the base pair opening at X⁶•T¹⁷ and protected the T¹⁷ imino proton from solvent exchange. The opening of base pair X⁶•T¹⁷ altered base stacking patterns at the crosslink site and induced slight unwinding of the DNA duplex. The structural data are interpreted in terms of biochemical data suggesting that this crosslink is bypassed by a variety of DNA polymerases, yet is significantly mutagenic [Kanuri, M., Nechev, L. V., Tamura, P. J., Harris, C. M., Harris, T. M., and Lloyd, R. S. (2002) *Chem. Res. Toxicol.* 15, 1572–1580].

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²The definitions of the prochiral protons at C_α and C_δ of the N⁶,N⁶-dA crosslink moiety are based upon the Cahn, Ingold, and Prelog nomenclature. The proton H_α is defined as the pro-*R* proton at C_α; H_{α'} is defined as the pro-*S* proton at C_α. Likewise, H_δ is the pro-*R* proton at C_δ; H_{δ'} is the pro-*S* proton at C_δ.

1,3-Butadiene (CAS RN 106-99-0) (BD)¹ is used in the manufacture of styrene-butadiene rubber (SBR) (1,2); several billion lbs/yr are produced in the United States. It is a combustion product from automobile emissions (3) and cigarette smoke (4). BD is genotoxic and is a carcinogen in rodents, particularly in mice (5–7) and also in rats (8). BD was classified by the United States Environmental Protection Agency as "carcinogenic to humans by inhalation" (9). The International Agency for Cancer Research (IARC) lists BD as a "probable human carcinogen" (Group 2A) (10–12). Chronic human exposure in the SBR industry may induce genotoxic effects (13–15) and is correlated with increased risk for leukemia (1,16–24).

BD is epoxidized primarily by cytochrome P450 2E1, but also by cytochrome P450 2A6, to form 1,2-epoxy-3-butenes (BDO) (Scheme 1) (25,26). These may be further oxidized by cytochrome P450 2E1 or 3A4 to form 1,2:3,4-diepoxibutenes (BDO₂) (25,27–31). Hydrolysis of BDO mediated by epoxide hydrolase forms 1,2-dihydroxy-3-butenes (29,32,33), which are metabolized by cytochrome P450 to hydroxymethylvinylketone (HMK) (34). Either BDO₂ or the 1,2-dihydroxy-3-butenes undergo cytochrome P450-mediated oxidation to form 1,2-dihydroxy-3,4-epoxybutanes (BDE) (29,32,35). Thus, proximate electrophiles arising from BD metabolism include BDO, BDO₂, and BDE, and potentially, HMK (36).

The diepoxide BDO₂ is highly genotoxic (2,10,37), probably due to its potential to form DNA-DNA (38–41) and DNA-protein crosslinks, the latter of which have been observed in mice (42,43). Mice have been shown to possess greater sensitivity to butadiene exposure than rats, and this is attributed to their efficient oxidation of BD to BDO₂ (44,45), presumably facilitating DNA crosslinking. Butadiene genotoxicity was further enhanced in knockout mice lacking a functional microsomal epoxide hydrolase gene (46). Polymorphisms in the human epoxide hydrolase gene may also contribute to differences in BD genotoxicity within the human population (47,48).

The predominant DNA crosslink induced by BDO₂ involves interstrand bis-alkylation at N7-dG in 5'-GNC-3' sequences (40). This crosslink was isolated from DNA and characterized by mass spectrometry (41). Recently, additional guanine-adenine DNA interstrand crosslinks induced by BDO₂, including an N7-dG-N⁶-dA crosslink, were characterized by mass spectrometry (49). The recovery of an interstrand crosslink involving N⁶-dA was perhaps unexpected since, with respect to alkylation by butadiene epoxide, the N⁶-dA exocyclic amine is less reactive than the N1 imine. The prevailing hypothesis posits that N⁶ alkylation products of butadiene epoxides result from initial alkylation at the N1 position, followed by Dimroth rearrangement (50,51). The BDT N1-dA adduct has been identified in humans exposed occupationally to BD (52). The N⁶-dA BDT adduct was identified in Chinese Hamster ovary cells (53).

The bis-alkylation of tandem deoxyadenosines to yield intrastrand N⁶,N⁶-dA *R,R* crosslinks is anticipated to be a rare occurrence. This adduct has not yet been reported from mass spectrometry analysis of DNA exposed to BDO₂ (49,54). However, its synthetic accessibility (55) facilitated biochemical structure-activity studies. As compared to N⁶-dA monoadducts arising from BDO or BDE (56,57), the N⁶,N⁶-dA *R,R* crosslink was significantly more mutagenic. It was examined as to site-specific mutagenesis, both in *Escherichia coli* and in

¹Abbreviations: BD, butadiene; BDE, 3,4-epoxy-1,2-butanediol; BDO, butadiene monoepoxide (1,2-epoxy-3-butene); BDO₂, butadiene diepoxide (1,2:3,4-diepoxibutane); BDT, butadiene triol; CPK, Corey-Pauling-Koltun space-filling models; DQF-COSY, double-quantum filtered correlation spectroscopy; HMBC, heteronuclear multiple bond correlation spectroscopy; HMK, hydroxymethylvinylketone; MALDI-TOF, matrix assisted laser desorption ionization time-of-flight mass spectrometry; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; R₁^X, sixth root residual; rMD, restrained molecular dynamics; rmsd, root mean square deviation; SBR, styrene-butadiene rubber; TBE, tris borate EDTA buffer; TOCSY, total correlation spectroscopy; TPPI, time-proportional phase increment.

COS-7 cells (55). In both systems, the primary mutations were A→G transitions observed at the 3' adenine of the crosslink. The levels of mutations observed in the COS-7 cells were significantly greater than were observed in the bacterial system.

Both the *R,R* and *S,S* stereoisomers of this N⁶,N⁶-dA intrastrand crosslink were bypassed by a variety of DNA polymerases *in vitro* (55), suggesting that they did not pose severe blocks to replication; mutagenesis in *E. coli* may be due to DNA polymerase II-mediated lesion bypass (58). It was proposed that these BDO₂-induced crosslinks were accommodated within the major groove, similar to several N⁶-dA adducts of styrene oxide, which also did not pose severe blocks to replication (59,60). This was in contrast to the corresponding N²,N²-dG (*R,R*) and (*S,S*) intrastrand crosslinks, which were severe replication blocks when examined *in vitro* (61).

The present work characterizes structural perturbation to the *ras61* oligodeoxynucleotide caused by the 1,4-bis(2'-deoxyadenosin-N⁶-yl)-2*R*,3*R*-butanediol crosslink. Molecular dynamics calculations restrained by interproton distances and torsion angle restraints obtained from NMR spectroscopy indicate that the crosslinked moiety is accommodated in the major groove of the DNA double helix. Watson-Crick base pairing is disrupted at position X⁶•T¹⁷, the 5'-side of the intrastrand crosslink. Helicoidal analysis suggests an opening of this base pair. Potential hydrogen bond formation between the β-hydroxyl group of the crosslink, and T¹⁷ O⁴, the nucleotide complementary to X⁶, shields the T¹⁷ imino hydrogen from exchange with solvent. In contrast, Watson-Crick base pairing is intact at position Y⁷•T¹⁶, the 3'-side of the crosslink.

MATERIALS AND METHODS

Sample Preparation

The oligodeoxynucleotides 5'-d(CGGACAAGAAG)-3' and 5'- d(CTTCTTGTCCG)-3' were synthesized by the Midland Certified Reagent Co. (Midland, TX) and purified by anion-exchange chromatography. The crosslinked oligodeoxynucleotide 5'-d(CGGACXYGGAAG)-3' was synthesized by a variation of the post-oligomerization method described for the preparation of monoadducts of butadiene epoxides (55,62). The concentrations of the single-stranded oligonucleotides were determined from their calculated extinction coefficients at 260 nm (63). The modified oligodeoxynucleotide and its complement were annealed in a buffer consisting of 10 mM NaH₂PO₄, 0.1 M NaCl, and 50 μM Na₂EDTA at pH 7.0. The modified duplex was eluted from DNA Grade Biogel hydroxylapatite (Bio-Rad Laboratories, Hercules, CA) with a gradient from 10 to 200 mM NaH₂PO₄, pH 7.0. It was desalted using Sephadex G-25.

Capillary gel electrophoresis

The purity of the modified duplex was analyzed using a PACE 5500 (Beckman Instruments, Inc., Fullerton, CA) instrument. Electrophoresis was conducted using an eCAP ssDNA 100-R kit applying 12,000 V for 30 min. The electropherogram was monitored at 254 nm.

Mass Spectrometry

MALDI-TOF mass spectra were measured on a Voyager-DE (PerSeptive Biosystems, Inc., Foster City, CA) instrument in negative reflector mode. The matrix contained 0.5 M 3-hydroxypicolinic acid and 0.1 M ammonium citrate.

Thermal Melting

Thermal melting of duplex DNA was monitored using UV spectroscopy at 260 nm in a buffer consisting of 1 M NaCl, 10 mM Na₂HPO₄, 10 mM NaH₂PO₄, 50 μM Na₂EDTA at pH 7.0.

Melting temperatures were determined by measuring the absorbance change as a function of temperature; the temperature range was 15° C to 85° C with a 1° C/min increment.

Electrophoretic Mobility

Adducted or unmodified oligodeoxynucleotides were combined individually with excess complement 5'-d(CGCTTCTTGTC). They were phosphorylated with 3.8 units T4 polynucleotide kinase and 20.8 µL of 10 µM ATP at 37 °C in 6 µL 10x DNA kinase buffer (70 mM Tris-HCl, pH 7.6, 10 mM MgCl₂, 5 mM dithiothreitol) (pH 7.6) and incubated overnight at 37 °C with an additional 1.9 units of kinase and 10.4 µL of 10 µM ATP. *Bam*H I linker [5'-d(CGGGATCCCG)₂-3'] was phosphorylated with 1.9 units T4 polynucleotide kinase in 2 µL of 10x kinase buffer and 10.4 µL of 10 µM ATP and incubated overnight at 37 °C. Fractions of each reaction (*Bam*H I: 0.025 A₂₆₀units; unmodified: 0.02 A₂₆₀ units; four carbon linker: 0.04 A₂₆₀ units) were removed and incubated with T4 DNA ligase (*Bam*H I: 5 units; unmodified: 2 units; four carbon linker: 2 units) in 2 µL of 10x T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM MgCl₂, 10 mM dithiothreitol, 1 mM ATP, 5 µg/mL bovine serum albumin) (pH 7.8) and 10 µL of 10 µM ATP overnight at 16 °C, and precipitated with absolute ethanol. They were suspended in 6 mL of nondenaturing loading buffer (Sigma). 1 µL of a pBR322 *Hae* III digest (0.4 µg/mL, 10 mM Tris-HCl, 0.1 mM Na₂EDTA) (pH 8.0) was mixed with nondenaturing loading buffer per each length marker lane. Electrophoresis was performed using a nondenaturing 8% polyacrylamide gel (mono:bis-acrylamide ratio 29:1, 3.3% catalyst). Gels were run at 1000 V for 2.5-3 h and stained for 30 min with 250 mL GelStar nucleic acid stain (10,000 x) (BioWhittaker Molecular Applications, Walkersville, Maine) in 250 mL 1 × TBE. Gels were imaged on a UV transilluminator (Alpha Innotech, San Leandro, CA). Control multimers formed by ligation of the 10-bp *Bam*H I linker were used to calculate apparent lengths of crosslinked multimers.

NMR

The modified duplex was prepared at a concentration of 2 mM. For observation of nonexchangeable protons, the sample was dissolved in 0.5 mL of 99.96% D₂O containing 10 mM NaH₂PO₄, 0.1 M NaCl, and 50 µM Na₂EDTA (pH 7.0). For observation of exchangeable protons, the sample was dissolved in 0.5 mL of 9:1 H₂O:D₂O in the same buffer. ¹H NMR spectra were recorded at 600.13 MHz and 800.23 MHz. The non-exchangeable protons were monitored at 25 °C; the exchangeable protons were monitored at 17 °C. Chemical shifts were referenced to water. Data were processed using the program FELIX2000 (Accelrys, Inc., San Diego, CA). NOESY spectra of non-exchangeable protons were recorded using TPPI phase cycling with mixing times of 150, 200, and 250 ms. Spectra for exchangeable protons were recorded using a 150 ms mixing time. These were recorded with 1024 real data points in the d1 dimension and 2048 real data points in the d2 dimension. A relaxation delay of 2.0 s was used. Water suppression was performed using the WATERGATE sequence (64). TOCSY experiments were performed with mixing times of 90 and 150 ms, utilizing homonuclear Hartman-Hahn transfer with the MLEV17 sequence (65) for mixing. DQF-COSY spectra were zero-filled to give a matrix of 1024 × 2048 real points. A skewed sine-bell square apodization function with a 90° phase shift and a skew factor of 1.0 was used in both dimensions.

Experimental Restraints

(a) Distance Restraints—Footprints were drawn around cross peaks obtained at a mixing time of 200 ms using FELIX2000. Identical footprints were transferred and fit to the crosspeaks obtained at the other two mixing times. The intensities of cross peaks were determined by volume integrations. These were combined as necessary with intensities generated from complete relaxation matrix analysis of a starting DNA structure to generate a hybrid intensity matrix (66,67). MARDIGRAS (68–70) was used to iteratively refine the hybrid intensity matrix

and to optimize the agreement between the calculated and experimental NOE intensities. Calculations were initiated using isotropic correlation times of 2, 3, and 4 ns, and with both IniA and IniB starting structures and the three mixing times, yielding eighteen sets of distances. Analysis of this data yielded the experimental distance restraints used in subsequent restrained molecular dynamics calculations, and the corresponding standard deviations for the distance restraints. The distance restraints were divided into five classes, reflecting the confidence level in the experimental data.

(b) Torsion Angle Restraints—Deoxyribose pseudorotation (57) was determined graphically using the sums of 3J 1H coupling constants (58), measured from DQF-COSY spectra. Discrete $J_{1',2''}$ and $J_{1,2'}$ couplings were measured from active and passive couplings, respectively, of the H2'' (d2) to H1' (d1) spectral region. The data were fit to curves relating the coupling constants to the deoxyribose sugar pseudo rotation angle (P), sugar pucker amplitude (ϕ), and the percentage S-type conformation. The sugar pseudo rotation angle and amplitude ranges were converted to the five dihedral angles ν_0 to ν_4 . Coupling constants measured from 1H - ^{31}P HMBC spectra were applied (71,72 1991 1991) to the Karplus relationship (73) to determine the backbone dihedral angle ϵ (C4'-C3'-O3'-P), related to the H3'-C3'-O3'-P angle by a 120° shift. The ζ (C3'-O3'-P-O5') backbone angles were calculated from the correlation between ϵ and ζ in B-DNA (60).

Restrained Molecular Dynamics Calculations

Classical A-DNA and B-DNA were used as reference structures to create starting structures for the refinement (74). The butadiene adduct was constructed at A⁶ using the BUILDER module of INSIGHT II (Accelrys, Inc., San Diego, CA). A-form and B-form structures of the appropriate sequence were energy-minimized by the conjugate gradients method for 200 iterations using the AMBER 7.0 force field (75) without experimental restraints to give starting IniA and IniB used for the subsequent relaxation matrix analysis and molecular dynamics calculations. The restraint energy function included terms describing distances and dihedral restraints as square-well potentials (76). Bond lengths involving hydrogens were fixed with the SHAKE algorithm (77). The generalized Born approach was used to model solvent (78, 79). The calculations utilized a salt concentration of 0.2 mM. A series of randomly seeded rMD calculations were performed over a time course of 40 ps. These used the SANDER module of AMBER 7.0, and the Cornell *et al.* force field (80), including the Parm94.dat parameter set. The simulated annealing protocol utilized a starting temperature of 25 K. In the first ps the temperature was increased to 600 K. This was maintained for 4 ps, followed by cooling to 298 K over 15 ps. During the final 20 ps the temperature was reduced to 0 K. Temperature was controlled by coupling to a temperature bath. During the first 1 ps of heating a coupling of 0.4 ps was used. During the next 4 ps of constant temperature dynamics a coupling of 1.0 ps was used. In the first 15 ps of cooling, a value of 1.0 ps was used, followed by a value 10 of 0.5 ps for the second 15 ps. During the final 5 ps of cooling, the coupling was ramped down to 0.01 ps. In the first 1 ps of heating, the experimental force constants were amplified by factors that ranged from 0.5 to 1.00. During the 4 ps of constant temperature dynamics and the first 15 ps of cooling, the amplification factor was increased to 1.75. In the final 20 ps of cooling, the amplification factor was reduced to 1.0. Structure coordinates were archived every 0.1 ps over the final 10 ps of the simulation. Structure coordinates extracted from the final 4 ps were averaged and energy-minimized for 200 iterations using the conjugate gradients algorithm. An average structure was obtained from eight randomly seeded rMD calculations. Back-calculation of 1H NOE data was performed using CORMA (v. 4.0) (66,67). Helicoidal parameters were examined using 3DNA (81).

RESULTS

Sample Properties

The purity of the (*R,R*) N⁶,N⁶-dA crosslinked adduct duplex was assessed using capillary gel electrophoresis. The electropherogram exhibited two peaks in a 1:1 ratio after correction for the respective absorbance coefficients. The identity of the duplex was verified using MALDI-TOF mass spectrometry. Mass measurement showed two signals that corresponded to mass units of 3484 and 3272. These peaks corresponded to the crosslinked adduct strand 5'-d(CGGACXYGAAG)-3' and to the complementary strand 5'-d(CTTCTTGTC CG)-3', respectively. The melting temperature of the sample was determined by UV spectroscopy as 50° C, less than the observed 57° C melting temperature of the unmodified *ras61* duplex. The crosslink sample yielded excellent NMR data in the temperature range of 10° C – 25 °C.

DNA ¹H Resonance Assignments

(a) Nonexchangeable Protons—The sequential NOEs between the aromatic and anomeric protons are displayed in Figure 1. These were assigned using standard methods (82,83). There were no breaks observed in connectivity in either the crosslinked strand or the complementary strand. With assignments of the sugar H1' protons in hand, the remainder of the deoxyribose sugar protons were assigned from DQF-COSY spectra. With the exception of several of the H5' and H5" protons, assignments of the sugar protons were made unequivocally. Table S1 in the Supporting Information details the complete non-exchangeable ¹H NMR assignments of the crosslinked adduct oligodeoxynucleotide duplex.

(b) Exchangeable Protons—An expanded region showing the far downfield region of the ¹H NMR spectrum, exhibiting cross-peaks between the hydrogen-bonded imino protons is shown in Figure 2. Sequential assignments of the imino protons from base pairs G²•C²¹→C⁵•G¹⁸ and X⁶•T¹⁷→A¹⁰•T¹³ were obtained, with a break in connectivity observed between base pairs C⁵•G¹⁸ and X⁶•T¹⁷. The NOE between T¹³ N3H and T¹⁴ N3H was weak. It was not observed at the contour level plotted in Figure 2. This crosspeak was also weak in the *ras61* spectrum (84) and presumably reflected the effects of strand fraying and resulting rapid exchange of this proton with solvent. Each of the peaks identified in the cytosine amino region of the ¹H NMR spectrum exhibited the anticipated cross peak with the appropriate deoxyguanosine imino proton as expected in Watson-Crick base pairing. With the exception of T¹⁶, each thymine N3H proton exhibited an NOE to the corresponding adenine H2 proton in the complementary strand. Cross peaks were observed between T¹⁷ N3H and the T¹⁶ CH₃ and T¹⁷ CH₃ resonances.

(c) Butadiene Protons—The protons of the butadiene crosslink were observed as separate resonances, with spectral line widths comparable to the oligodeoxynucleotide protons (Figure 3). At a mixing time of 200 ms, NOEs were observed between the butadiene crosslink protons. The H_α' proton at 3.51 ppm showed NOEs to H_β at 4.03 ppm and H_α" at 2.72 ppm. The H_β proton showed NOEs to H_α' and H_α", along with a weak NOE to H_γ at 3.62 ppm and H_δ' at 3.26 ppm. The H_γ proton showed a weak NOE to H_β (not observed in Figure 3) along with NOEs to H_δ' at 3.01 ppm and H_δ", respectively. A cross peak was observed between T¹⁷ N3H and the H_δ· proton of the crosslink (Figure 4). This was the only cross peak observed between the butadiene crosslink and the oligodeoxynucleotide.

Torsion Angle Measurements

Analysis of DQF-COSY data suggested that all deoxyribose pseudorotation angles remained in the C2'-endo range anticipated for B-family DNA. The glycosyl torsion angles were monitored using ¹H NOESY experiments. These revealed weak NOEs between the purine H8 or pyrimidine H6 protons and the anomeric H1' protons of the attached deoxyribose sugars,

consistent with glycosyl torsion angles in the normal *anti* conformational range. There were also no unusual chemical shifts or $^3J_{\text{H-31P}}$ couplings observed in the ^{31}P spectrum. Data obtained from $^3J_{\text{H-31P}}$ experiments indicated that the torsion angles associated with the backbone phosphodiester linkages were not significantly perturbed by the presence of the crosslink. Karplus analysis yielded values within the standard ranges of $165.0^\circ \pm 10^\circ$ and $235^\circ \pm 10^\circ$ for the backbone torsion angles ϵ and ζ , respectively (85).

Chemical Shift Effects

The ^1H chemical shifts of the crosslinked duplex were compared to those of the unmodified *ras61* oligomer (84). Figure 5 shows that chemical shift differences were localized in the crosslinked strand at the two crosslinked nucleotides X^6 and Y^7 . The $\text{X}^6\text{H}1'$ resonance shifted 0.6 ppm upfield, while the $\text{Y}^7\text{H}8$ resonance shifted 0.4 ppm downfield. In the far downfield region of the ^1H spectrum, the $\text{T}^{17}\text{N}3\text{H}$ imino resonance shifted upfield approximately 1.0 ppm. The complementary strand did not exhibit significant changes in ^1H chemical shifts.

Structural Refinement

The structural refinement incorporated 338 experimental distance restraints. There was one NOE observed between the crosslinked butadiene moiety and the DNA. The distance restraints were evenly distributed over the length of the crosslinked oligodeoxynucleotide. In addition, 80 deoxyribose pseudorotation restraints were included. There were 106 phosphodiester backbone torsion angle restraints. The phosphodiester backbone angles ϵ and ζ were restrained at angles of $165^\circ \pm 10^\circ$ and $245^\circ \pm 10^\circ$ respectively. Empirical Watson-Crick hydrogen-bonding restraints were used for all base pairs except for $\text{X}^6\cdot\text{T}^{17}$, as NMR data indicated no base pairing at this site. Inspection of the structures that emerged from an initial series of randomly seeded rMD calculations suggested that the $\text{C}\beta$ hydroxyl of the crosslink and T^{17}O^4 were within hydrogen bonding distance. It was therefore decided to add an empirical restraint for this hydrogen bond in subsequent calculations.

A stereoview of six energy minimized structures emergent from the rMD calculations is shown in Figure 6. These rMD calculations were initiated from both the A-form and B-form starting structures. These two starting structures exhibited an rmsd of 5.92 Å. The rMD calculations converged to similar ensembles of structures irrespective of starting structure. The maximum pairwise rmsd between these emergent structures was 0.90 Å, suggesting that the experimental distance and torsion angle restraints, combined with the additional empirical hydrogen bonding restraints, enabled the rMD calculations to converge to a well-defined set of structures. The average structure emergent from the rMD calculations exhibited a rmsd of 2.50 Å as compared to the IniB starting structure. This deviation from canonical B-form DNA resulted primarily from structural variation associated with the crosslinked base pairs $\text{X}^6\cdot\text{T}^{17}$ and $\text{Y}^7\cdot\text{T}^{16}$.

The validities of the structures emergent from the rMD calculations were evaluated using complete relaxation matrix calculations (66,67). Figure 7 shows R_1^x values as a function of nucleotide position. The residual values were consistent over the length of the modified oligodeoxynucleotide and ranged from $3.1\text{--}14.8 \times 10^{-2}$. The inclusion of the empirical hydrogen bonding restraint between the $\text{C}\beta$ hydroxyl of the crosslinked adduct and T^{17}O^4 resulted in a 10% improvement in the R_1^x residual for base step $\text{C}^5\rightarrow\text{X}^6$, as compared to the corresponding calculations in which this empirical restraint was not utilized.

Structure of the (R,R)-BD-(61-2, 3) Crosslinked Adduct

The structures that emerged from the rMD calculations indicated that the N^6,N^6 -dA butadiene crosslink oriented in the major groove of the DNA, as shown in Figure 8. Watson Crick base pairing was disrupted at $\text{X}^6\cdot\text{T}^{17}$. In contrast, base pairing at $\text{Y}^7\cdot\text{T}^{16}$ was maintained. Helicoidal analysis (81) suggested a change of the phosphodiester backbone torsion angle β $\text{P}5'\text{-O}5'\text{-C}5'$ -

C4', at X⁶. In the presence of the N⁶,N⁶-dA crosslink, angle β at X⁶ increased to approximately 200°, as compared to the expected range of 165–180°. The emergent structures suggested the formation of a hydrogen bond between the C β -hydroxyl of the butadiene crosslink and T¹⁷ O⁴, thus stabilizing nucleotide T¹⁷ in the complementary strand in the absence of Watson-Crick hydrogen bonding with crosslinked nucleotide X⁶.

The structures that emerged from the rMD calculations suggested that the four-carbon N⁶,N⁶-dA crosslink did not bend the DNA duplex. To confirm this observation, a series of electrophoretic mobility assays were conducted on ligated multimers of an oligodeoxynucleotide containing the non-hydroxylated analog of the N⁶,N⁶-dA crosslink. Thermal melting studies monitored by UV spectroscopy indicated that the non-hydroxylated alkyl tether also lowered the T_m of the duplex by approximately 6°, as observed for the hydroxylated N⁶,N⁶-dA crosslink, suggesting that the non-hydroxylated alkyl tether provided a reasonable model for the N⁶,N⁶-dA crosslinked oligodeoxynucleotide studied by NMR. The mobilities of the ligated crosslinked multimers were analyzed by non-denaturing polyacrylamide gel electrophoresis (86). The electrophoretic migration data corroborated NMR data suggesting that this crosslinked oligodeoxynucleotide did not undergo significant bending.

The base stacking patterns at the crosslink site are shown in Figure 9. Helicoidal analysis suggested a decrease in the base pair opening parameter at base pair X⁶•T¹⁷, from the expected range of $0 \pm 5^\circ$ (base pair closed) to a value of approximately -13° . Intrastrand crosslink formation was accompanied by a decrease in the twist angle between base pairs C⁵•G¹⁸ and X⁶•T¹⁷, resulting in unwinding of the duplex. Consequently, in the crosslinked structure, nucleotides X⁶ and Y⁷ did not exhibit the typical 36° twist as was observed for the monodentate butadiene triol adduct located at position X⁶, or for the unmodified *ras61* oligodeoxynucleotide. The opening of the duplex at X⁶•T¹⁷ resulted in the six-carbon pyrimidine ring of the X⁶ purine stacking above the imidazole ring of the Y⁷ purine. In the complementary strand, the base stacking of the T¹⁶ and T¹⁷ nucleobases appeared unperturbed by the presence of the N⁶,N⁶-dA crosslink.

DISCUSSION

The genotoxicity of butadiene may be related to its ability to form crosslinks in DNA via its diepoxide oxidation product. Animal studies revealed species differences between mice and rats with regard to BD genotoxicity, which mice exhibiting greater sensitivity (7,8,87). This was explained by the observation that in mice the conversion of BD to BDO₂ was more efficient than in rats, an observation which also pointed to BDO₂ as the key proximate electrophile in BD-mediated genotoxicity (45). This suspicion was confirmed by studies showing that BDO₂ was indeed considerably more mutagenic than the mono-epoxide EB (37)—probably due to its crosslinking ability. The primary evidence suggesting the presence of BDO₂-induced DNA crosslinks *in vivo* comes from studies in which human lymphoblastoid TK6 and splenic T cells were exposed to low levels of BDO₂ (37,88,89). These experiments revealed transitions and transversions at both GC and AT sites, suggesting the presence of both dG and dA adducts arising from BDO₂. They also revealed the presence of significant levels of deletion mutants, consistent with the presence of DNA crosslinks.

Structural Perturbations Associated with the N⁶,N⁶-dA Intrastrand Crosslink

This intrastrand crosslink, tethering nucleotides X⁶ and Y⁷ in the *ras61* oligodeoxynucleotide, oriented in the major groove. That there were no interruptions in DNA sequential NOE connectivities (Figure 1), and all Watson-Crick base pairs, with the exception of base pair X⁶•T¹⁷, were intact (Figure 2), suggesting that the crosslink did not substantially perturb the right-handed DNA helix. Bending of the intrastrand crosslinked duplex was not observed. This

was consistent with the fact that the major groove of B-family DNA had capacity to accommodate the four-carbon crosslink. One NOE was observed between the (*R,R*) N⁶,N⁶-dA crosslinked adduct and the DNA, an observation which was consistent with the major groove orientation of the adduct, since the major groove contains few protons as potential sources of dipolar relaxation with the BD protons. An approximate 1 ppm upfield shift of the T¹⁷ N3H imino proton resonance was consistent with a loss of hydrogen bonding and supported the conclusion that Watson-Crick base-pairing was disrupted at base pair X⁶•T¹⁷. The formation of a hydrogen bond between the β-OH group of the crosslink and T¹⁷ O⁴, predicted by the rMD calculations, possibly explained the observation of the T¹⁷ N3H resonance. It suggested that in the crosslinked adduct, this non-hydrogen bonded proton was sheltered from fast exchange with the solvent. The rMD calculations predicted that the crosslinked duplex was characterized by an increase in the β torsion angle at X⁶. This facilitated opening of the duplex and enabled an approximate 5° unwinding of the duplex at base pair X⁶•T¹⁷. The resulting changes in stacking between crosslinked nucleotides X⁶ and Y⁷ possibly accounted for the upfield shift of the Y⁷ H8 resonance (Figure 5).

Mechanistic Insights into Formation of N⁶,N⁶-dA Intrastrand Crosslinks

This N⁶,N⁶-dA crosslinked adduct has not yet been reported from mass spectrometry analysis of DNA exposed to BDO₂ (49,54). It is generally argued that N⁶-dA alkylation products of butadiene oxides arise not by direct attack at the exocyclic amine, but rather by initial alkylation at the more nucleophilic N1 imine, followed by Dimroth rearrangement to yield the N⁶-dA product (50,51,90). One might predict that formation of an intrastrand N⁶,N⁶-dA crosslink would require tandem N1-dA alkylation by BDO₂ at the neighboring adenines, followed by tandem Dimroth rearrangements of both alkylated dA nucleotides. Alternatively, formation of a X⁶ N1-dA adduct by BDO₂, followed by Dimroth rearrangement, would result in a N⁶-dA BDO adduct (91,92). Orientation of the X⁶ N⁶-dA BDO adduct similar to N⁶-dA butadiene triol adducts (93,94) might facilitate direct attack on the BDO epoxide by the less nucleophilic N⁶-dA at the neighboring A⁷. To examine this possibility, the N⁶-dA BDO adduct was modeled at position X⁶ based upon the experimentally determined solution structures of the corresponding N⁶-dA BDT adducts (93,94). The modeling study (Figure 10) suggested that this might be a reasonable hypothesis. However, the initially formed N1-monoalkylated intermediate might also reorient about the glycosyl bond, into the *syn* conformation. Although this has not been confirmed for the N1-dA monoalkylation product of BD, the N1-(1-hydroxy-3-buten-2(*S*)-yl)-2'-deoxyinosine adduct arising from deamination following N1-dA alkylation at N1-dA by BDO was accommodated in duplex DNA by rotation about the glycosyl torsion angle into the *syn* conformation (58). Overall, formation of the N⁶,N⁶-dA intrastrand crosslink seems likely to represent a rare event, with hydrolysis of the monoalkylated BDO intermediates prevailing. Indeed, the BDT N1-dA adduct was identified in humans exposed occupationally to BD (52). The N⁶-dA BDT adduct was identified in Chinese Hamster ovary cells (53).

Structure-Activity Relationships

The major groove orientation and the small conformational perturbation of the DNA structure induced by the crosslink are possibly correlated with facile bypass of this lesion by a variety of DNA polymerases. It was of interest to compare this crosslink structure with those of two N⁶-dA butadiene triol adducts, which are products of monoalkylation at N⁶-dA by BD (93, 94). The monoalkylated N⁶-dA BDT adducts also did not pose blocks to replication (56). Perhaps significantly, the *R*(61,2) and both the *R*- and *S*(61,3) N⁶-dA α adducts of styrene oxide also did not pose blocks to replication (59,60), and also oriented in the major groove (95–97). The *R*- and *S*(61,2) β adducts of styrene oxide behaved similarly (60,97). Collectively, these results fit an emerging pattern in which lesion bypass by DNA polymerases is minimally affected by the presence of small major groove N⁶-dA adducts, although it should be noted

that the $R(61,2)$ α adduct of styrene oxide did block replication (59). In contrast, the (R,R) and (S,S) N^2,N^2 -dG intrastrand crosslinks were severe replication blocks when examined *in vitro* (61).

The (R,R) N^6,N^6 -dA intrastrand crosslink was significantly more mutagenic than two N^6 -dA BDT mono adducts previously examined as to mutagenesis (56) and structure (93,94). In the mammalian COS-7 site-specific mutagenesis assay the most frequent mutations were A→G transitions, followed by A→C and A→T transversions, located at the 3'-adducted dA in the tandem crosslink. In *Escherichia coli* wild type and AB2480 (*uvrA*-, *recA*-) cells the (R,R) N^6,N^6 -dA crosslink was significantly less mutagenic, yielding A→G transitions (55). Recent data suggests that in *E. coli*, DNA polymerase II is responsible for the mutagenic bypass of the (R,R) N^6,N^6 -dA crosslink (58).

The observation of A→G transitions at the 3'-position of the tandem crosslink suggested that this (R,R) N^6,N^6 -dA crosslink facilitated incorrect incorporation of dCTP opposite crosslinked nucleotide Y⁷. It will be of interest to examine the structure of a mismatched dC opposite the crosslink. NMR studies on mismatched dC opposite the $S(61,2)$ α adduct of styrene oxide (98), which induced low levels of A→G mutations (59), and opposite the R - and $S(61,3)$ α adducts of styrene oxide (99) which were non-mutagenic (59), revealed both sequence- and stereospecific differences in the mismatched duplexes.

The corresponding (S,S) N^6,N^6 -dA crosslink was less mutagenic in the COS-7 system (55, 61). This may predict stereospecific structural differences between the (R,R) and (S,S) N^6,N^6 -dA crosslinks, and it will be of interest to compare the structure of the (S,S) N^6,N^6 -dA crosslink with that of the (R,R) N^6,N^6 -dA crosslink.

Biological Significance

When B6c3F1 *lacI* transgenic mice were exposed to butadiene, the primary adenine-specific point mutations were A→T transversions (100–104). The site specific mutagenesis experiments on the (R,R) N^6,N^6 -dA intrastrand crosslink, yielding primarily A→G mutations (55) suggest that it does not represent the source of the predominant A→T BD-induced mutations in bacterial or in mammalian cells. Nonetheless, butadiene was reported to induce A→G transitions in the *H-ras* codon 61 (105). The (R,R) N^6,N^6 -dA crosslink may contribute to these. The N1-deoxyinosine adduct represents another potential source of A→G transitions. This adduct arises from deamination of the initially formed N1-dA adduct, and is strongly mutagenic with respect to A→G transitions (55). The adducts responsible for BD-induced A→T transversions (100–104) remain to be determined. BD-induced crosslinks involving monoalkylation of N7-dG (106–109) followed by crosslinking with dA (49) represent potential candidates.

The major BDO₂-induced DNA crosslink is the N^7,N^7 -dG interstrand crosslink (41). It occurs in 5'-GNC-3' sequences (40), as opposed to nearest neighbor 5'-GC-3' sequences. In this regard, BDO₂-induced N^7,N^7 -dG interstrand crosslinking appears to be similar to that of the nitrogen mustard mechlorethamine (110). It will be of considerable interest to examine structures of N^7,N^7 -dG interstrand crosslinks.

Summary

The intrastrand (R,R) N^6,N^6 -dA crosslink induced by BDO₂ in duplex DNA oriented in the major groove of the duplex. It resulted in an increase in base pair opening at base pair X⁶•T¹⁷, accompanied by a shift in the phosphodiester backbone torsion angle β at nucleotide X⁶. The DNA helix was not bent by the presence of the four-carbon crosslink. A hydrogen bond between the hydroxyl group located on the β -carbon of the four-carbon crosslink, and

T¹⁷O⁴ perhaps stabilized base pair opening at X⁶•T¹⁷ and protected the T¹⁷ imino proton from solvent exchange. The opening of base pair X⁶•T¹⁷ altered base stacking and induced unwinding of the duplex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Expanded plots of a NOESY spectrum at a mixing time of 200 ms showing sequential NOE connectivities from aromatic to anomeric protons of the (*R,R*)-BD-(61,2-3) crosslinked adduct. A. Nucleotides C¹→G¹¹ of the modified strand. B. Nucleotides C¹²→G²² of the complementary strand.

Figure 2.

Expanded plot of a NOESY spectrum at a mixing time of 200 ms showing NOE connectivities for the imino protons for the base pairs from G²•C²¹ to A¹⁰•T¹³. Note that there is a break in the walk between the imino resonances for base pairs G²•C²¹ and X⁶•T¹⁷. The resonance observed for base pair X⁶•T¹⁷ is shifted in the spectrum.

Figure 3. Expanded NOESY spectrum at 200 ms mixing time exhibiting the assignment of crosslink protons in the (*R,R*)-BD-(61,2-3) crosslinked adduct duplex. The experiment was at 800.23 MHz and 25 °C.

Figure 4. NOE observed between $\text{T}^{16} \text{N}3\text{H}$ and the H_δ proton of the (R,R) -BD-(61,2-3) crosslinked adduct. The experiment was at 800.23 MHz and 17 °C.

Figure 5. Chemical shift differences of protons of the (*R,R*)-BD-(61,2-3) crosslinked adduct duplex relative to the unmodified *ras61* oligodeoxynucleotide. A. The modified strand of the crosslinked adduct. B. The complementary strand of the crosslinked adduct. Grey bars represent the deoxyribose H1' protons; black bars represent the purine H8 or pyrimidine H6 protons, respectively.

Figure 6.

A stereoview of six superimposed structures emergent from the simulated annealing rMD protocol; the structures resulted from randomly seeded calculations.

Figure 7.

R_1^x values as a function of position in the (*R,R*)-BD-(61,2-3) crosslinked adduct. A. The crosslinked strand. B. The complementary strand.

Figure 8.

A close up view of the *(R,R)*-BD-(61,2-3) crosslinked duplex. View from the major groove of X6•T17 and Y7•T16 and the flanking base pairs C5•G18 and G8•C15. The duplex DNA is shown in blue, the crosslink is in red.

Figure 9.

Base stacking orientations of the *(R,R)*-BD-(61,2-3) crosslinked duplex. A. The crosslinked duplex detailing base stacking of the X⁶ and Y⁷ base pairs. B. The crosslinked duplex detailing base stacking of the X⁶ and A⁷ base pairs. C. The unmodified *ras61* oligodeoxynucleotide duplex detailing base stacking of the A⁶ and A⁷ base pairs.

Figure 10.

Molecular modeling of the N⁶-dA BDO adduct at position X⁶ of the *ras61* oligodeoxynucleotide. The X⁶ BDO adduct is predicted to orient in the 3'-direction, toward the exocyclic amino group of A⁷. This might facilitate direct attack by the exocyclic amine on the epoxide, leading to formation of an N⁶,N⁶-dA crosslink. Only nucleotides X⁶ and A⁷ in the modified strand of the duplex DNA are shown. The BD moiety is in red; the DNA is in blue.

Scheme 1.
Reactive Metabolites of Butadiene.

A.

B.

Scheme 2.

The *ras61* oligodeoxynucleotide (A), and the chemical structure of (B) the (2*R*,3*R*)-*N*⁶-(2,3-dihydroxybutyl)-2-deoxyadenosyl cross link adduct and nomenclature.

Table 1Analysis of the rMD-Generated Structures of the *ras61* (R,R) N⁶,N⁶-dA crosslinked adduct.

NMR restraints	
total no. of distance restraints	338
interresidue distance restraints	91
intraresidue distance restraints	247
DNA-crosslink distance restraints	0
torsion angle restraints	
sugar pucker restraints	80
backbone torsion angle restraints	71
empirical restraints	
H-bonding restraints	46
dihedral planarity restraints (in XPLOR)	20
structural statistics	
NMR R-factor (R_1^x) ^{a,b,c}	1.12×10^{-1}
rmsd of NOE violations (Å)	2.15×10^{-2}
no. of NOE violations > 0.15 Å	9
pairwise rmsd (Å) over all atoms	
IniA vs. IniB	5.92
IniA vs. rMDA _{avg}	4.75
IniB vs. rMDB _{avg}	2.50
rMDA vs rMDB	0.90
rMDA vs rMDA	0.51
rMDB vs rMDB	0.81
<rMDA> vs. rMDA _{avg}	0.33 ± 0.11
<rMDB> vs. rMDB _{avg}	0.52 ± 0.21

^aThe mixing time was 150 ms.^b $R_1^x = \sum |(a_o)_i|^{1/6} - (a_c)_i|^{1/6} / \sum |(a_o)_i|^{1/6}|$, where a_o and a_c are the intensities of observed (non-zero) and calculated NOE cross-peaks.^c<rMDA>, represents a group of six converged structures starting from IniA; <rMDB>, represents a group of six converged structures starting from IniB. rMDA_{avg} represents the potential energy minimized average structure of all six rMD calculations starting with A-form DNA rMDB_{avg} represents the potential energy minimized average structure of all eight rMD calculations starting with B-form DNA. The comparisons: rMDA vs rMDB, rMDA vs rMDA, rMDB vs. rMDB, represent the maximum observed pairwise rmsd over all atoms between these groups.