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The influence of cytosine methylation on the chemoselectivity of benzo[a]pyrene diol epoxide-oligonucleotide adducts determined using nanoLC/MS/MS

James Glick^a, Wennan Xiong^a, Yiqing Lin^a, Anne M. Noronha^b, Christopher J. Wilds^b, and Paul Vouros^{a,*}

^a Barnett Institute and Department of Chemistry, Northeastern University, Boston, MA 02115, USA

^b Department of Chemistry & Biochemistry, Concordia University, Montreal, Quebec H4B 1R6, Canada

Abstract

Benzo[a]pyrene is a major carcinogen implicated in human lung cancer. Almost 60% of human lung cancers have a mutation in the *p53* tumor suppressor gene at several specific codons. An on-line nanoLC/MS/MS method using a monolithic nanocolumn was applied to investigate the chemoselectivity of the carcinogenic diol epoxide metabolite, (±)-(7R,8S,9S,10R)-benzo[a]pyrene 7,8-diol 9,10-epoxide [(±)-*anti*-benzo[a]pyrene diol epoxide (BPDE)], which was reacted *in vitro* with a synthesized 14-mer double stranded oligonucleotide (5'-ACCCG₅CG₇TCCG₁₁CG₁₃C-3'/5'-GCGCGGGCGCGGGT-3') derived from the *p53* gene. This sequence contained codons 157 and 158, which are considered mutational 'hot spots' and have also been reported as chemical 'hot spots' for the formation of BPDE-DNA adducts. In evaluating the effect of cytosine methylation on BPDE-DNA adduct binding, it was found that codon 156, containing the nucleobase G₅ instead of the mutational hot spot codons 157 (G₇) and 158 (G₁₁), was the preferential chemoselective binding site for BPDE. In all permethylated cases studied, the relative ratio for adduction was found to be G₅ ≫ G₁₁ > G₁₃ > G₇. Permethylation of CpG dinucleotide sites on either the nontranscribed or complementary strand did not change the order of sequence preference but did enhance the relative adduction level of the G₁₁ CpG site (codon 158) approximately two-fold versus the unmethylated oligomer. Permethylation of all CpG dinucleotide sites on the duplex changed the order of relative adduction to G₅ ≫ G₇ > G₁₁ > G₁₃. The three- to four-fold increase in adduction at the mutational hot spot codon 157 (G₇) relative to the unmethylated or single-stranded permethylated cases suggests a possible relationship between the state of methylation and adduct formation for a particular mutation site in the *p53* gene. Using this method, only 125 ng (30 pmol) of adducted oligonucleotide was analyzed with minimal sample cleanup and high chromatographic resolution of positional isomers in a single chromatographic run.

Keywords

DNA adducts; BPDE; oligonucleotides; methylation; monolithic column; LC-MS

Introduction

Benzo[a]pyrene (B[a]P), a polycyclic aromatic hydrocarbon (PAH) environmental carcinogen, is strongly implicated as a causative agent of human lung cancers associated with tobacco smoke. These tobacco-smoke-related cancers are attributed to mutations in the *p53* tumor suppressor gene due to the high affinity of PAH adduction for specific sequences in this gene and the distinct mutagenicity of this class of compounds. After metabolism by cellular monooxygenases, B[a]P is transformed to the ultimate active carcinogenic metabolite, ($\pm$)-*anti*-benzo[a]pyrene diol epoxide (BPDE), which binds to DNA and forms predominantly covalent ($\pm$)-*trans* adducts at the N2 position of guanine (N²-BPDE-dG) or N6 position of adenine (N⁶-BPDE-dA)[1–3]. Most human lung cancers contain mutations in the *p53* tumor suppressor gene, in particular at codons 157, 158, 248 and 273. However, only codon 157 seems to be a specific mutational ‘hot spot’ for lung cancer with the majority of these mutations occurring as G $\rightarrow$ T transversions.

The CpG dinucleotide sequence is of special significance in mammalian DNA. Sixty to ninety percent of these CpG sites in human DNA contain 5-methylcytosine[4]. Over 50% of human lung cancers have a mutation at CpG dinucleotide sequences in certain codons of the *p53* tumor suppressor gene and various bulky chemical carcinogens form DNA adducts preferentially at these C5 cytosine methylated CpG sites[5–10]. It has been reported that methylated CpG sites not only greatly increases carcinogen-DNA adduct formation at CpG sites but also affects carcinogen-DNA adduct formation at surrounding sequences as well[9,11]. Studies have shown that the reactivity of BPDE is influenced by the methylation status of CpG sequences in the *p53* gene. Using a nuclease excision method with the repair enzyme UvrABC, Denissenko *et al.* had found that the major mutational ‘hot spots’ in the *p53* tumor suppressor gene in human lung cancers were codons 157, 248, and 273 and are the preferential binding sites for ($\pm$)-*anti*-BPDE[6,8]. However, the adduct profile obtained from plasmid DNA containing genomic *p53* sequences was different from that in actual genomic DNA[12]. Among all genetic alterations in the *p53* gene, a remarkably high number of somatic mutations are found at methylated CpG dinucleotides. When the cytosines at CpG sequence in plasmid DNA were converted to 5-methylcytosines, adduct hot spots were detected which were similar to those seen in genomic DNA.[8]

The influence of C5 cytosine methylation of CpG dinucleotide on BPDE-DNA conformation in the *p53* gene has been reported by several research groups[13–16]. Weisenberger *et al.* used polyacrylamide gel electrophoresis (PAGE) analysis to study the effect of methylation on the reactivity of BPDE at CpG sequences. Huang and coworkers used ultraviolet (UV) absorption and fluorescence methods to investigate the conformational change of BPDE-dG adducts induced by 5'-flanking-5-methyl-substituted cytosine in a CpG double stranded oligonucleotide sequence[17]. While both of these techniques provided information on the presence of adduction in a sequence, the exact location or quantity of adducts was not determined. In order to better quantify the relationship between nucleotide sequence and adduct location in the sequence, Tretyakova *et al.* developed a stable isotope labeling HPLC-ESI-MS/MS method to analyze the distribution of BPDE-DNA adducts by detecting the BPDE-nucleoside adducts level[15,16,18,19]. The method of Tretyakova and coworkers represented a dramatic improvement in both sensitivity and adduct quantitation over traditional bioanalytical methods such as PAGE and fluorescence. However, the extensive sample preparation which required 5 nmol of oligonucleotide for each reaction step for each individual preparation of every isotopically tagged base[15] suggests that a direct on-line method would be more suitable to elucidate the carcinogen-binding spectrum at the oligonucleotide sequence level directly and more efficiently. More recent work by Chowdhury and Guengerich using LC-MS/MS for the direct analysis of BPDE-adducted oligonucleotides demonstrated the utility of this technique for oligonucleotide sequencing[20]. However, because no separation was

done on the adducted oligonucleotide, assessment of adduction at different positions within the strand was not possible preventing a full assessment of the relative adduction frequencies among all possible adduction sites within the strand.

We recently reported on the development of an ion-pair reversed-phase nanohigh-performance liquid chromatography (IP-RP-nanoHPLC) method using a monolithic poly(styrene-divinylbenzene) (PS-DVB) column coupled to nanoelectrospray ionization mass spectrometry (nESI-MS) to separate and identify isomeric oligonucleotide adducts[21]. In the present study, this method was used to map the distribution of DNA adducts induced by ($\pm$)-*anti*-BPDE along a 14-mer sequence from the *p53* gene which contained the mutation 'hot spot' codons 157 and 158. Only data for the nontranscribed strand was analyzed due to its significance in mutations occurring in the *p53* gene[6]. In addition, the effect of cytosine methylation at CpG sites on the formation of BPDE adducts was also determined. The interfacing of LC separations with on-line MS and MS/MS analysis eliminated several sample preparation steps and the need for isolation of individual analytes. This direct LC/MS method decreases the total sample requirement, increases the MS and MS/MS sensitivity and improves the throughput of the assay.

Experimental Procedures

Chemicals and oligodeoxynucleotide samples preparation

Caution: ($\pm$)-*anti*-BPDE and several of its derivatives are carcinogenic to rodents and should be handled carefully.

Reagents—Methanol and water (all HPLC grade) were obtained from Fisher Scientific (Pittsburgh, PA, USA). Triethylammonium bicarbonate (TEAB) and sodium chloride–tris–EDTA (STE) buffer were obtained from Fluka (Milwaukee, WI, USA). Divinylbenzene (synthesis grade), styrene (synthesis grade), decanol (synthesis grade), tetrahydrofuran (THF) (analytical reagent grade), azobisisobutyronitrile (synthesis grade), caffeine (analysis grade) and triethylamine (TEA) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Styrene and divinylbenzene were distilled before use. ($\pm$)-*anti*-BPDE was obtained from the National Cancer Institute Chemical Carcinogen Reference Standard Repository (Midwest Research Institute, Kansas City, MO, USA). The 5-methyl dC phosphoramidite was prepared according to published procedures by Divakar *et al.*[22] and then incorporated into oligonucleotides, using an Applied Biosystems 3400 synthesizer on a 1- μ mol scale using standard cyanoethylphosphoramidite chemistry supplied by the manufacturer. Long chain alkylamine controlled pore glass (CPG) was used as the solid support. The concentration of the phosphoramidites was 0.1 M for the deoxymonomers and 0.15 M for the 5-methyl dC phosphoramidite. The oligomers were deprotected using concentrated ammonium hydroxide : ethanol (0.3 ml : 0.1 ml) for 4 h at 55 °C, purified by strong anion exchange HPLC using the following gradient: linear 0–50% B over 30 min, (buffer A: 0.1 M Tris HCl (pH 7.5) 10% acetonitrile; and buffer B: 0.1 M Tris HCl (pH 7.5) 10% acetonitrile, 1 M NaCl), and finally desalted using C-18 Sep Pak cartridges (Waters Inc., Milford, MA, USA). The oligonucleotides were dissolved in water to a concentration of 0.15 μ g/ml, based on either calculated extinction coefficients or molecular weights. To obtain double stranded (ds) DNA, equimolar amounts of the two DNA strands in STE buffer (10-mM Tris pH 7.8, 50-mM NaCl, 1-mM EDTA) were heated to 90 °C for 10 min, and gradually cooled to room temperature. The completeness of duplex formation was confirmed by HPLC under nondenaturing conditions.

($\pm$)-*anti*-BPDE modified oligonucleotide adduct synthesis: 1.5 mg of ($\pm$)-*anti*-BPDE in 1.0 ml THF was added gradually over 2 h to a solution of 1 μ g/ μ l ds 14-mer oligonucleotide in 2 ml of TE buffer (10-mM Tris-Cl, pH 7.5 and 1-mM EDTA, pH 7). The reaction mixture was incubated at 37 °C for 48 h. The reaction mixture was extracted three times with 3 ml of ethyl

acetate to remove unreacted diol epoxide. The solvent was evaporated under vacuum. Samples were reconstituted in 200 μ l deionized (DI) water (final concentration of 0.5 μ g/ μ l) and incubated at 95°–100 °C for 10 min, then chilled on packed ice for 30 min to denature. The samples were stored at –20 °C until analysis.

High-performance liquid chromatography

A HP1100 liquid chromatograph was used to generate solvent flow at a rate of 0.2 ml/min (Agilent Technologies, Palo Alto, CA, USA). A microTee (Upchurch Scientific, Oak Harbor, WA, USA) and polyimide-coated fused silica capillary tubing (2 m length, 360 μ m o.d., 50 μ m i.d., Polymicro Technologies, Phoenix, AZ, USA) was used to split the flow rate to 200 nl/min, resulting in a split flow ratio of 1000/1. The flow from the splitter was then directed to the integrated capillary LC/ μ ESI system. A 6-port microbore valve (VICI, Valco, Houston, TX, USA) equipped with 250-nl PEEK internal sample loop was used for manual sample introduction. A monolithic polystyrene divinyl benzene (PS-DVB) nanocolumn (75 μ m i.d. $\times$ 360 μ m o.d. $\times$ 20 cm) was prepared by thermally initiated copolymerization of styrene and divinylbenzene inside a silanized 75- μ m i.d. fused silica capillary using a mixture of THF and decanol as porogen according to a published protocol[23]. The mobile phase was filtered through a 0.22- μ m filter before use in the HPLC analysis. A low-pressure in-line filter was placed between the microTee and the pump to avoid clogging the spray tip.

Separation of ($\pm$)-BPDE modified oligonucleotides was achieved using a binary mobile phase consisting of 25-mM TEAB pH 8.3 and methanol. For the first 3 min of each run, the mobile phase was held isocratic at 100% 25 mM TEAB to ensure the sample loop was flushed completely and the analyte was enriched at the head of the capillary column to avoid peak broadening. A gradient was run from 0 to 10% MeOH over 1 min. The methanol was increased linearly from 10 to 40% over 60 min and then stepped to 100% and held isocratic at 100% methanol for 5 min to purge the capillary column of late eluting species. The capillary column was then allowed to reequilibrate by holding 100% 25-mM TEAB for 45 min prior to subsequent analyses. All samples were injected in triplicate and only data for the nontranscribed strand was analyzed.

Electrospray ionization mass spectrometry

ESI-MS was performed on an LCQ classic quadrupole ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA) equipped with a homemade integrated nanospray ion source, which was similar in design to an interface reported previously[24]. Both the HPLC and MS instruments were controlled by Xcalibur software Version 1.4 (Thermo Finnigan, San Jose, CA, USA). Total ion chromatograms and mass spectra were recorded on a personal computer with the LCQ Xcalibur software. Data from the MS/MS spectra for the adducted oligonucleotides were sequenced manually. A syringe pump (Harvard Apparatus, Holliston, MA, USA) equipped with a 250 μ l glass syringe (Hamilton, Reno, NV, USA) was used for direct infusion. Mass calibration and coarse tuning were performed in the positive ion mode by direct infusion of a solution of caffeine, methionylarginylphenylalanylalanine and Ultramark 1621 (all from Finnigan, San Jose, CA). Subsequently, the parameters of the ion optics were tuned at m/z 1394 for maximum ion transmission in the negative ion mode by infusion of 200 nl/min of a 20-pmol/ μ l solution of single-stranded 14-mer oligonucleotide in 25 mM TEAB in 50 : 50 (v/v) water : methanol.

To analyze the oligonucleotide samples, the mass spectrometer was operated in full scan, negative ion detection mode to assess all masses present. The scan range for these experiments was m/z 1000–2000. The samples were analyzed by LC/MS/MS, producing fragmentation data for the modified oligonucleotides. The scan range for the MS/MS spectra was m/z 415–2000. The LC/MS and LC/MS/MS interface conditions were held constant, and are as follows: spray

voltage: 1.8 kV, capillary voltage: -4V, capillary temperature: 210 °C and tube lens voltage: -25V. The LC/MS/MS experiments were conducted at a collision width of 3 Da and relative collision energy of 30%.

Results and Discussion

Guanine nucleobases within the *p53* gene of codons 157 and 158 are specific mutation hot spots for smoking-induced lung cancer. To investigate the extent of ($\pm$)-*anti*-BPDE adduct formation at these sites, a series of cytosine methylated 14-mer DNA duplexes 5'-ACCCGCGTCCGCGC-3'/5'-GCGCGGGCGCGGGT-3' representing codons 156–159 were synthesized for these experiments. The DNA duplexes were incubated with ($\pm$)-*anti*-BPDE and liquid–liquid extraction was used to remove the unreacted BPDE. The crude reaction mixtures were used for analysis without further purification. An ion-pair reversed-phase nanoHPLC method using a monolithic PS-DVB column coupled to nESI-MS/MS was utilized to map the distribution of BPDE-guanine adducts within DNA duplexes. LC/MS was employed for on-line separation of *in vitro* generated ($\pm$)-*anti*-BPDE-modified single-stranded oligonucleotides. LC/MS/MS was used to assign the sites of modification within the target sequence with MS/MS spectra recorded above the peak half-width of any peak for data interpretation to minimize interference from flanking peaks. A series of fragment ions a-B (derived from the 5' end of the oligonucleotides) and w (derived from the 3' end) were used to identify the sites of adduct formation. These fragment ions arose from characteristic decompositions occurring on modified oligonucleotides as described by McLuckey *et al.*[25, 26]. By using a monolithic column combined with the ion-pairing reagent TEAB, several positional isomeric oligonucleotides were easily separated. The chromatographic resolution of the positional isomers allowed estimation of both the extent of overall adduct formation and the effect of sequence context on adduct formation. The extent of modification was determined directly from the LC/MS peak area for each peak under the assumption that all positional isomers had the same ionization efficiency within their narrow chromatographic elution window. Because the gradient was shallow (0.5% B per minute) we expect that the signal intensity should be relatively similar across the elution window for each of the individual positional isomers resolved in the run. The good reproducibility of the data as indicated by the relative standard deviations for the triplicate analyses suggests this assumption is valid but variations in the ionization efficiency could have an impact on the quantitation and should be evaluated further.

Distribution of BPDE adducts in an unmethylated ds 14-mer oligonucleotide

The composite extracted ion chromatogram for the unmodified and modified nontranscribed strands obtained from the LC/MS analysis of the crude reaction mixture of ds oligonucleotide with ($\pm$)-*anti*-BPDE is shown in Fig. 1. The unmodified oligonucleotide was characterized by its m/z 1394 $[M-3H]^{3-}$ triply charged molecular ion detected as the major multiply charged species at a retention time of 41.6 min. Three peaks eluting between 64 and 77 min (II-1, II-2 and II-3) were consistent with the mass of the adducted nontranscribed strand sequence 5'-ACCCG₅CG₇TCCG₁₁CG₁₃C-3', containing only one covalent ($\pm$)-BPDE modification with a m/z 1495 $[M-3H]^{3-}$. For the incubation of ($\pm$)-*anti*-BPDE with the 14-mer oligonucleotide, a molar ratio of 1 : 12 BPDE to oligonucleotide was used. This ratio is higher than that reported by others in which Matter *et al.* used a 1 : 5 molar ratio[16] and Chowdhury and Guengerich used a 1 : 0.3 molar ratio[20]. While the ratio reported here is higher than what others have used, no doubly adducted oligonucleotide was detected. While it is possible that the excess BPDE could have affected the relative adduction profile and masked alternate sequence preferences for adduction, the lack of multiple adducts and the results of Tretyakova *et al.* in which 1 : 1 and 5 : 1 [BPDE] : [DNA] molar ratios were used with no double adduction detected [15] strongly suggests that the excess BPDE did not affect the sequence preference.

The on-line MS/MS spectra for all three peaks from the ds oligonucleotide are shown in Fig. 2. The different fragment ions observed from this MS/MS analysis are summarized in Table 1, in which the modified oligonucleotides are indicated with an asterisk on the bases bearing the modification. All chromatographic peaks derived from the modified ds oligonucleotides were identified by the analysis of the MS/MS fragmentation data.

Peak II-1, ACCCG₅CG₇TCCG₁₁*CG₁₃C (64.71 min): diagnostic pairs of fragments $[w_3]^-$ and $[a_{11}*B_{11}]^{2-}$ as well as $[w_4*]^-$ and $[a_{10}B_{10}]^{2-}$ were obtained. Thus one component of a possible mixture must have had an adduct on G₁₁, which was retained in $[w_4*]^-$ and was lost along with G₁₁ upon formation of the $[a_{11}*B_{11}]^{2-}$ fragment. The absence of modification in the $[a_{11}*B_{11}]^{2-}$ fragment, as well as the lack of any unmodified $[w_4]^-$ fragment, indicated that no component was present with an adduct 5' to G₁₁. Another diagnostic fragment observed was $[a_{12}*B_{12}]^{2-}$. The absence of any unmodified $[a_{12}B_{12}]^{2-}$ fragment and the absence of modification in the $[w_3]^-$ fragment indicated that there was no component with an adduct on the 3' side of G₁₁. Thus, peak II-1 in Fig. 1 consisted of a single compound with the BPDE adduct at G₁₁. Assignment of the location of the adduct on the other modified oligonucleotides in the chromatograms was similarly made by analysis of the MS/MS data as described above with identification of diagnostic pairs of ions allowing for the following adduct assignments:

Peak II-2, ACCCG₅*CG₇TCCG₁₁CG₁₃C (71.66 min) and **Peak II-3**,

ACCCG₅CG₇*TCCG₁₁CG₁₃C (76.66 min). There was no adduct found at the G₁₃ base and each chromatographic peak corresponded to a single positional isomer. No doubly adducted oligomers were detected. The approximate ratio for the different modification sites was 0.86 (G₅), 0.04 (G₇) and 0.11 (G₁₁). When analyzed in triplicate, the relative standard deviations were 3%, 25% and 9% respectively. These standard deviations were typical for all of the replicate samples tested in this study in which higher standard deviations were observed for sites with lower adduction frequencies. These initial results show that BPDE binds significantly at codon 156 (G₅) on the selected sequence for an unmethylated oligonucleotide instead of the reported mutational hot spots on codon 157 (G₇) and 158 (G₁₁). Codon 158 showed moderate binding affinity while codon 157 showed a weak binding affinity and codon 159 (G₁₃) showed no binding affinity toward BPDE.

Cytosine permethylation of nontranscribed or complementary strands and the influence of adduction on the nontranscribed strand

In order to determine the sequence specificity for ($\pm$)-anti-BPDE adduction for highly methylated ds oligomers, permethylated nontranscribed strand (5'-ACCC^MCG₅^MCG₇TC^MCG₁₁^MCG₁₃C-3') and permethylated complementary strand (3'-TGGG^MCG^MCAGG^MCG^MCG-5') were annealed with their unmethylated complements and incubated with ($\pm$)-anti-BPDE. After removal of the unreacted BPDE the permethylated nontranscribed strands were analyzed by LC-MS/MS as reported above and the data are summarized in Table 2. As can be seen in Table 2, permethylation of either strand resulted in an approximately 24% decrease in adduct formation at the G₅ position of the nontranscribed strand relative to the unmethylated oligomer. The decrease at the G₅ position was accompanied by a doubling of adduct formation at the G₁₁ position and the formation of adduct at the G₁₃ position when compared to the unmethylated oligomer. Despite the decrease at G₅, this position remained the major site of adduct formation. It is not surprising that adduct formation occurred at the G₁₃ position in the permethylated cases as it had been previously reported that CpG methylation stabilized the double strand thus promoting adduct formation[9]. The poor relative rate of adduction at G₁₃ in the unmethylated case may be an artifact of the short length of the oligomers at the 3' end which could result in conformational flexibility and thus reduced overall reactivity at this site. If this is true, the increased adduct enhancement when the CpG G₁₃ site was methylated lends additional support to the premise that adduct formation at CpG methylation sites is inherently more conformationally restrained than at unmethylated sites.

Cytosine permethylation of nontranscribed and complementary strands and the influence of adduction on the nontranscribed strand

Since 60–90% of CpG sites in human DNA contain 5-methylcytosine, including all CpG sites along exons 5–8 of the *p53* tumor suppressor gene[4], it is necessary to evaluate the effect of cytosine methylation on the chemical selectivity of BPDE with DNA when all the cytosines of CpG dinucleotides on both the nontranscribed and complementary strands were methylated.

One key component of this work is the ability to sufficiently resolve oligonucleotides that contain only one covalent modification in order to obtain detailed sequencing information about the adduct position within the strand. Figure 3 demonstrates the chromatographic separation of the isomeric BPDE adducts of the permethylated nontranscribed strand following incubation of the duplex with ($\pm$)-*anti*-BPDE. With the enhanced chromatographic resolution achieved with the monolithic PS-DVB nanocolumn, detailed MS/MS spectra were obtained for the four major positional isomeric adducted peaks detected between 51 and 63 min. In addition, two low-intensity peaks with the diagnostic ion of m/z 1513 for a monoadducted oligonucleotide were detected at retention times of 48 and 68 min but their intensities were too low for MS/MS sequencing. From previous experience with these columns, it is possible that the low-intensity peaks represent the resolution of stereoisomers related to adducts at the same adduct site on the oligomers[21]. However, without sufficient MS/MS data and authentic standards, the peak assignments are unknown.

The relative contribution of CpG dinucleotide permethylation on the formation of N²-BPDE-dG adducts in the nontranscribed strand sequence is shown in Table 2. The relative abundance of BPDE adducts in the nontranscribed strand is: $G_5 \gg G_7 > G_{11} > G_{13}$. A three- to five-fold enhancement of BPDE binding is seen at the G_7 CpG site relative to all of the other systems examined but the G_5 CpG site still remained the preferential site of adduction. The enhanced relative adduction level observed at G_7 does not correlate with the effects seen with permethylation of the individual strands and suggests an alternative mechanism for selectivity at this site. Perhaps the changes in stereo-structure and chemical environment cited by Chen *et al.*[9] that are directly related to CpG methylation can better explain the enhanced selectivity at G_7 in the permethylated duplex case. Additional modeling studies would be needed to investigate the nature of adduction enhancement under the experimental conditions presented in this work.

Conclusions

The introduction of 5-methylcytosine within CpG dinucleotides on the same oligonucleotide sequence significantly changed the ratio of BPDE-dG formation at different bases relative to the unmethylated case. Cytosine permethylation on either the nontranscribed or complementary strands significantly stimulated diol epoxide binding to codon 158 (G_{11}) in the selected sequence. Nevertheless, the major modification was still located at codon 156 (G_5). Permethylation of both strands dramatically increased adduction at codon 157 (G_7) relative to codon 158 (G_{11}) with the major adduction site remaining at codon 156 (G_5). As shown in Fig. 4, the pattern of ($\pm$)-*anti*-BPDE chemoselectivity within the selected 14-mer oligonucleotide did not correlate with the mutational spectra in the *p53* gene reported for lung cancer[27]. For other codons within the *p53* gene, namely 245, 248 and 273, the chemoselectivity and mutational spectra of ($\pm$)-*anti*-BPDE are more clearly associated[15,16]. In the case of codon 156 examined in this work, Tang *et al.*[16,28] and Matter *et al.*[16] had previously shown using two different labor intensive techniques that the highest adduction frequency occurred at codon 156 (G_5) with the same trend reported in this work for the relative rates of adduction: $G_5 \gg G_7 > G_{11} > G_{13}$. While the particular application described here does not address the quantitation of diastereomers, the use of nanoLC-MS/MS for the analysis of intact adducted oligonucleotides can provide valuable data on positional isomer analysis using only 30 pmol

of sample per injection and minimal sample preparation. The fact that these three different techniques could yield the same adduction profiles supports the notion that the direct analysis of the oligomers as presented in this work is suitable for evaluating the adduction profile in important gene sequences. That the chemoselective 'hot spot' is not the mutational 'hot spot' for codon 156 highlights the need for additional work in the area of DNA adduction and mutation and points to the possibility that the adduct conformation flanking sequences of known mutational sites may help destabilize this region of the *p53* gene and increase the likelihood of mutation for BPDE. The data also suggest that a threshold adduction level may exist below which the incidence of mutation is much higher relative to other codon sites in the *p53* gene for a majority of human lung cancers. The exact nature of this threshold level and its overall significance to mutation remains to be established.

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References

1. Cheng SC, Hilton BD, Roman JM, Dipple A. DNA adducts from carcinogenic and noncarcinogenic enantiomers of benzo[a]pyrene dihydrodiol epoxide. *Chemical Research in Toxicology* 1989;2:334. [PubMed: 2519824]
2. Schurter EJ, Yeh HJ, Sayer JM, Lakshman MK, Yagi H, Jerina DM, Gorenstein DG. NMR solution structure of a nonanucleotide duplex with a dG mismatch opposite a 10R adduct derived from trans addition of a deoxyadenosine N6-amino group to (-)-(7S,8R,9R,10S)-7,8-dihydroxy-9,10-epoxy-7,8,9,10- tetrahydrobenzo[a]pyrene. *Biochemistry* 1995;34:1364. [PubMed: 7827084]
3. Yeh HJ, Sayer JM, Liu X, Altieri AS, Byrd RA, Lakshman MK, Yagi H, Schurter EJ, Gorenstein DG, Jerina DM. NMR solution structure of a nonanucleotide duplex with a dG mismatch opposite a 10S adduct derived from trans addition of a deoxyadenosine N6-amino group to (+)-(7R,8S,9S,10R)-7,8-dihydroxy-9,10-epoxy-7,8,9,10- tetrahydrobenzo[a]pyrene: an unusual syn glycosidic torsion angle at the modified dA. *Biochemistry* 1995;34:13570. [PubMed: 7577946]
4. Tornaletti S, Pfeifer GP. Complete and tissue-independent methylation of CpG sites in the *p53* gene: implications for mutations in human cancers. *Oncogene* 1995;10:1493. [PubMed: 7731703]
5. Feng Z, Hu W, Rom WN, Beland FA, Tang MS. N-hydroxy-4-aminobiphenyl-DNA binding in human *p53* gene: sequence preference and the effect of C5 cytosine methylation. *Biochemistry* 2002;41:6414. [PubMed: 12009904]
6. Denissenko MF, Pao A, Tang M, Pfeifer GP. Preferential formation of benzo[a]pyrene adducts at lung cancer mutational hotspots in *P53*. *Science* 1996;274:430. [PubMed: 8832894]
7. Smith LE, Denissenko MF, Bennett WP, Li H, Amin S, Tang M, Pfeifer GP. Targeting of lung cancer mutational hotspots by polycyclic aromatic hydrocarbons. *The Journal of the National Cancer Institute* 2000;92:803.
8. Denissenko MF, Chen JX, Tang MS, Pfeifer GP. Cytosine methylation determines hot spots of DNA damage in the human *P53* gene. *Proceedings of the National Academy of Sciences U S A* 1997;94:3893.
9. Chen JX, Zheng Y, West M, Tang MS. Carcinogens preferentially bind at methylated CpG in the *p53* mutational hot spots. *Cancer Research* 1998;58:2070. [PubMed: 9605744]
10. Feng Z, Hu W, Rom WN, Beland FA, Tang MS. 4-aminobiphenyl is a major etiological agent of human bladder cancer: evidence from its DNA binding spectrum in human *p53* gene. *Carcinogenesis* 2002;23:1721. [PubMed: 12376482]
11. Li VS, Reed M, Zheng Y, Kohn H, Tang M. C5 cytosine methylation at CpG sites enhances sequence selectivity of mitomycin C-DNA bonding. *Biochemistry* 2000;39:2612. [PubMed: 10704210]

12. Puisieux A, Lim S, Groopman J, Ozturk M. Selective targeting of p53 gene mutational hotspots in human cancers by etiologically defined carcinogens. *Cancer Research* 1991;51:6185. [PubMed: 1933877]
13. Weisenberger DJ, Romano LJ. Cytosine methylation in a CpG sequence leads to enhanced reactivity with Benzo[a]pyrene diol epoxide that correlates with a conformational change. *The Journal of Biological Chemistry* 1999;274:23948. [PubMed: 10446162]
14. Dong H, Bonala RR, Suzuki N, Johnson F, Grollman AP, Shibutani S. Mutagenic potential of benzo [a]pyrene-derived DNA adducts positioned in codon 273 of the human P53 gene. *Biochemistry* 2004;43:15922. [PubMed: 15595848]
15. Tretyakova N, Matter B, Jones R, Shallop A. Formation of benzo[a]pyrene diol epoxide-DNA adducts at specific guanines within K-ras and p53 gene sequences: stable isotope-labeling mass spectrometry approach. *Biochemistry* 2002;41:9535. [PubMed: 12135376]
16. Matter B, Wang G, Jones R, Tretyakova N. Formation of diastereomeric benzo[a]pyrene diol epoxide-guanine adducts in p53 gene-derived DNA sequences. *Chemical Research in Toxicology* 2004;17:731. [PubMed: 15206894]
17. Huang X, Colgate KC, Kolbanovskiy A, Amin S, Geacintov NE. Conformational changes of a benzo [a]pyrene diol epoxide-N(2)-dG adduct induced by a 5'-flanking 5-methyl-substituted cytosine in a (Me)CG double-stranded oligonucleotide sequence context. *Chemical Research in Toxicology* 2002;15:438. [PubMed: 11896693]
18. Rajesh M, Wang G, Jones R, Tretyakova N. Stable isotope labeling-mass spectrometry analysis of methyl- and pyridyloxobutylguanine adducts of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in p53-derived DNA sequences. *Biochemistry* 2005;44:2197. [PubMed: 15697245]
19. Ziegel R, Shallop A, Upadhyaya P, Jones R, Tretyakova N. Endogenous 5-methylcytosine protects neighboring guanines from N7 and O6-methylation and O6-pyridyloxobutylation by the tobacco carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Biochemistry* 2004;43:540. [PubMed: 14717610]
20. Chowdhury G, Guengerich FP. Direct detection and mapping of sites of base modification in DNA fragments by tandem mass spectrometry. *Angewandte Chemie (International ed. in English)* 2008;47:381. [PubMed: 18022988]
21. Xiong W, Glick J, Lin Y, Vouros P. Separation and Sequencing of Isomeric Oligonucleotide Adducts Using Monolithic Columns by Ion-Pair Reversed-Phase Nano-HPLC Coupled to Ion Trap Mass Spectrometry. *Analytical Chemistry* 2007;79:5312. [PubMed: 17579370]
22. Divakar KJ, Reese CB. 4-(1,2,4-Triazol-1-yl)- and 4-(3-nitro-1,2,4-triazol-1-yl)-1-(b-D-2,3,5-tri-O-acetyl-arabinofuranosyl)pyrimidin-2(1H)-ones. Valuable intermediates in the synthesis of derivatives of 1-(b-D-arabinofuranosyl)cytosine (ara-C). *Journal of the Chemical Society, Perkin Transactions 1: Organic and Bio-Organic Chemistry (1972-1999)* 1982:1171.
23. Premstaller A, Oberacher H, Huber CG. High-performance liquid chromatography-electrospray ionization mass spectrometry of single- and double-stranded nucleic acids using monolithic capillary columns. *Analytical Chemistry* 2000;72:4386. [PubMed: 11008774]
24. Gangl ET, Turesky RJ, Vouros P. Determination of in vitro- and in vivo-formed DNA adducts of 2-amino-3-methylimidazo[4,5-f]quinoline by capillary liquid chromatography/microelectrospray mass spectrometry. *Chemical Research in Toxicology* 1999;12:1019. [PubMed: 10525280]
25. McLuckey SA, Van Berkel GJ, Glish GL. Tandem Mass-Spectrometry of Small, Multiply Charged Oligonucleotides. *Journal of the American Society for Mass Spectrometry* 1992;3:60.
26. McLuckey SA, Habibigoudarzi S. Ion-Trap Tandem Mass-Spectrometry Applied to Small Multiply-Charged Oligonucleotides with a Modified Base. *Journal of the American Society for Mass Spectrometry* 1994;5:740.
27. Petitjean A, Mathe E, Kato S, Ishioka C, Tavtigian SV, Hainaut P, Olivier M. Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: lessons from recent developments in the IARC TP53 database. *Human Mutation* 2007;28:622. [PubMed: 17311302]
28. Tang MS, Zheng JB, Denissenko MF, Pfeifer GP, Zheng Y. Use of UvrABC nuclease to quantify benzo[a]pyrene diol epoxide-DNA adduct formation at methylated versus unmethylated CpG sites in the p53 gene. *Carcinogenesis* 1999;20:1085. [PubMed: 10357792]

Figure 1.

LC/MS chromatograms obtained from the injection of a crude reaction mixture of ($\pm$)-BPDE with ds oligonucleotide 5'-ACCCGCGTCCGCGC-3'/3'-GCGCGGGCGCGGGT-5': composite extracted ion chromatogram for the $[M-3H]^{3-}$ ion of (I) the unmodified (m/z 1394) (II) the singly modified oligonucleotides (m/z 1495).

Figure 2. MS/MS fragment ion spectra for the singly modified oligonucleotides with m/z 1495 $[M-3H]^{3-}$ ion obtained from on-line LC/MS/MS for the unmethylated nontranscribed strand 14-mer oligonucleotide 5'-ACCCGCGTCCGCGC-3'.

Figure 3.

LC/MS chromatograms obtained from the injection of a crude reaction mixture of (±)-BPDE with ds oligonucleotide 5'-ACC^MCG^MCGTC^MCG^MCGC-3'/3'-G^MCG^MCGGG^MCG^MCGGGT-5': extracted ion chromatogram for the [M-3H]³⁻ ion of the singly modified nontranscribed strand oligonucleotides (bold) (*m/z* 1513).

Figure 4.

Relative formation of BPDE-dG adducts at different guanine positions (shaded bars) within the ds sequence derived from the *p53* gene and their relationship to G → T transversions (solid black bar) in human lung cancer obtained from the IARC *p53* mutation database[27]. A-E in each set represents the four different methylation conditions evaluated. The IARC database version used was Ver. R12, November 2007.

Table 1

Assignment of fragment ions from MS/MS collision-induced dissociation of m/z 1495 $[M-3H]^{3-}$ ion from the unmethylated nontranscribed 14-mer strand. Bold entries represent critical pairs used to identify the adduct position. Italicized entries indicate ions containing the adduct. ND = not detected

Peak II-1 (ACCCG ₅ CG ₇ TCCG ₁₁ *CG ₁₃ C)			
w_1^-	ND	$(a_{13}-B_{13})^{3-}$	ND
w_2^-	635.1	$(a_{12}-B_{12})^{2-}$	1868.3
w_3^-	924.1	$(a_{11}-B_{11})^{2-}$	1552.8
w_4^-	1555.3	$(a_{10}-B_{10})^{2-}$	1408.2
w_5^-	<i>1844.3</i>	$(a_9-B_9)^{2-}$	ND
w_6^-	ND	$(a_8-B_8)^{2-}$	ND
w_7^{2-}	<i>1218.2</i>	$(a_7-B_7)^-$	1895.3
w_8^{2-}	<i>1382.7</i>	$(a_6-B_6)^-$	1606.3
w_9^{2-}	<i>1527.3</i>	$(a_5-B_5)^-$	1277.2
w_{10}^{2-}	<i>1691.8</i>	$(a_4-B_4)^-$	988.2
w_{11}^{2-}	<i>1836.3</i>	$(a_3-B_3)^-$	699.1
w_{12}^{2-}	<i>1980.8</i>	$(a_2-B_2)^-$	ND
w_{13}^{3-}	<i>1416.6</i>	$(a_1-B_1)^-$	ND
Peak II-2 (ACCCG ₅ *CG ₇ TCCG ₁₁ CG ₁₃ C)			
w_1	ND	$(a_{13}-B_{13})^{3-}$	<i>1341.6</i>
w_2^-	635.1	$(a_{12}-B_{12})^{2-}$	<i>1868.3</i>
w_3^-	924.1	$(a_{11}-B_{11})^{2-}$	<i>1703.8</i>
w_4^-	1253.2	$(a_{10}-B_{10})^{2-}$	<i>1559.3</i>
w_5^-	1542.2	$(a_9-B_9)^{2-}$	<i>1414.8</i>
w_6^-	ND	$(a_8-B_8)^{2-}$	ND
w_7^{2-}	1067.2	$(a_7-B_7)^-$	<i>1098.4</i>
w_8^{2-}	1231.7	$(a_6-B_6)^-$	1908.4
w_9^{2-}	1376.2	$(a_5-B_5)^-$	1277.2
w_{10}^{2-}	1691.8	$(a_4-B_4)^-$	988.2
w_{11}^{2-}	<i>1836.3</i>	$(a_3-B_3)^-$	699.1
w_{12}^{2-}	<i>1980.8</i>	$(a_2-B_2)^-$	ND
w_{13}^{3-}	<i>1416.6</i>	$(a_1-B_1)^-$	ND
Peak II-3 (ACCCG ₅ CG ₇ *TCCG ₁₁ CG ₁₃ C)			
w_1	ND	$(a_{13}-B_{13})^{3-}$	<i>1341.6</i>
w_2^-	635.1	$(a_{12}-B_{12})^{2-}$	<i>1868.3</i>
w_3^-	924.1	$(a_{11}-B_{11})^{2-}$	<i>1703.8</i>
w_4^-	1253.2	$(a_{10}-B_{10})^{2-}$	<i>1559.3</i>
w_5^-	1542.2	$(a_9-B_9)^{2-}$	<i>1414.8</i>
w_6^-	ND	$(a_8-B_8)^{2-}$	ND
w_7^{2-}	1067.2	$(a_7-B_7)^-$	1895.3

w_8^{2-}	1382.7	$(a_6-B_6)^-$	1606.3
w_9^{2-}	1527.3	$(a_5-B_5)^-$	1277.2
w_{10}^{2-}	1691.8	$(a_4-B_4)^-$	988.2
w_{11}^{2-}	1836.3	$(a_3-B_3)^-$	699.1
w_{12}^{2-}	1980.8	$(a_2-B_2)^-$	ND
sw_{13}^{3-}	1416.6	$(a_1-B_1)^-$	ND

Table 2

Relative formation of N²-BPDE-dG on nontranscribed strand sequences (bold) derived from *p53* gene exon 5. Each sample was injected in triplicate

Sequences	Ratio			
	G ₅	G ₇	G ₁₁	G ₁₃
<u>Unmethylated CpG dinucleotides both strands</u>				
5'-ACCCG ₅ CG₇ TCCG ₁₁ CG₁₃C-3' 3'-TGGGC GC AGGC GC G-5'	0.86 ± 0.03	0.04 ± 0.01	0.11 ± 0.01	n.d.
<u>Permethylated CpG dinucleotides nontranscribed strand</u>				
5'-ACC ^M C ₅ ^M CG₇ TC ^M CG₁₁ ^M CG₁₃C-3' 3'-TGG GC GC AG GC GC G-5'	0.62 ± 0.01	0.07 ± 0.01	0.21 ± 0.03	0.11 ± 0.02
<u>Permethylated CpG dinucleotides complementary strand</u>				
5'-ACCC G ₅ C G₇ TC C G ₁₁ C G₁₃C-3' 3'-TGGG ^M C G ^M C AG G ^M C G ^M C G-5'	0.62 ± 0.01	0.04 ± 0.01	0.26 ± 0.02	0.09 ± 0.01
<u>Permethylated CpG dinucleotides both strand</u>				
5'-ACC ^M C G ₅ ^M CG₇ TC ^M C G ₁₁ ^M C G₁₃C-3' 3'-TGG G ^M C GC AG G ^M C G ^M C G-5'	0.53 ± 0.07	0.21 ± 0.03	0.17 ± 0.02	0.09 ± 0.03